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Short communication

Characterization of *Listeria monocytogenes* and *Listeria innocua* from a vegetable processing plant by RAPD and REA

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Abstract

The incidence of *Listeria monocytogenes* in a vegetable processing plant was investigated over a 23-month period. Frozen ready-to-eat vegetable samples, well as the plant environment, were sampled. The molecular subtyping techniques, Random Amplified Polymorphic DNA (RAPD) and Restriction Endonuclease Analyses (REA), were performed to help investigate the origin and routes of *Listeria* dissemination.

The low and sporadic incidence of *L. monocytogenes* made it impossible to establish an epidemiological sequence in the processing plant, though a case of cross-contamination between tomato and ratatouille was detected. *Listeria innocua* subtyping, however, allowed us to determine the prevalence of several strains in vegetables, and their presence on machinery samples suggested the possibility of cross-contamination during processing.

The low incidence of *L. monocytogenes* indicated that the risk of listeriosis transmission by vegetable consumption is low. On the other hand, the isolation of the same strain of *L. innocua* in several surveys pointed out the risk of colonisation on surfaces and machinery. The persistence of *Listeria* spp. is a cause for concern as can lead to future contamination of vegetables processed in the plant and to a possible increased risk for health. Therefore, periodic controls for the presence of *Listeria* spp. and a further review of the cleaning and disinfection procedures used in frozen vegetable plants are recommended.

Keywords: *Listeria monocytogenes*; *Listeria innocua*; RAPD; REA; Subtyping frozen vegetables

1. Introduction

The last few decades have seen an increasing interest in the foodborne pathogen *Listeria monocytogenes*. Listeriosis is considered a serious health problem due to the severity of symptoms and its high mortality rate. Listeriosis outbreaks that have occurred in the last years have highlighted contaminated food as the main source of transmission (Farber and Peterkin, 1991). A variety of food products have been involved in these outbreaks, including soft cheese (Linnan et al., 1988; Bille, 1990) and cooked meat products (McLauchlin, 1991; Tjomb, 1993; Anonymous, 1999; Valk et al., 2000). These are considered of special risk due to the ability of *Listeria* to grow and survive in them. However, there are other products, traditionally considered of low risk, which have recently been linked to listeriosis transmission, e.g. the large listeriosis outbreak reported in Italy by

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Aureli et al. (2000) due to the consumption of corn. Though no fatalities occurred, more than 1500 people were affected.

The risks of acquiring foodborne listeriosis has obliged food producers and distributors to adhere to strict hygienic control measures to minimize contamination by *L. monocytogenes*. Although preventative measures by the USA have been effective in reducing the number of listeriosis cases in that country (Tappero et al., 1995), the production of food free of *L. monocytogenes* is still unrealistic, particularly for certain foods. Part of this difficulty stems from the ubiquitous nature of this organism and the possibility of cross-contamination between one or several products during processing.

Venables (1989) remarked on the difficulties in eliminating *Listeria* due to its ability to colonise surfaces by forming a biofilm that remains attached to equipment surfaces (Wong, 1998; Arizcun et al., 1997).

Though on several occasions vegetable consumption has been linked to listeriosis outbreaks (Schlech et al., 1983; Ho et al., 1986; Allerberger and Guggenbichler, 1989), few studies have been done to investigate the incidence of *L. monocytogenes* in this kind of food. Incidence rates reported in these studies have been, in general, below 10% (Brackett, 1999), despite the ubiquity of this microorganism, and the fact that vegetables seem to be a good substrate for growth of *L. monocytogenes* (Farber et al., 1998).

This study focuses on the incidence of *Listeria* in a vegetable processing plant by monthly analyses of final product and regular testing of the processing plant and equipment. Molecular subtyping of *Listeria* isolated was carried out to elucidate the possible sources of contamination and routes of spread of the bacteria.

2. Materials and methods

2.1. Sampling

The occurrence of *Listeria* spp. was investigated in a total of 906 samples of frozen vegetables, as final product, from a processing plant. Samples were collected monthly, from November 1997 to September 1999, and kept frozen until the analyses were performed.

During the last 9 months of the study, the presence of *Listeria* spp. was also investigated in machinery and on floors of the processing plant. Samples were taken by swabbing the surfaces with a sterile swab moistened with saline and transporting swabs at refrigeration temperature to the laboratory where analyses were performed within 24 h.

2.2. Isolation and identification of Listeria species

The analysis of samples was carried out following method NF V08-055, a simplified version, adopted by the French normative, of procedure EN ISO 11290-1 (Anonymous, 1997). A 25-g sample was homogenized in 225 ml of Fraser 1 broth (Oxoid, Madrid, Spain) and incubated at 30 ± 1 °C for 24 h. Next, 0.1 ml of the incubated broth was transferred to 10 ml of Fraser 2 broth (Oxoid) and incubated at 30 ± 1 °C. After 48 h of incubation, broth was streaked for isolation on Palcam agar plates (Merck, Madrid, Spain) at 37 ± 1 °C for 24–48 h.

Suspect colonies were classified to the species level using morphological characteristics and biochemical tests (motility, catalase, xylose, rhamnose, hemolysis and CAMP).

2.3. Serotyping

Serotyping was carried out using commercial specific antisera (Denka Seiken, Tokyo, Japan) following the manufacturer's instructions. Agglutination patterns were linked to *Listeria* serotypes following the criteria established by Seeliger and Höhne (1979).

2.4. RAPD

This technique was performed as previously described (Wernars et al., 1996), with minor modifications. Briefly, the reaction mixtures were prepared in 10 Mm Tris–HCl, pH 8.3; 50 Mm KCl; 1.2 Mm $MgCl_2$; and contained 100 μM of each dATP, dTTP, dCTP and dGTP; 0.3 μM primer and 0.625 units of Amplitaq® DNA Polymerase (Perkin-Elmer). The bacterial suspension was calibrated by spectrophotometry (A_{600}) and diluted to a final concentration of $7.5 \pm 0.5.10^6$ CFU/ml in the 25-μl reaction mixture (Mazurier and Wernars, 1992). The amplification reactions were carried out in a Perkin-Elmer Cetus

2400® Thermal Cycler following a 45-cycle program: a first cycle at 94 °C/4 min; 39 °C/45 s; 72 °C/1 min, followed by 43 cycles at 94 °C/15 s; 39 °C/45 s; 72 °C/1 min, and finally one cycle at 94 °C/15 s; 39 °C/45 s; 72 °C/10 min.

Primers HLWL74 (5′-ACGTATCTGC-3′), HLWL85 (5′-ACAACTGCTC-3′) and OMP-01 (5′-GTTGGTGGCT-3′) were employed for all *L. monocytogenes* subtyping. The different profiles produced with each primer were named with capital letters and, by combining all of them, the RAPD subtypes were obtained and designed as LmI to LmVI.

Only primer OMP-01 was employed in *Listeria innocua* subtyping, as in previous studies, it was shown to be most discriminatory for this species (data not shown). Subtypes obtained were designated LiI to LiIV.

In both cases, randomly selected strains were analysed twice and controls were added in all the reactions to ensure reproducibility.

2.5. REA

Microrestriction patterns of genomic DNA were also studied for all *L. monocytogenes* strains isolated. DNA was extracted following the procedure of Moyra et al. (1996). Approximately 10 μg of bacterial DNA was digested with 20 units of *Cfo*I (Roche Diagnostics, Barcelona, Spain) in a total volume of 20 μl at 37 °C overnight. The fragments obtained were separated by electrophoresis in agarose gel 0.8% at 44V/cm for 14 h. A control strain was included in every gel to help pattern normalization using the software Gel Compar (Applied Maths, Kortrijk, Belgium).

The comparison was established by the band patterns observed in the region between 23.1 and 3.1 kb, and a single band difference was considered enough to distinguish two strains (Gerner-Smidt et al., 1996). Using these criteria, REA subtypes design at i to vi, were defined.

2.6. Discrimination index

The discriminatory power of the subtyping methods employed in this study was calculated according to Hunter (1990), as the discrimination index at the 95% confidence level (D_{95}).

3. Results

3.1. Incidence of Listeria species in vegetables and surface samples

The incidence of *L. monocytogenes* in frozen vegetables was 1.2% (Table 1), with 11 strains being isolated from the 906 samples collected over the 23-month survey. The pathogen was mainly found in green beans and tomato products. Isolated samples of cauliflower, peas and artichoke were also contaminated, while broccoli, carrot and spinach samples did not contain the organism. *L. innocua* was detected in a total of 77 (8.5%) samples, while other non-pathogenic *Listeria* species were also isolated in three of the samples tested.

During the last 9 months of this study, a total of 11 (6.6%) *L. innocua* isolates were identified in samples taken at different points of the processing chain, such as washing tunnels, food conveyor belts and floors. As for the vegetable samples, the incidence of *L. monocytogenes* in the plant environment was lower than that of *L. innocua* (Table 1).

3.2. Subtyping of the Listeria isolates

The distribution of subtypes of *Listeria* strains, isolated from frozen vegetables and from environ-

Table 1
Incidence of *Listeria* species in different types of samples

Source	No. of samples	Samples positive (%)			
		L. monocytogenes	*L. innocua*	*L. welshimeri*	*L. seeligeri*
Frozen vegetables	906	11 (1.21)	77 (8.49)	2 (0.22)	1 (0.11)
Processing machinery	166	2 (1.20)	11 (6.62)	–	–
Total	1072	13 (1.21)	88 (8.20)	2 (0.18)	1 (0.09)

Table 2
Listeria subtypes isolated from frozen vegetables

Sampling distribution[a]		No. of samples	*L. monocytogenes*							*L. innocua*	
			No. strains	Serotype	HLWL74	HLWL85	OMP-01	RAPD[b]	REA[c]	No. strains	RAPD[d]
1997	4th	27	–							5	LiII
1998	1st	164	–							11	LiI ($n=6$), LiII ($n=5$)
	2nd	240	1	1/2a	A	B	B	LmI	iii	38	LiI ($n=31$), LiII ($n=2$), LiIII ($n=5$)
	3rd	95	4	1/2a	B	A	C	LmII ($n=1$)	iv	8	LiI ($n=7$), LiIV ($n=1$)
					A	A	A	LmIII ($n=3$)	i		
	4th	107	1	1/2a	C	A	D	LmIV	v	7	LiI ($n=2$), LiII ($n=5$)
1999	1st	124	1	1/2b	D	C	B	LmV	vi	8	LiI
	2nd	95	3	1/2a	A	A	A	LmIII ($n=2$)	i	–	
					E	A	E	LmVI ($n=1$)	ii		
	3rd	54	1	1/2a	A	A	A	LmIII	i	–	

[a] Year and quarter.
[b] RAPD subtypes obtained after using three different primers.
[c] REA subtypes.
[d] RAPD subtypes obtained by using only primer OMP-01.

mental samples surveyed in the same processing plant, is shown in Tables 2 and 3, respectively. Most *L. monocytogenes* strains isolated from frozen vegetables belonged to serotype 1/2a ($n=10$) while one, from cauliflower, was found to be a serotype 1/2b. RAPD analysis, revealed the presence of six different subtypes, one subtype (LmIII) comprising six strains (all of them from green beans or tomato products), while the other five subtypes were isolated on only one occasion from unrelated products. Significantly, the two *L. monocytogenes* strains from equipment samples belonged to the most common subtype LmIII. Using REA, *L. monocytogenes* isolates were also classified in six subtypes, correlating with the RAPD results (Tables 2 and 3).

Two major RAPD patterns were found after RAPD-typing *L. innocua* using primer OMP-01, of which LiI accounted for 70% (54/77) of the isolates from vegetables (Table 2) and 100% (11/11) of machinery samples (Table 3). Of the remaining food isolates, 17 (22%) belonged to RAPD type LiII, and six (7.8%) to two minor subtypes, LiIII and LiIV. Pattern LiIII was found in strains isolated from five samples of a single product (minimally processed tomato) on the same date.

The D_{95} was calculated as the discrimination index for 95% confidence, according to Hunter (1990). For primers HLWL74, HLWL85 and OMP-01 used alone, the D_{95} was 0.62, 0.34 and 0.71, respectively. Using all three primers, the D_{95} was calculated to be 0.72

Table 3
Listeria subtypes isolated from machinery samples of the vegetables processing plant

Sampling distribution[a]		No. of samples	*L. monocytogenes*							*L. innocua*	
			No strains	Serotype	HLWL74	HLWL85	OMP-01	RAPD[b]	REA[c]	No. strains	RAPD[d]
1999	1st	15	–							5	LiI
	2nd	119	2	1/2a	A	A	A	LmIII	i	3	LiI
	3rd	32	–							3	LiI

[a] Year and quarter.
[b] RAPD subtypes obtained after using three different primers.
[c] REA subtypes.
[d] RAPD subtypes obtained by using only primer OMP-01.

(one difference), 0.72 (two differences) and 0.22 (for three differences). For REA, this index was calculated to be 0.72; concordance between RAPD and REA was 100%.

The D_{95} for *L. innocua* from vegetables was 0.46 after RAPD with primer OMP-01 and zero for the strains isolated from the plant.

4. Discussion

The incidence of *L. monocytogenes* from frozen vegetables in this study has been scarce with the pathogen being detected in only 1.2% of samples tested. Previous studies on the incidence of *Listeria* in vegetables focussed on fresh-cut unprocessed or minimally processed vegetables and ready-to-eat salads. While Petran et al. (1988) reported to not find any *L. monocytogenes* in samples of fresh and frozen vegetables analysed, other investigators have found incidence rates of 5% (Hitchins, 1996), 7.8% (De Simón et al., 1992) and 82% (Harvey and Gilmour, 1993). The incidence of *L. innocua* was more infrequent than *L. monocytogenes* in the latter two studies, in contrast with our results in which the presence *L. innocua* was more prevalent. Although most authors have reported on incidence rate of *L. monocytogenes* below 10% in vegetables, substantial differences were found by Heisick et al. (1989) depending on the type of vegetable involved. In their study, up to 21% of potato samples was found to be contaminated with the pathogen, while *L. monocytogenes* was undetected in other produce such as cauliflower, tomatoes, carrots and lettuce.

With regards to the processing plant, the presence of the pathogen in the plant environment leads to the possibility that the organism was introduced into the plant from contaminated vegetables. Few studies are available on environmental contamination of vegetables with *Listeria*; however, Cox et al. (1989) found an incidence of 3% of *L. monocytogenes* in a potato-processing plant. In the latter study, the occurrence of *Listeria spp* in the plant environment was 47%, with most of the bacteria being detected in stagnant water on floors.

The low number of *L. monocytogenes* strains isolated over the 23-month survey made it difficult to trace the contamination route of this microorganism in the plant. In fact, six different patterns were found among the 11 strains investigated by RAPD and REA. Only RAPD subtype LmIII, corresponding to REA subtype *i*, was consistently observed by six isolates from three of the samplings carried out. Three of the isolates come from green bean samples analysed on the same day, which leads us to postulate a possible contamination route. The prevalence of particular *L. monocytogenes* subtypes in a particular type of food, namely smoked salmon, has been previously described (Aguado et al., 2001). Finally, the last two isolates belonging to the prevalent subtype, i.e., RAPD LmIII, REA *i*, were found on tomato and ratatouille processed on the same day.

Although tomatoes have not been reported to be a good growth substrate for *L. monocytogenes* at temperatures ranging from 5 to 15 °C, the pathogen can remain viable on this product for periods beyond its shelf-life (Beuchat and Brackett, 1991). The presence of the same subtype in two different tomato products (chopped tomato and ratatouille) and in the machinery samples during the same survey suggests to the processing plant was the most likely source of this strain. The abundance of a particular subtype in vegetable products may be attributable to at least three mutually non-exclusive causes: (i) an abundance of this strain in a particular vegetable; (ii) its resistance to the cleaning and disinfection procedures employed; and (iii) an unusually high ability to produce biofilms. *L. innocua* was a useful indicator to determine how widespread the contamination was in the processing plant, as throughout the survey, it was isolated more frequently than *L. monocytogenes*. Previous studies have pointed out the usefulness of *L. innocua* as an indicator of contamination by *L. monocytogenes* (Greenwood et al., 1991; Gravani, 1999). The fact that two major subtypes could be detected in several samplings throughout this study indicates that these could be prevalent strains in vegetables, or that the survival of the bacteria on the processing equipment is still possible despite the daily cleaning carried out. Fenlon et al. (1996) demonstrated that contamination of the raw material is rare, and that there is a considerable rise in contamination in the final product, supporting the notion that the dissemination of *Listeria* takes place during processing.

In summary, we can conclude that the incidence of *L. monocytogenes* was low. However, concurrent iso-

lation of non-pathogenic *Listeria* species can be considered as a warning indicator, which reinforces the necessity for corrective measures to avoid contamination of the plant and processing machinery. Following these results, a programme of periodic testing of critical control points in the processing plant was established. The characterization of the strains isolated was also recommended in order to identify the sources of contamination and dissemination routes.

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