

Characterization of *Listeria monocytogenes* isolated from production lines of fresh and cold-smoked fish

M. Vaz-Velho^{1,3}, G. Duarte¹, J. McLauchlin⁴ and P. Gibbs^{1,2}

¹Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal, ²Leatherhead Food Research Association, Surrey, England, ³Escola Superior de Tecnologia e Gestão, Instituto Politécnico de Viana do Castelo, Portugal, ⁴Food Safety Microbiology Laboratory, Division of Gastrointestinal Infections, Public Health Laboratory Service Central Public Health Laboratory, London, UK

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Aims: The aims of this study were to characterize strains of *Listeria monocytogenes* isolated from cold-smoking fish plants to establish possible routes of contamination through the processing chain.

Methods and Results: *Listeria monocytogenes* from fresh fish suppliers, raw materials, factory sites and finished products isolated in Portugal (162 isolates) and England (28 isolates) were characterized by serotyping, phage typing, tetracycline, cadmium and arsenic resistance, and plasmid profiling. On the basis of serotyping and phage typing, the isolates were categorized into eight groups. Although cultures within some of the groups could be further differentiated on the basis of plasmid profiling and cadmium and arsenite typing, consideration of all typing data predominantly clustered together isolates from a single location. *L. monocytogenes* strains: from fresh salmon suppliers were not found in the processing lines; from fresh salmon from different locations differed; and from the water where salmon trout were farmed differed from those isolated from the fish samples.

Significance and Impact of the Study: No clear source or route of contamination in the cold-smoked processing chain could be established; however, these results highlight the complexity in tracking this bacterium through food chains.

INTRODUCTION

Human listeriosis is predominantly a food-borne disease caused by *Listeria monocytogenes* and although rare, has a high mortality. The disease most often affects unborn or newly delivered infants, pregnant women and the immunocompromised (Farber and Peterkin 1991). A wide range of food types has been associated with transmission, and this includes a number of small outbreaks associated with smoked fish and shellfish (Misrachi *et al.* 1991; Mitchell 1991; Ericsson *et al.* 1997; Hall *et al.* 1995; Brett *et al.* 1998; Miettinen *et al.* 1999). Although *L. monocytogenes* has been isolated from production lines of fresh to cold-smoked fish in various different countries (Farber 1991; Dillon *et al.* 1992; Gibson 1992; Ben Embarek 1994; Fuchs and

Nicolaides 1994; Jemmi and Keusch 1994; Duarte *et al.* 1995, 1999; Eklund *et al.* 1995; Rørvik *et al.* 1995; Vaz-Velho *et al.* 1998a, 1998b, 2000; Johansson *et al.* 1999), methods of controlling the bacterium within fish production environments together with the relative role of these food types in disease transmission are poorly understood. Neither the smoke temperature (below 30°C), nor the salt content (approximately 3–5% of sodium chloride in the water phase) and the storage temperatures (between 4 and 6°C), are sufficient to ensure the absence or inhibit the multiplication of *L. monocytogenes*. Hence it is important to investigate the ecology of *L. monocytogenes* within these environments to identify potential sources of contamination and to trace the spread of the organism within cold-smoked fish production plants.

Advances in typing methods for this bacterium offer powerful tools for tracking of *L. monocytogenes* in food processing plants (Bille and Rocourt 1996) and combinations of these methods offers systems of high discrimination.

However, the establishment of these typing systems requires a considerable investment in time, personnel and equipment, and are usually practicable only in national reference laboratories with a special interest in *Listeria*. McLauchlin *et al.* (1997) described simple typing methods, based on arsenic and cadmium susceptibility together with plasmid profiling, which can be utilized easily in laboratories without a specialized expertise for this bacterium.

The aim of this study was to differentiate strains of *L. monocytogenes* isolated from cold-smoking fish plants, and to further evaluate these simple typing methods by comparison with the results obtained from serotyping and phage typing. This information may allow the tracking of this organism within cold-smoking fish plants and may assist in the identification of areas where control measures can be applied.

MATERIALS AND METHODS

A total of 190 *L. monocytogenes* isolates from Portuguese and English production lines of fresh to cold-smoked fish were tested in this study. One hundred and sixty-two *L. monocytogenes* isolates recovered from environmental and fish samples (salmon, salmon trout, swordfish and tuna) collected during a 3-year period in three Portuguese factories in different locations were analysed (Duarte *et al.* 1995, 1999; Vaz-Velho *et al.* 1998a, 2000, 2001). Fish and environmental samples were analysed, after and just prior to each step of the processing chain within each of the plants, together with fresh fish and environmental samples from the salmon trout and salmon suppliers. Two different isolation procedures for *L. monocytogenes* were used independently for the Portuguese survey. The first comprised a Bactometer capacitance-based method (Rodrigues *et al.* 1995; Capell *et al.* 1995) followed by the conventional procedure (Jemmi and Keusch 1994). The second used the ISO 11290-1 protocol together with mini-VIDAS *L. monocytogenes* immunoassay (BioMérieux, BioMérieux Vitek, Inc., MI, USA) together with enhanced haemolysis agar (Beumer *et al.* 1997). Twenty-five *L. monocytogenes* isolates recovered from cold-smoked and fresh salmon trout samples, collected in a single sampling from a smoking plant and its fresh fish supplier in England, were also analysed. The strains were isolated following the ISO 11290-1 protocol.

All *L. monocytogenes* cultures were stored on Lab-Lemco agar slopes (Oxoid CM17, Unipath Limited, Basingstoke, UK) in the dark at room temperature, and were identified by: Gram stain; catalase test; oxidase test; tumbling motility; API *Listeria* (BioMérieux 10300); CAMP test with *Staphylococcus aureus* (ATCC 25923, Food Quality Centre, Escola Superior de Biotecnologia, Porto, Portugal) and *Rhodococcus equi* (NCTC 1691, Leatherhead Food Research Association-LFRA, Leatherhead, Surrey, UK) on sheep

blood agar plates (BioMérieux); and haemolysis on horse blood agar (McLauchlin 1997a).

All isolates were further characterized in the Food Safety Microbiology Laboratory in London. Cultures were sero-typed using in-house produced antisera and phage typed as described previously (Seeliger and Höhne 1979; Rocourt *et al.* 1985; McLauchlin *et al.* 1986; McLauchlin and Shah 1992a; McLauchlin 1996a). Resistance to cadmium and arsenic was determined as described previously (McLauchlin *et al.* 1997). Resistance to tetracycline was determined using a similar method by using the overnight growth from a Nutrient Broth (Oxoid) inoculated onto an Isosensitest agar plate (Oxoid) containing 8 µg ml⁻¹ of tetracycline-HCl (Sigma T3383, Aldrich, Poole, Dorset, UK). Growth was compared with an Isosensitest plate without tetracycline after overnight incubation at 37°C. Plasmid DNA profiling was performed on 46 Portuguese and 10 English selected isolates using the method described by McLauchlin *et al.* (1997). Control strains for the cadmium, arsenic, tetracycline and plasmid analysis were included in each batch of tests.

RESULTS

Results of characterization of 162 Portuguese and 28 English *L. monocytogenes* isolates are shown in Tables 1 and 2. All isolates were confirmed as *L. monocytogenes*, and 184 (97%) gave an API *Listeria* profile as 6510, two isolates in group 1 and one in group 3 gave profiles of 6410 (D-arabitol negative), and three in group 2 gave profiles of 2510 (mannosidase negative). To evaluate their reproducibility, the unusual isolates – 6410 and 2510 API profiles – were tested again and the same biochemical profiles were obtained. All isolates were haemolytic on horse blood agar and CAMP test positive with *S. aureus* but not with *R. equi* except for all 10 isolates in group 2: all 10 isolates were non-haemolytic on horse blood agar and CAMP negative except for one culture which was CAMP positive with *S. aureus*. On the basis of serotyping and phage typing, the isolates were categorized into eight groups (designated 1–8; Tables 1 and 2), two of which were serovar 1/2a, one 1/2b, three 1/2c, 1 serovar 4b and one serogroup 4 but not 4b. Groups 1–6 occurred at 64%, 6%, 6%, 2%, 6% and 16%, respectively, among the 162 Portuguese isolates and groups 7 and 8 at 11% and 89%, respectively, among the 28 English isolates.

Plasmid profiling was performed on 56 (29%) of the 190 cultures, and a single large plasmid of 50–60 Md was detected in 20 of the isolates. Nineteen of the 20 cultures where plasmid DNA was detected were also resistant to cadmium (Tables 1 and 2). Plasmid profiles were identical within representatives from five of the six groups tested (no isolates from groups 5 and 6 were examined). Seven of the

Table 1 Characterization of 162 *L. monocytogenes* isolated from cold-smoking fish plants and associated sites in Portugal

Group	Plasmid DNA detected/Total number tested	Source				
		Plant A	Plant B	Plant C	Trout farm 2	Salmon importer
1. Serovar 4b						
Phage type A						
a. Cd ^r As ^r	0/14	80	—	3	1*	—
b. Cd ^s As ^r	0/4	5	—	1	—	—
c. Cd ^s As ^s	0/8	—	—	8	6+*	—
2. Serogroup 4						
Non-phage typable						
a. Cd ^r As ^s	6/9	4	5†	—	—	—
b. Cd ^s As ^r	1/1	1	—	—	—	—
3. Serovar 1/2b						
Phage type B						
a. Cd ^s As ^r	0/1	—	1	—	—	—
b. Cd ^s As ^s	0/6	—	9	—	—	—
4. Serovar 1/2c						
Phage type D						
Cd ^r As ^s	3/3	—	3	—	—	—
5. Serovar 1/2a						
Phage type E						
Cd ^r As ^s	—	9***	—	—	—	—
6. Serovar 1/2a						
Non-phage typable						
Cd ^s As ^s	—	—	—	8**	18*****	—
Total number of isolates	10/46	90	27	12	15	18

Phage types indistinguishable from the following reactions: A–1444, 1317, 3274; B–46, 1652, 351; D–21; E–575, 881, 1652 1967.

†Plasmid DNA detected in all isolates;

*from fresh salmon trout;

**from the dam water;

***from fresh salmon from Faroe Islands;

****from fresh salmon from Norway.

Cd^r = resistant to cadmium chloride; Cd^s = sensitive to cadmium chloride; As^r = resistant to sodium arsenite; As^s = sensitive to sodium arsenite.

10 isolates tested from group 2 contained plasmid DNA: 2/5 and 5/5 isolates from Plants A and B, respectively, contained plasmid DNA. All 190 isolates were sensitive to tetracycline.

In 34 of 36 samples where more than one isolate was recovered using different media, the same serotype and phage-type profiles were obtained. In one sample groups 1a and 2a were simultaneously recovered; in another sample groups 1a, 2a and 2b were simultaneously recovered. In seven Portuguese samples and in one English sample, either groups 1a and 1b, 3a and 3b or 7a and 7b were detected simultaneously.

Within five of the eight groups identified by serotyping and phage typing, patterns of cadmium and arsenite

resistance differentiated different types. However, isolates from a single location, already clustered together by serotyping and phage typing, were usually clustered together in a predominant group by arsenite and cadmium susceptibilities. For example, most of the group 1 isolates from Plant A were clustered together, as were those in group 3 from Plant B, group 6 from the salmon importer and group 7 from the cold smoked trout.

Consideration of the distribution of *L. monocytogenes* types from the smoking plants, trout farm and salmon importer, as well as the frequencies of types found (Tables 1 and 2) may indicate the probable source of contamination. Isolates within group 1 were the predominant strain recovered from fresh fish, processed fish and environmental

Table 2 Characterization of 28 *L. monocytogenes* isolated from cold-smoked and fresh trout in England

Group (based on serotype and phage type)	Plasmid DNA detected/ total number tested	Source	
		Fresh trout	Cold smoked trout
7. Phage type C			
a. Cd ^r As ^r	7/7	–	22
b. Cd ^s As ^r		–	3
8. Serovar 1/2c			
Non-phage typable			
a. Cd ^r As ^r	2/2	2	–
b. Cd ^r As ^s	1/1	1	–
Total number of isolates	10/10	3	25

Phage type C, indistinguishable from reactions with phages 10, 43 (21), 19, 387 1967 (4477), 575, 12029 (881). Figures in parenthesis indicate weak reactions. Cd^r = resistant to cadmium chloride; Cd^s = sensitive to cadmium chloride; As^r = resistant to sodium arsenite; As^s = sensitive to sodium arsenite.

samples collected in Plants A and C, and from two fresh salmon trout samples collected in trout farm 2. This is consistent with salmon trout from farm 2 as the original or continued source of *L. monocytogenes* contamination into smoking plants A and C. Plant C, however, did not process cold-smoked salmon trout, but used small salmon trout for hot smoking from the same farm. Plant B, despite processing raw material from farm 2, was not contaminated by group 1 isolates.

Group 2 isolates were recovered from salmon and salmon trout samples after filleting and from salmon samples after washing and after smoking in plant A, and from salmon samples after filleting and from knives used for filleting collected in plant B. Since these types were not isolated from the raw material, the source of contamination was not known. Isolates within groups 3 and 4 were recovered from one wooden working surface and from a sample of frozen swordfish, both from plant B, indicating the raw swordfish as a probable source of contamination. Group 5 isolates were recovered on a single occasion from fresh salmon in Plant B, and this salmon was from a different origin (the Faroe Islands) to that most usually received from the Norwegian supplier to this smoking factory.

Isolates of *L. monocytogenes* group 6 were recovered from the water where the salmon trout was farmed and from fresh salmon from Norway, but since isolates of this group were not recovered from any of the plants, these can be excluded as a source of contamination.

The isolates from England were categorized into two groups, both of which differed from those recovered in Portugal (Tables 1 and 2). All isolates from cold smoked

trout (group 7) differed from those recovered from the fresh trout (group 8) supplied to this factory (Table 2).

DISCUSSION

The recovery of *L. monocytogenes* from fish and shellfish products in a wide variety of countries highlights the continuing problematic nature of contamination of these food types (especially smoked fish and shellfish) by this bacterium (Rørvik and Yndestad 1991; Dillon and Patel 1992; Dillon *et al.* 1992; Ben Embarek 1994; Fuchs and Nicolaides 1994; Jemmi and Keusch 1994; McLauchlin and Nichols 1994; Arnold and Coble 1995; Duarte *et al.* 1995, 1999; Rørvik *et al.* 1995; Destro *et al.* 1996; Boerlin *et al.* 1997, 1998b; Vaz-Velho *et al.* 1998a; Jørgensen and Huss 1998; Johansson *et al.* 1999). In the United States, seafood was either the largest (30% of recalls during 1991–2) or second largest (12% of recalls during 1994–8) food-type involved with recalls because of microbiological contamination: during both periods (1991–2 and 1994–8) *L. monocytogenes* was the most common microbial contaminant responsible for recalls (Venugopal *et al.* 1996; Wong *et al.* 2000). In addition, cases of listeriosis have been implicated with consumption of seafood products (Misrachi *et al.* 1991; Mitchell 1991; Ericsson *et al.* 1997; Hall *et al.* 1995; Brett *et al.* 1998; Miettinen *et al.* 1999). Hence the presence of *L. monocytogenes* in processed fish and shellfish, together with an understanding of the sources and routes of contamination, is of worldwide concern to those involved with food production, retailing and regulation, as well as public health microbiologists. The application of epidemiological typing systems for *L. monocytogenes* is essential for such an understanding.

Apart from a small proportion of isolates of *L. monocytogenes* (for example those in this study from group 2), this bacterium is remarkably uniform in its biochemical characteristics (McLauchlin 1997a), and hence biotyping is not useful for analysis of this bacterium. There is, however, a range of discriminatory epidemiological typing systems available for *L. monocytogenes*, and similarly to previous studies, of all the methods used here phage typing was the most discriminatory (Bille and Rocourt 1996; McLauchlin *et al.* 1996). In this study phage typing identified eight groups, two within serovar 1/2a, one within serogroup 1/2b, three within serovar 1/2c and two within serogroup 4. Although cultures from within some of the groups could be differentiated further on the basis of plasmid profiling and cadmium and arsenite typing, consideration of all typing data predominantly clustered isolates together from a single location (see below).

The use of cadmium and arsenite sensitivities allows the recognition of only four different 'types'; however, it was suggested previously that this (together with plasmid

analysis) would provide a useful screening test for the analysis of large numbers of cultures (McLauchlin *et al.* 1997). This is particularly important since samples from foods or the environment can be contaminated simultaneously by multiple strains of *L. monocytogenes* (McLauchlin and Shah 1992b; Danielsson-Tham *et al.* 1993; Loncarevic *et al.* 1996). Consideration of results from plasmid profiling and cadmium and arsenite typing alone would have, at least in part, identified the major groups from each location. For example, these data alone would have allowed the conclusion that Plants A and C were predominantly colonized by a single *L. monocytogenes* type, and that a more heterogeneous mixture of strains occurred in Plant B. This study illustrates the utility of these simple typing methods, which can be established easily in laboratories without a specialist interest in this bacterium.

McLauchlin *et al.* (1997) reported previously that the resistance to cadmium and arsenic and the presence of plasmid DNA varied markedly with the serotype, and that plasmid DNA was strongly associated with cadmium resistance in serogroup 1/2 cultures but not within those of serogroup 4. The results of this study, predominantly using isolates of *L. monocytogenes* from a different country to that described previously, are consistent with these observations.

The occurrence of *L. monocytogenes* in smoked fish products results either from contamination of the raw material that is not eradicated during processing, or from cross-contamination during processing from raw materials or from sites and equipment within the factory. Evidence from outbreaks of listeriosis has highlighted the importance of contamination of foods from factory sites by *L. monocytogenes* (Farber and Peterkin 1991; McLauchlin 1996b). Analysis of *L. monocytogenes* in smoked fish factories may, however, be more complicated than some other foods since the process may not contain listericidal steps to eradicate the organism from the raw product. Hence foods may be contaminated as a result of the original microflora of their constituents as well as from contact with their manufacturing environment. This is consistent with results from other studies indicating contamination by multiple strains of *L. monocytogenes* of fish smoking plants (Jemmi and Keusch 1994; Rørvik *et al.* 1995; Autio *et al.* 1999; Johansson *et al.* 1999) and a shrimp processing plant (Destro *et al.* 1996). The results obtained here indicated that samples of fresh fish, processed fish and environmental sites from Plants A and C were contaminated predominantly by a single strain of *L. monocytogenes*, suggesting a single source of contamination which was further cross-contaminated between products within the factories. This observation may indicate raw material as the initial source of contamination, and indeed isolates of *L. monocytogenes* indistinguishable from those in Plants A and C were recovered from salmon trout from farm 2. However, Plant C did not process salmon trout, but used small rainbow

trout for hot smoking purposes, albeit that these came from the same source (farm 2). However, some of the raw materials can be excluded as the source of contamination in Plants A and C since the *L. monocytogenes* strains isolated from the fresh salmon at the salmon importer and the dam water from trout farm 2 were never found among isolates from these factories. Isolates from Plant B, however, comprised a heterogeneous mixture of at least four *L. monocytogenes* strains, which differed from almost all of those recovered from Plants A and C as well as those from the trout farm and salmon importer. The recovery of at least four *L. monocytogenes* strains from this factory suggests either simultaneous contamination of multiple factory sites by different strains, or contamination from multiple source materials. This is consistent with the recovery of indistinguishable isolates from: freshly filleted salmon and the filleting knives (group 2); from wooden working surfaces and swordfish (groups 3 and 4); and fresh salmon from the Faroe Islands (group 5). With respect to the isolates from England, the differences between those from cold smoked trout and the fresh trout can exclude this raw material as the source of contamination.

All isolates from England differed from those recovered in Portugal, and this further highlights the lack of an association between any food type or environmental source and specific strains of *L. monocytogenes* (Farber and Peterkin 1991; McLauchlin 1997b).

From the typing results described here on isolates of *L. monocytogenes* over a 3-year time period, it was not possible to identify the exact contamination routes of *L. monocytogenes* in these particular cold-smoked processing chains. However, it was clear that the plants investigated had a different microbial ecology and that the *L. monocytogenes* isolated from two European countries were markedly different. Further surveys on the occurrence of *L. monocytogenes* along the cold-smoked fish processing chain, as well as the detailed characterization of the strains, using phenotypic and molecular typing systems are likely to assist in the identification of the sources of contamination which will allow the establishment of effective controls over the presence of *L. monocytogenes* in the final product.

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