ORIGINAL ARTICLE

Occurrence, Antibiotic Resistance and Molecular Characterization of *Listeria monocytogenes* in the Beef Chain in the Republic of Ireland

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Impacts

- *Listeria monocytogenes* was isolated from bovine hides (27%), pre-chill carcasses (14%) and ground beef (29%), but not from ready-to-eat beef products, and the concentration of the pathogen in the majority (95%) of contaminated samples was low and detectable only after an enrichment step.
- The most commonly isolated serotype group was 1/2a (58%) followed by 4b (12%)
- A small portion (<5%) isolates had demonstrated resistance to certain key anti-microbials used in clinical treatment of *Listeria* infections.

Keywords:
Listeria monocytogenes; beef; hide; carcass; ground meat

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Summary

This study investigated the occurrence, concentration and key characteristics of *Listeria monocytogenes* in beef chain samples (n = 1100) over a 2-year period (July 2007–June 2009). *Listeria monocytogenes* was isolated from bovine hides (27%), pre-chill carcasses (14%) and ground beef (29%), but not from ready-to-eat (RTE) beef. The concentration of the pathogen in the majority (95%) of contaminated samples was low and detected by enrichment only. The highest concentrations recovered (100–200 CFU/g) were in ground beef samples. The most commonly isolated serotype group was 1/2a (58%) followed by 4b (12%), 1/2b (10%) and 1/2c (6%). A small portion (<5%) isolates had demonstrated resistance to key anti-microbials including ampicillin, vancomycin and gentamycin which are recommended treatment options for *listeriosis*. Pulsed-field gel electrophoresis showed indistinguishable profiles for a number of isolates recovered from the hide and carcass (after slaughter and dressing) of the same animals, highlighting the role of hides as a source of contamination. Equally, indistinguishable pulsotypes for isolates recovered at different stages and time points (up to 6 months apart) in the beef chain demonstrated the persistence of specific clones in the factory, process and distribution environments. Overall, the study demonstrated a high prevalence of clinically significant *L. monocytogenes* entering and progressing along the beef chain and highlights the needs to control cross-contamination during beef processing and distribution and the need for thorough cooking of raw beef products.

Introduction

Although cases of listeriosis are relatively rare in the EU (0.32 cases per 100 000 population in 2011) (EFSA, 2013) and in the Republic of Ireland (0.15 cases per 100 000 in 2011) (HPSC, 2012), they pose a public health concern for the elderly, persons with underlying illness and other vulnerable groups such as pregnant women and neonates because of the severity of infection and the associated high fatality rate. While *L. monocytogenes* has 13 known serotypes, four serotypes 4b, 1/2a, 1/2b and 1/2c are responsible for more than 95% of human cases of listeriosis. Over a

ten-year period in the US (1998–2008) outbreaks of *listeriosis* were associated with a range of foods vehicles (Cartwright et al., 2013), primarily foods of animal origin (meat and dairy) but fresh produce (sprouts, taco/nacho and salads) were associated with more outbreaks later in the study period. As *Listeria* is a ubiquitous organism and widely distributed in the environment, sources of food contamination include soil, forage, water and animal faeces. Studies in the USA have shown that the bovine hide is a key source of *Listeria* contamination into the beef slaughter house (Rivera-Betancourt et al., 2004; Guerini et al., 2007). However, there are limited European studies (Wieczorek et al., 2012) and no previous Irish studies on the role of bovine hide as a source of *L. monocytogenes,* or that have looked at transfer of the pathogen from the hide to carcass during slaughter and dressing. The aim of the present study was to determine the prevalence, concentration, serotype group, antibiotic resistance profile and molecular characteristics of *Listeria* spp. isolated at key stages along the Irish beef chain.

Materials and Methods

Samples

Over a 2-year period (July 2007–June 2009), at a large commercial export, beef slaughter plant in the Republic of Ireland. Hides (n = 400) and carcasses (prior to evisceration and chilling) (n = 400) of the same tracked animal were swab sampled. The swabs (polyurethane (100 mm × 100 mm × 10 mm) (Sydney Heath & Son Ltd, Staffordshire, UK), which had been previously placed in autoclavable bags with 10 ml Maximum Recovery Diluent (MRD) (Oxoid, Basingstoke, UK) and sterilized, were passed 10 times vertically and 10 times horizontally over a defined 400 cm^2 areas of the brisket surface of hides/carcasses site using uniform pressure. Swabs were then held at 2–4°C, transported to the laboratory within 1 h and analysed for the presence of *L. monocytogenes* within 24 h of collection.

Ground beef (n = 100) and a range of ready-to-eat (RTE) beef products (n = 200) including roast beef (n = 68), corned beef (n = 86), ox tongue (n = 18), pastrami (n = 21) and cured or smoked beef (n = 7) were collected from retail outlets in the Dublin region between July 2008 and May 2009. It was not possible in the study to track samples from the slaughter house to the retail outlet. All samples were stored, transported and analysed as described above.

Microbiological analysis

Enumeration of Listeria spp

Abattoir swab samples and retail beef samples (10 g) were placed in stomacher bags (Sparks Lab Supplies, Dublin, Ireland) with 200 ml of MRD and 90 ml half Fraser broth, respectively, (Oxoid) and analysed according to ISO-11290-2. Following homogenization in a stomacher (IUL Instrument, Barcelona, Spain) for 3 min, aliquots of 1 ml and 0.1 ml were plated in duplicates onto Palcam Agar (Merck, Germany) and incubated at 37°C for 48 h microaerobically (GenBox gas sachets, Biomérieux, France). Typical green colonies surrounded by a black zone were enumerated and subcultured onto Tryptone Soya Agar (TSA) (Oxoid). After 48 h of incubation at 37°C, biochemical confirmation was carried out using the KOH test to establish gram reaction (Halebian et al., 1981) and the catalase reaction (Stopforth et al., 2005).

Detection of Listeria spp

Detection of the *Listeria* spp. was carried out in accordance to the ISO-11290-1 with some minor modification. Aliquots (40 ml) of the remaining homogenates were transferred into sterile tubes and centrifuged at 6300× ***g*** (Eppendorf 5810R, Hamburg, Germany) for 15 min. Pellets were resuspended in 100 ml half Fraser broth (Oxoid) and incubated microaerobically at 30°C for 24 h. Following enrichment, 0.1 ml of each sample was transferred to 10 ml full Fraser broth and incubated at 30°C for a further 48 h. A loopful of overnight culture was spread onto both Palcam and Oxford Agar (Merck) and incubated at 37°C for 48 h under microaerobic and aerobic conditions, respectively. Biochemical confirmation of the presumptive colonies was carried out as described above.

Characterization

Speciation and serotyping

Confirmed *Listeria* isolates were grown in Tryptone Soya Broth (TSB) (Oxoid) at 37°C overnight and recovered by centrifugation (1 ml) at 6200× ***g*** for 10 min (Eppendorf 5810R). DNA was extracted from cell pellets using a DNeasy kit, following the protocol for Gram-positive organisms (Qiagen, Crawley, UK). The multiplex PCR assay described by Doumith et al. (2004) was used to (i) confirm isolates as *Listeria* spp., (ii) identify *L. monocytogenes* and (iii) allocated isolates into four distinct *L. monocytogenes* serotype groups. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide (0.5 mg/l) (Sigma-Aldrich, Wicklow, Ireland) and visualized using the Bio-Rad GelDoc 2000 documentation system (Bio-Rad Laboratories, Italy).

Pulsed-field gel electrophoresis

A selection of the *L. monocytogenes* isolates (73 of 191 isolates) were selected for analysis by pulsed-field gel electrophoresis. The isolates were chosen based on serotype group

results, to explore epidemiologically interesting linkages such as the same serotype recovered on the hide of carcass of the same animal, the same serotype from different animals slaughtered on the same day, or the same serotype on different beef samples purchased from the same shop, as well as isolates recovered at different chronological times in the study to explore seasonal variation and strain persistence. Restriction digestions were initially carried out using *Asc*I enzyme (ISIS, Ireland) and further differentiated using *Apa*I enzyme (Roche, Mannheim, Germany) in accordance with the Pulsenet standardized protocol (Graves and Swaminathan, 2001). The generated DNA fragments were resolved by electrophoretic separation in a 1% agarose gel (Seakem Gold agarose, Lonza Biologics, UK), using the CHEF DR III system (Bio-Rad Laboratories) with 2.7 L 0.5X Tris–Boric–EDTA buffer (Roche) and 50 mM Thiourea (Sigma) for 18 h at 6 V, in accordance with the PulseNet protocol. The gel was stained for 30 min in 400 ml of deionized water with 40 μl of ethidium bromide (10 mg/ml), then washed three times with distilled water every 20 min before being visualized using the Gel Doc Scanner 2000. The resultant banding patterns were normalized against the size standard strain *Salmonella enterica* serotype *Braenderup* H9812, which was restricted with 10 U *Xba*I for 2 h and loaded into wells 1, 7, 14 and 20. BIONUMERICS software version 5 (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyse PFGE banding patterns and antibiotic resistance profiles. Dendrogram of PFGE profiles was generated based on the Dice correlation co-efficient for similarity and the unweighted-pair group method with arithmetic means (UPGMA) was employed for cluster analysis. Band matching was carried out at an optimization of 1% and position tolerance of 2%. A cut-off level of ≥80% similarity was established for the definition of macrorestriction profile in the pulsogroup (Gianfranceschi et al., 2009).

Anti-microbial resistance

A selection of the *L. monocytogenes* isolates (73 of 191 isolates) were selected for analysis. The isolates were chosen based on serotype group results and were representative of the isolates of different origin, sample type, season, sample time, etc. The minimum inhibitory concentrations (MIC's) of a range of antibiotics against the recovered *L. monocytogenes* isolates were determined by a micro-broth dilution method using Sensititre® Gram-positive susceptibility plates (Trek Diagnostics, Limerick, Ireland). Bovine isolates (n = 77,) and a control strain of *L. monocytogenes* 4b (NCTC 11994) were grown overnight on TSA at 37°C. Colonies were suspended into phosphate-buffered saline (PBS) (Oxoid), adjusted to an optical density of 0.132 at 600 nm, diluted 1 : 1000 with Muller–Hinton broth (Oxoid) and dispensed in 50 μl aliquots into Sensititre® plate wells. Plates were sealed with sterile adhesive covers and incubated at 37°C for 24 h. The resultant patterns of presence/absence of bacterial growth were used to determine MIC of each isolates. MIC is defined as the lowest concentration of antibiotic that prevents the bacterial growth or at which bacterial growth was visibly inhibited. The clinical breakpoints for ampicillin and penicillin were defined according to the Clinical and Laboratory Standards Institute guideline recommended for *L. monocytogenes* (Clinical and Laboratory Standards Institute (CLSI), 2006). The guidelines do not incorporate clinical breakpoint for other anti-microbials; therefore, the clinical breakpoint for staphylococci was used (Conter et al., 2009).

Imo2821 gene

The same selection of *L. monocytogenes* isolates (73 of 191 isolates) were screened for the presence of putative internalin lmo2821 gene by the method of Liu et al. (2003), using PCR assay mixtures (25 μl) composed of 25 pmol of each forward and reverse primer, 0.5 U Taq PCR polymerase, 50 μm dNTPs, 1 × PCR buffer and 3 μl template DNA.

Results

Listeria monocytogenes was detected in 27%, 14% and 29% of hide, carcasses and ground beef samples, respectively (Table 1), but was not recovered from RTE beef products.

Of the carcasses testing positive for *L. monocytogenes* (n = 56), the majority (62%) were derived from animals which also had a *L. monocytogenes* positive hide.

The majority of contaminated samples (95%) contained the pathogen in low concentrations and below the limit of detection of the enumeration method. The limit of detection for enumeration was 0.5 CFU/cm^2 for hide and carcass

Table 1. Prevalence and concentration of *Listeria monocytogenes* along the beef chain

Sample type	Sample number	Number positive samples (%)	Number (%) positive samples in concentration range (Log_{10} CFU cm^2/g)		
			Enrichment	0.25 –1.0	100–200
Hide	400	106 (27)	102 (96)a	3 (3) a	1 (1) a
Carcass	400	56 (14)	55 (98) a	1 (2) a	0
Ground beef	100	29 (29)	24 (82)b	4 (14) b	1 (3) b
Ready-to-eat beef	200	0	0	0	0
Total	1100	191 (17)	181 (95)	8 (4)	2 (1)

[a]Count unit is CFU/cm^2.
[b]Count unit is CFU/g.

surface samples and 0.25 CFU/g for ground beef samples. *Listeria* in these samples was not detected by direct plating and was only recovered after enrichment. Across all samples, 4% had counts in the range Log_{10} 0.25–1.00 CFU/cm^2/g, and most of these, 4/8 samples, were ground beef. Two samples (1 hide and 1 ground beef) had counts in the range of 100–200 CFU/cm^2/g. A range of *L. monocytogenes* serotype groups were recovered with 1/2a (58%) most commonly isolated followed by 4b (12%) (Table 2). This pattern of 1/2a as the dominant serotype group was observed across all samples types (52–67%). The presence of the putative virulence gene *lmo*2821 was highly variable among isolates (33–100%) regardless of the serotype group or sample of origin (Table 3). Anti-microbial profiles for the *L. monocytogenes* isolates (Table 4) showed a low percentage of strains (<5%) were resistant to a range of antibiotics including some of which are used to treat human listeriosis; ampicillin, gentamycin and penicillin. PFGE profiling showed isolates fitted into 14 different pulsogroups, shown as A–N in Fig. 1. Strains (D16H310, D16C310), (D17C346 and D17H346) within pulsogroup A, strains (D16C309 and D16H309) in pulsogroup F, and strains (D18C356 and D18H356) within pulsogroup M were all isolated from the hides and dressed carcass of the same animals and had a similarity level ranging from 92 to 100%. Some of the strains isolated from ground beef clustered at 81–93% similarity with strains from hide and/or carcass isolated over a 6-month period.

Table 2. Percentage (%) of Irish beef chain samples containing different serotype groups of *Listeria monocytogenes*

Serotype group	All samples (%)	Hide (%)	Carcass (%)	Ground beef (%)
1/2a	58	67	54	52
4b	12	10	6	19
1/2b	10	6	17	7
1/2c	6	0	0	17
Mixed serotypes	15	17	23	5

Table 3. Percentage (%) *Listeria monocytogenes* serotype group isolates from the Irish beef chain containing the *lmo*2821 putative virulence gene

Serotype group	Total	Hide	Carcass	Ground beef
1/2a	70	74	51	86
4b	78	83	100	50
1/2b	64	75	83	33
1/2c	24	0	0	71
Mixed serotypes	NT	NT	NT	NT

NT, not tested.

Table 4. Anti-microbial profiles of selected *Listeria monocytogenes* (n = 73) recovered from the beef chain in the Republic of Ireland

Anti-microbials	Range (μg/ml)	Number of isolates (%)		
		Susceptible	Intermediate	Resistant
Ampicillin	0.12–16	75 (97)	0	2 (3)
Ciprofloxacin	0.5–2	77 (100)	0	0
Clindamycin	0.12–2	28 (36)	0	49 (64)
Daptomycin	0.25–8	7 (9)	0	70 (91)
Erythromycin	0.25–4	77 (100)	0	0
Gentamycin	2–16, 500	73 (95)	0	4 (5)
Oxacillin + 2%NaCl	0.25–8	12 (16)	0	65 (84)
Penicillin	0.06–8	74 (96)	0	3 (4)
Quinupristin/ dalfopristin	0.12–4	71 (92)	6 (8)	0
Rifampicin	0.5–4	75 (96)	0	3 (4)
Tetracycline	2–16	76 (99)	0	1 (1)
Vancomycin	1–128	75 (97)	0	2 (3)

Discussion

This study is one of the first European studies and the first study in Ireland to report on *L. monocytogenes* contamination of bovine hides. The results showed that 27% of hides were contaminated with *L. monocytogenes* which is considerably higher than previously reported levels of the pathogen on hides of 9.9% and 13.3% in 2 US studies (Rivera-Betancourt et al., 2004 and Guerini et al., 2007) and 10.8% in a study from Poland (Wieczorek et al., 2012). The reasons for such a difference is unclear, but may be related to a range of diverse factors influencing the beef production systems (feedlots in other studies versus a grass-based system in Ireland), transport and lairage-related conditions or indeed to geographical variations in patterns of bovine carriage and shedding of *L. monocytogenes*. Nonetheless, despite the high occurrence noted, in this study, the concentration of the pathogen when present was very low with the majority of positive hides (96%) having counts <0.5 CFU/cm^2 and detectable by enrichment only. Overall, the study highlights that the hide is an important source of *L. monocytogenes* contamination into beef plants and it is a source of beef carcass contamination during slaughter and dressing as confirmed by indistinguishable pulsotypes for a number of isolates recovered from the hide and the carcass of the same animal or other carcasses slaughtered on the same day. The observed prevalence of 14% *L. monocytogenes* on carcasses is comparable to that reported in the Polish study (10%) at the same stage of slaughter, using similar swab and detection method (Wieczorek et al., 2012). US studies on carcasses at the pre-eviseration stage have reported *L. monocytogenes* at levels of 0 to 23.2 depending on the plant and the season with levels

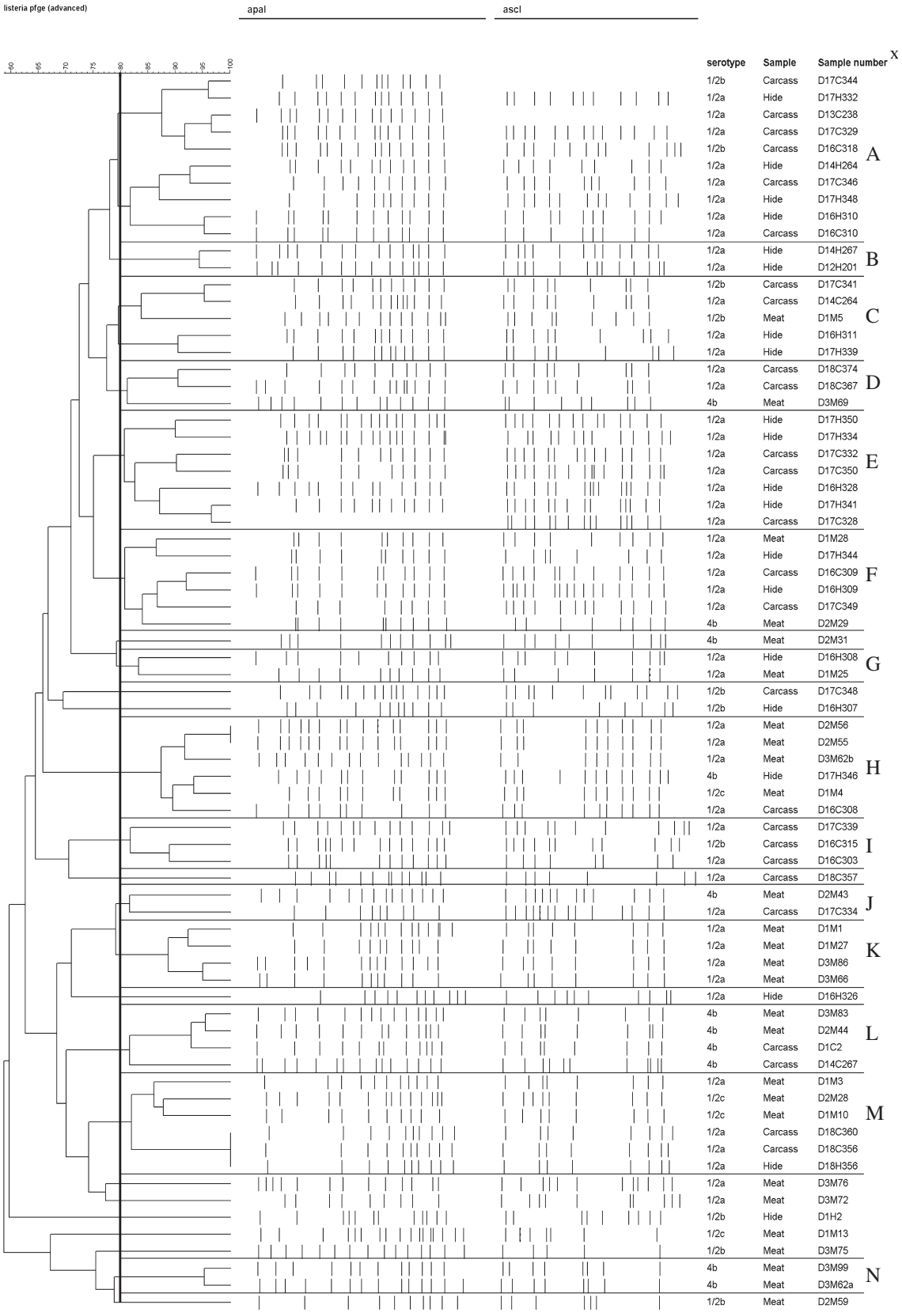

Fig. 1. Composite dendrogram of *Asc*I and *Apa*I macrorestriction pattern for selected *Listeria monocytogenes* beef chain isolates (n = 73).

generally higher in the winter than the summer (Rivera-Betancourt et al., 2004; Guerini et al., 2007). Contrary to the results from the US, in this study, the recovery of *L. monocytogenes* from both the hide and the carcass was highest in spring, with 5% and 0% of hides and carcasses testing positive in the winter. The impact of chilling on the micro-flora is variable, and while reductions in numbers of *Escherichia coli* and coliforms on carcass have been noted after chilling (McEvoy et al., 2004), *Listeria* as a psychrotropic organism is likey to survive well and to grow slowly at chill temperatures. This may explain the large increase in prevalence as beef progress through the production chain to ground beef products (29%), and the higher concentration of the pathogen also noted in these products (17% with counts in region 0.25–200 CFU/g). The increased prevalence is also reflective of the opportunities for cross-contamination of ground beef as a result of mechanical processing of the meat and cross-contamination during distribution. PFGE showed some of the strains isolated from ground beef clustered at 81–93% similarity with strains isolated from hide and/or carcass at different time periods (pulsogroup F, D1M28 and D17H344) and (pulsgroup G, D16H308 and D1M25). Also isolates of serotype group 1/2a, grouped with >85% similarity, were recovered from a single butcher shop over a 6-month period (pulsogroup K), demonstrating the persistence of particular clones in the factory, process and distribution environments.

Listeria monocytogenes serotypes were determined using a multiplex PCR assay as described by Doumith et al. (2004). Although the method does not distinguish serotype 1/2a, 1/2b, 1/2c and 4b from its corresponding serotype-complex (1/2a, 3a), (1/2a, 3b, 7), (1/2c, 3c) and (4b, 4d, 4e), it has been widely used as an alternative to conventional serotyping (Guerini et al., 2007; Ayaz and Erol, 2010). Serotype 1/2a was the most frequently isolated serotype, constituting 58% of all isolates. The prevalence of serotype 4b in 12% of all samples and 19% of ground beef samples is of particular concern as it is the most clinically significant serotype in relation to human illness and outbreaks internationally (Cartwright et al., 2013) and in Ireland (HPSC, 2011). This study also confirms previous reports of the recovery of multiple serotypes of *L. monocytogenes* from a single sample (Guerini et al., 2007).

Listeria monocytogenes isolates were examined for the presence of the *Imo*2821 gene. This gene has been reported to be exclusively associated with virulent strains of *L. monocytogenes* as compared to other virulence-associated genes such as *hly*A (encoding LLO), *plc*A (encoding PI-PLC) and *plc*B (encoding PC-PLC), which have been found in both virulent and avirulent strains (Liu et al., 2003). The occurrence of the gene was variable and had no direct relationship to serotype or sample of origin. There are number of studies assessing potential virulence of *L. monocytogenes* isolates recovered from food. Many putative virulence markers in *L. monocytogenes* have been identified, and the surface-associated internalins are claimed to play a role in the pathogenesis of human listeriosis. Wieczorek et al. (2012) reported that genes (*inlA*, *inlC* and *inlJ*) as well as the *lmo2672* marker were detected in all *L. monocytogenes* isolates recovered from the beef chain. Neves et al. (2008) reported that all *L. monocytogenes* isolates from food and food-related environments were potentially virulent or highly virulent on the basis of plaque-forming assay (PFA). It highlights that beef is a vehicle for clinically relevant *L. monocytogenes*.

While the majority of isolates were susceptible to all anti-microbials examined (Table 3), a small portion (<5%) had demonstrated resistance to certain key clinical used anti-microbials including ampicillin, vancomycin and gentamycin which are recommended treatment option of listeriosis infection in pregnancy (Janakiraman, 2008). Wieczorek et al. (2012) also reported low-level resistance to gentamycin (1.9%), although observed no resistance to vancomycin or ampicillin in *L. moncytogenes* isolated from bovine hide and carcasses in Poland. A Portugese study (Barbosa et al., 2013) on *L. monocytogenes* isolates from foods ($n = 353$) and human listeriosis ($n = 95$), showed 28.3% of food and 21.0% of clinical isolates, were resistant to at least one antibiotic. Even such low level of resistance to these drugs is of concern as it may be representative of an emerging pattern of developed resistance.

The data obtained in this study demonstrate the prevalence and potential for transfer of clinically significant *L. monocytogenes* to consumer through the beef chain. It highlights the need to implement effective food safety management approaches and interventions at all stages of the beef slaughter/processing/retail and consumer level (handling and cooking practices) to reduce the risk of cross-contamination and lower the burden of consumer exposure to *L. monocytogenes*.

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