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Characterization of *Listeria monocytogenes* from food and food-related settings

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Abstract

Listeriosis is a serious public health concern due to its high fatality rates. The present investigation screened for the occurrence of *Listeria monocytogenes* in food and food-related environments by analyzing samples from a cheese making factory, a slaughterhouse and a supermarket. Among the eighty-seven samples analyzed, thirty-four isolates were recovered and subsequently evaluated regarding genomic diversity, antibiotic susceptibility and virulence. Twenty-one *L. monocytogenes* were allocated to serogroup 1/2a-3a, ten assigned to serogroup 4b-4e-4ab and three to serogroup 1/2c-3c. Results obtained also detected the presence of *L. monocytogenes* isolates resistant to two clinically relevant antibiotics for the treatment of listeriosis, ampicillin and penicillin. Overall, data gathered disclosed the occurrence of listeria isolates with high pathogenicity potential at all very distinct sampling sites, reinforcing the need for an effective surveillance network designed for detecting the presence of *L. monocytogenes* in food products and food processing environments.

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Introduction

The genus *Listeria* comprises several ubiquitous species, from which only a few cause listeriosis in humans and animals. This foodborne illness is relatively rare but severe, leading to long hospitalization stays and resulting in high mortality rates. In the European Union 1640 human cases were reported in 2012, with an associated mortality rate of 17.8%. The route of transmission to humans occurs through consumption of contaminated food, responsible for 99% of human listeriosis (EFSA 2014).

L. monocytogenes is the species responsible for the majority of human listeriosis, being classified in thirteen serotypes associated with distinct pathogenicity potential: six responsible for epidemic listeriosis (lineage I: 1/2b, 4b, 4d, 4e, 3b, 7), three associated with sporadic listeriosis (lineage II: 1/2a, 1/2c, 3a) and the last three (lineage III: 4a, 4c, 7) primarily associated with animal listeriosis (Velge *et al*., 2010).

L. monocytogenes is known to persist and multiply in food and food-processing environments, being frequently isolated from fish, seafood, meat and particularly from ready to eat (RTE) food (O'Connor, 2010). The purpose of this work was to screen for the presence of *L. monocytogenes* in food and food-related samples collected from three distinct settings: a cheese making factory, a swine slaughterhouse and a supermarket. The isolates were further evaluated regarding their pathogenicity potential and resistance to antibiotics representing various drug classes.

Materials and Methods

Sampling and microbial isolation

Samples including water, food and food-contact surfaces were retrieved from a cheese making factory (n=29), a swine slaughterhouse (n=20) and a supermarket (n=38); in the Lisbon area, Portugal. Isolation procedures were performed following the standard double enrichment method as described by ISO 11290:1 (Anon., 1997). All isolates considered *L. monocytogenes* were stored at -80 ºC in Brain Heart Infusion -BHI- (Scharlau, Barcelona, Spain) containing 20% (v/v) glycerol until further use.

Molecular identification and typing

To confirm genus and species identification and achieve serogroup allocation, the isolates were subjected to multiplex-PCR according to the method described by Kérouanton *et al*. (2010). Control strains, representing all human associated serogroups, were kindly provided by Instituto Oswaldo Cruz (IOC)-Laboratório de Zoonoses Bacterianas-Brazil and included at all times.

Subsequently, the genomic diversity

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of the *L. monocytogene*s was assessed by PCR fingerprinting using primers csM13 (5'-GAGGGTGGCGGTTCT-3') and ERIC1 (5'-ATGTAAGCTCCTGGGGATTCAC-3')/ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') in independent reactions (Cocolin, 2005; Soni *et al*., 2013). Regarding data analysis, the BioNumerics software (version 6.6, Applied Maths, Kortrijk, Belgium) was used to register fingerprinting patterns, normalize densitometric traces, calculate the Pearson product–moment correlation coefficient, and perform cluster analysis by the unweighted pair group method with arithmetic mean algorithm (UPGMA).

Antibiotic susceptibility

Eleven antimicrobial agents (Oxoid Limited, Cambridge, United Kingdom) were used in this study: ampicillin 10 µg, ciprofloxacin 5 µg, clindamycin 2 µg, chloramphenicol 30 µg, erythromycin 15 µg, gentamicin 10 µg, penicillin G 10 units, rifampicin 5 µg, trimethoprim/sulfamethoxazole 25 µg, tetracycline 30 µg and vancomycin 30 µg. Susceptibility was evaluated by the disk diffusion method, using breakpoints of resistance previously established by the Clinical and Laboratory Standards Institute for *Staphylococcus* spp. (2013). For quality control, *Staphylococcus aureus* ATCC 25923 was also included.

Virulence traits

The presence of key virulence genes *(plcA, actA, hlyA, iap, inlA, inlB, inlC and inlJ)* was screened by PCR amplification using primers and conditions previously described by Liu *et al*. (2007) and Rawool *et al*. (2007).

Results and Discussion

The overall prevalence of *L. monocytogene*s in all studied samples/settings was of 39% (34/87). In the supermarket the occurrence was 53% (20/38), 30% (6/20) in the swine slaughterhouse and 28% (8/29) in the cheese making factory. Though slightly higher than previous reports, these numbers are consistent with the average prevalence in studies from other countries which analyzed similar samples (Leite *et al*., 2005; Jallewar *et al.*, 2007; Kovacevic *et al*., 2012 and Wang *et al*., 2012). Regarding serogroup allocation, 62% of the *L. monocytogenes* belong to serogroup 1/2a-3a, 29% could be included in serogroup 4b-4e-4ab and 9% in serogroup 1/2c-3c. Previous studies also reported serogroup 1/2a as the predominant in food/ environmental isolates (Boerlin *et al.* 1997; Lukinmaa *et al*. 2003; Gudbjornsdottir

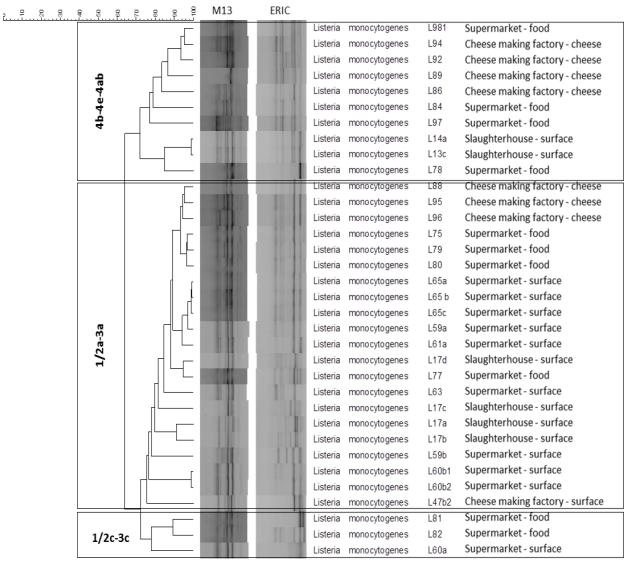

Figure 1. Dendrogram based on PCR-fingerprinting patterns. Similarity was calculated with Pearson product-moment correlation coefficient -r- and clustering performed with UPGMA

et al., 2004; Gilbreth *et al.*, 2005; Corcoran *et al*., 2006), probably due to the ability to survive more adequately in certain food matrixes (O'Connor *et al.* 2010).

Results obtained during the present study are summarized in Figure 1, which includes data regarding origin, serogroup and genomic typing. The dendrogram clearly depicts the distribution of the isolates under analysis among the serogroups observed, with members of the serogroup 4b-4e-4ab being visibly divergent from those belonging to 1/2a-3a and 1/2c-3c. Likewise, high similarity levels were observed between several of the food-related *L. monocytogenes*, as observed for example, for isolates L13c and L14a (from the same slaughterhouse surface) and L60b1/L60b2 (from the same supermarket surface). This indicates that the sampling procedure led to the recovery of genomic clones, which can help explain the high prevalence of *L. monocytogenes* obtained from the sampled settings. Additionally, the dendrogram also shows high genomic similarities (above 90%) between isolates from distinct environments, namely *L. monocytogenes* L88, L95 and L96 (cheese making factory) with L75, L79 and L80 (supermarket), suggesting the dissemination of successful clones among diverse food-related locations.

Furthermore, antibiotic susceptibility patterns showed an overall low level of resistance with all food-related isolates being considered susceptible to ciprofloxacin, chloramphenicol, erythromycin, sulfamethoxazole-trimethoprim, tetracycline and vancomycin. Resistance was observed for ampicillin

(35%), penicillin (24%), clindamycin, gentamycin and rifampicin (3% each). These results are especially relevant due to the importance of β-lactams in the treatment of human listeriosis. According to recent reports by Lungu *et al.* (2011) and Soni *et al.* (2013), since the first report of resistant Listeria isolates, a continuous pattern of resistant isolates from water, food, processing-food environments and clinical samples has been emerging.

The presence of genes coding for known virulence factors were assessed by two multiplex-PCR reactions. Reports by Rawool *et al*. (2007) combine amplification of four genes *(actA, hlyA, iap e plcA*), while Liu *et al.* (2007) screen for the presence of another four virulence-related determinants *(inlA, inlB, inlC and inlJ)*. Results showed a variable combination of virulence genes among the isolates under analysis, demonstrating the polymorphism already attributed to these genomic regions by other authors (Jallewar *et al*., 2007; Rawool *et al.*, 2007). The frequency of virulence determinants was as follows: 38% for *iap* and *hlyA*, 35% for *inlC* and *inlJ*, 32% for *inlA*, 26% for *inlB*, 18% for *actA* and 15% for *plcA*. One of the most critical features of *Listeria* infectious process is cell invasion mediated by InlA and InlB and vacuole exit, facilitated by listeriolysin O. Since some of the studied isolates lacked amplification for these three virulence genes, this could mean they lack the ability to surpass intestinal barrier and progress with the infectious process. Nonetheless, when immune defenses are suppressed or when the infection is caused intravenously (Liu *et al.*, 2007) these strains can still maintain their pathogenic potential. Likewise, strains which lack *inlA, inlB* and *inlC* genes but produce hemolysin maintain the ability to invade host cells.

Overall, the presence of *L. monocytogenes* in food or food-processing environments represents a risk for public health safety, especially in what regards RTE food products. The presence of this foodborne pathogen in samples obtained at the cheese making factory and supermarket represent a higher risk for the consumer in general, once the sampled food products can be readily consumed. Additionally, *L. monocytogenes* representing three serogroups were isolated from the supermarket samples, including those responsible for epidemic listeriosis, pointing to the need for effective surveillance networks. Moreover, a low risk is probably associated with the *L. monocytogenes* recovered from the swine slaughterhouse, since all positive samples were obtained from food-related surfaces and the meat products which may contact with them will be cooked prior to ingestion.

Conclusion

In general, data obtained showed that the three sampling sites under study harbor food-related *L. monocytogenes* belonging to the most virulent lineages (I and II). Some of which were found to be resistant to therapeutically relevant antibiotics, thus, pointing to a health risk associated with these settings, in particular where RTE products are produced.

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