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Listeria monocytogenes in pork slaughtering and cutting plants use of RAPD, PFGE and PCR–REA for tracing and molecular epidemiology

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Abstract

In order to determine the origin of pork cuts contamination by *Listeria monocytogenes*, 287 isolates, collected from five French pork slaughtering and cutting plants, from live pigs to pork cuts, were characterised using three molecular typing methods: random amplification of polymorphic DNA (RAPD) carried out with five different primers, genomic macrorestriction using *ApaI* with pulsed-field gel electrophoresis (PFGE) and a PCR–restriction enzyme analysis (PCR–REA) based on the polymorphism existing within the *inl*A and *inl*B genes. Results obtained from RAPD and PFGE were closely related and distinguished respectively 17 RAPD types (*r*1–*r*17) and 17 PFGE types (*a*1–*a*17) among the 287 isolates, whereas the PCR–REA analysis only yielded two profiles (*p*1 and *p*2). Considering the combined results obtained with the three molecular typing methods, 19 *Listeria monocytogenes* genotypes (1–19) were distinguished. Serotyping led at least four serotypes being distinguished: 1/2a, 3a, 1/2c and 3c. The application of genotyping identified the predominance of a *Listeria monocytogenes* strain of type (1) and other very closely related ones (5, 9, 10, 12, 13, 14, 16 and 19) which were present on pork as well as in the environment within the five investigated plants. This study also pointed out the presence of these closely related *Listeria monocytogenes* strains over a 1-year period in the environments of two plants, even after cleaning and disinfection procedures. This highlights the possibility for some *Listeria monocytogenes* strains to persist in pork processing environments and raises the problem of the efficiency of cleaning and disinfection procedures used in pork slaughterhouses, chilling and cutting rooms.

Keywords: *Listeria monocytogenes*; Genotyping; Traceability; Pork; Slaughterhouses and cutting processing plants

1. Introduction

Listeria monocytogenes, established as a food-

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borne pathogen since the early 1980s (Schlech et al., 1983), has become of major concern for public health authorities and food industry. Listeriosis, mainly caused by the consumption of contaminated foodstuffs, can occur as a sporadic disease or as part of an outbreak. It may result in very severe manifestations for human health, with a high mortality rate, especially for pregnant women, neonates, immuno-compromised people and the elderly. Moreover, the contamination of raw or processed food products by this micro-organism may result in drastic economic losses for the food industry.

Recent events in Europe have shown that pork meat and processed pork products, such as delicatessen, can be sources of listeriosis, as occurred in France in 1992 and 1993 (Jacquet et al., 1995; Goulet et al., 1998) and in other European countries (Jay, 1996; Loncarevic et al., 1997). *Listeria monocytogenes* has been found in every part along the pork processing industry. With a reduced prevalence, *Listeria monocytogenes* can be isolated at the farm level either from faeces and the skin of pigs, which may be healthy carriers and harbour *Listeria monocytogenes* in the intestine and tonsils, or from pig herds' environment with respect to the telluric origin of the bacterium (Skovgaard and Nfrrung, 1989; Adesiyun and Krishnan, 1995). The incidence of this micro-organism increases when going further in the pork processing industry, from the slaughterhouse to the cutting room (Boerlin and Piffaretti, 1991; Van der Elzen and Snijders, 1993; Nesbakken et al., 1996). The delicatessen industry also acts as a dissemination factor of *Listeria monocytogenes* (Salvat et al., 1995; Jay, 1996). Several studies have raised the fact that the first major amplification source of pork products contamination with *Listeria monocytogenes* might be the cross contaminations which occur within the slaughterhouses, and more precisely, in the environment of the chilling and cutting rooms (Grahan and Collins, 1991; Van der Elzen and Snijders, 1993; Wendtland and Bergann, 1994; Nesbakken et al., 1996). This hypothesis is enhanced by the psychrotrophic nature of this micro-organism and its ability to attach to various surfaces (Mafu et al., 1990a) and survive on these surfaces (Helke and Wong, 1994). Hence, *Listeria monocytogenes* is able to colonize working contact surfaces forming biofilms (Spurlock and Zottola, 1991; Ronner and Wong, 1993; Blackman and Frank, 1996), representing a source of potential contamination for food products (Cox et al., 1989; Sammarco et al., 1997).

During the last decade, powerful bacterial molecular typing methods have been developed. These techniques, which usually exhibit a higher discriminatory power than phenotyping methods, such as serotyping, are useful to assess the distribution of *Listeria monocytogenes* strains within various food processing environments (Lawrence and Gilmour, 1995; Destro et al., 1996; Ojeniyi et al., 1996; Unnerstad et al., 1996). Especially, genomic fingerprinting via random amplification of polymorphic DNA (RAPD) (Mazurier and Wernars, 1992; Farber and Addison, 1994; Kerr et al., 1995; Lawrence and Gilmour, 1995; Wagner et al., 1996) and genomic macrorestriction using rare cutting endonucleases such as *Sma*I or *Apa*I, followed by pulsed-field gel electrophoresis (PFGE) (Brosch et al., 1991; Buchrieser et al., 1991; Buchrieser et al., 1993) are considered as powerful typing methods for studying sets of epidemiologically related *Listeria monocytogenes* strains belonging to identical or different serovars.

The aim of this study was to extend the knowledge about the origin of *Listeria monocytogenes* present on pork cuts by tracing the bacterium within pork processing environments. For this purpose, 287 isolates of *Listeria monocytogenes* collected in 1995 and 1996 from five pork slaughtering and cutting plants, from live pigs entering the slaughterhouses to pork cuts, were characterised using three molecular typing methods: RAPD carried out with five different primers, *Apa*I genomic macrorestriction with PFGE and a PCR–restriction enzyme analysis (REA) technique according to Ericsson et al. (1995), based on the study of the polymorphism existing within the *inl*A and *inl*B genes, which has proven its usefulness to distinguish *Listeria monocytogenes* strains within the serotype 4b. Additional epidemiological information was added by serotyping 33 randomly chosen isolates belonging to each *Listeria monocytogenes* genotype.

2. Materials and methods

2.1. Listeria monocytogenes isolates

2.1.1. Origin

A total of 287 isolates of *Listeria monocytogenes*

collected from five pork slaughtering and cutting plants were studied. The plants (plants I–V) were located in the same production area in France, except for plant IV. In 1995, *Listeria monocytogenes* isolates were collected from the five plants on pork from various locations, including live animals, carcasses immediately after slaughtering, carcasses after chilling, pork cuts (rind and meat) and from the environment, including working surfaces, equipment and various areas in the slaughterhouses, such as the chilling and cutting rooms, which happened to be in contact with pork meat (conveyor belts, carcass breaking tables, etc.) or not (ceilings, ventilators, conveyor rails, etc.), both after cleaning and disinfection and during activity. There was a time difference of two weeks from two months between the isolation of *Listeria monocytogenes* from the slaughterhouse and the chilling or cutting room in each plant. In order to evaluate the persistence of *Listeria monocytogenes* strains in the pork environment, plants III and V were also investigated in 1996, after sanitizing operations, which allowed the collection of 34 isolates. Isolates collected in 1995 were not all preserved and only 253 of the total number (data not shown) were characterised. Details about the origin of characterised *Listeria monocytogenes* isolates in each plant are listed in Table 1.

2.2. Growth conditions

Listeria monocytogenes cultures obtained from a single picked colony were grown in Tryptic Soy

Table 1
Distribution of *Listeria monocytogenes* genotypes (*1–19*), obtained via genomic macrorestriction using *Apa*I with PFGE, RAPD and PCR–REA, in relation to the source and date of isolation, from five pork slaughterhouses and cutting processing plants

Genotype	Plant	Date	Sources of *Listeria monocytogenes* isolates	
			Environment	Pork
1	I	09/1995	Slaughterhouse after sanitizing and during activity	Live pigs, Carcasses after slaughtering and chilling
2	I	09/1995		Live pigs
1	I	11/1995	Cutting room during activity	Pork cuts
4	I	11/1995	Cutting room during activity	
5,6	I	11/1995		Pork cuts
1	II	10/1995	Slaughterhouse during activity	Live pigs, Carcasses after slaughtering and chilling
11,18	II	10/1995	Slaughterhouse during activity	
15,16	II	10/1995		Carcasses after chilling
11,18,19	II	10/1995	Cutting room during activity	Pork cuts
5,17	II	10/1995	Cutting room during activity	
10	II	10/1995		Pork cuts
5	III	10/1995	Slaughterhouse after sanitizing and during activity	
12	III	10/1995	Slaughterhouse during activity	Carcasses after slaughtering
14	III	10/1995		Carcasses after slaughtering
5,12	III	12/1995	Chilling room before and after sanitizing	
10	III	12/1995	Chilling room before sanitizing	
13	III	12/1995	Chilling room after sanitizing	
9,14	III	11/1996	Slaughterhouse and chilling room after sanitizing	
5	III	11/1996	Slaughterhouse after sanitizing	
10,12,13	III	11/1996	Chilling room after sanitizing	
1,11	IV			Pork cuts
1	V	10/1995	Slaughterhouse after sanitizing and during activity	Carcasses after chilling
7	V	10/1995	Slaughterhouse during activity	
8	V	10/1995		Live pigs
1	V	09/1995	Cutting room after sanitizing and during activity	Carcasses before cutting and pork cuts
9	V	09/1995	Cutting room after sanitizing and during activity	
2	V	09/1995	Cutting room after sanitizing	Pork cuts
5	V	09/1995	Cutting room after sanitizing and during activity	
3	V	09/1995		Carcasses before cutting
10	V	11/1996		Pork cuts
1,10	V	11/1996	Chilling room after sanitizing	
5,9	V	11/1996	Cutting room after sanitizing	

broth (Difco Laboratories, Detroit, MI, USA) for 24 h at 37°C. The cultures, supplemented with 15% glycerol, were maintained at −80°C. Prior to each DNA preparation, a loopful of frozen culture was streaked onto Tryptic Soy Agar (Difco Laboratories, Detroit, MI, USA) plates which were incubated overnight at 37°C.

2.3. DNA preparation for PCR and RAPD and macrorestriction with ApaI analysis

Bacterial lawns obtained from overnight cultures were harvested, washed twice in 2 ml sterile 0.01 mol l^{-1} Tris–HCl pH 7.6, 1 mol l^{-1} NaCl (TN–buffer) and pelleted by centrifugation at 5000 *g* (Biofuge Pico, Hereaus, France) for 10 min.

For the PCR–REA technique, bacterial DNA extraction was performed following the Fisher Genomic Mini FG (Fisher Scientific, Osi, France) instructions. The concentration and the purity of the DNA solutions were respectively estimated by $OD_{260\ nm}$ and the ratio $OD_{260\ nm}/OD_{280\ nm}$.

Listeria monocytogenes DNA preparation for both RAPD and genomic macrorestriction with *Apa*I prior to PFGE was based on the principle of lysis of whole cells embedded in agarose according to the procedure of Moore and Datta (1994) with some modifications. The OD read at 600 nm of washed cells was adjusted to 4 with TN–buffer. Bacterial suspensions were mixed with an equal volume of 1% agarose (Agarose standard, Eurobio, France) solution in TN–buffer. The mixture was dispensed in slotformers of 100 μl aliquots (Bio-Rad Laboratories, Richmond, USA). Agarose plugs were incubated in a lysis solution (1 ml per agarose plug) containing 6 mmol l^{-1} Tris–HCl pH 7.6, 0.1 mol l^{-1} EDTA, 1% (w/v) lauryolsarcosine (Sigma ChemicalCo., St. Louis, Mo, USA), 10 g l^{-1} lysosyme (Boehringer GmbH, Mannheim, Germany), for 16 h at 37°C. After removing the lysis solution, plugs were incubated in a 0.5 mol l^{-1} EDTA pH 9, 1% (w/v) lauryolsarcosine and 1 g l^{-1} Proteinase K (Boehringer GmbH, Mannheim, Germany) solution for at least 40 h at 50°C under soft agitation. In order to inactivate Proteinase K, agarose plugs were washed twice with 10 mmol l^{-1} Tris–HCl pH 7.6, 1 mmol l^{-1} EDTA (TE–buffer) for 30 min at room temperature and then incubated with 2 mmol l^{-1} amino-ethyl–benzesulfonyl (Benjamin and Datta, 1995) (Pefabloc®, Boehringer GmbH, Mannheim, Germany) at 37°C for 2 h under soft agitation. Finally, the Pefabloc® solution was removed and plugs were washed twice with TE–buffer for 30 min at room temperature. Agarose plugs were then cut into 25-μl mini-plugs and stored in TE–buffer for short-term use (less than a month) or in 0.5 mol l^{-1} EDTA for preservation at 4°C for at least 1 year.

2.4. RAPD analysis

Aliquots of 325 μl of TE–buffer were added to each 25-μl mini-plug containing *Listeria monocytogenes* DNA. The mixture was heated for 15 min at 95°C, vigorously vortexed and ice-cooled for 5 min. Aliquots of 100 μl of the DNA sample were stored at 4°C for immediate use or at −20°C for ulterior use. The primers employed in this study were selected as candidate RAPD primers after having tested their performance in pilot experiments (data not shown). Finally, five 10-mers were separately used: HLWL74 (5′-ACGTATCTGC-3′) of Mazurier and Wernars (1992), PB4 (5′-AAGGATCAGC-3′) of Boerlin et al. (1995), UBC127 (5′-ATCTGGCAGC-3′) of Farber and Addison (1994), Lis5 (5′-GCTGGAGTCA-3′) (this work) and Lis11 (5′-AGCCAGGTCA-3′) (this work). Each 20-μl PCR reaction mixture contained 1 mmol l^{-1} Tris–HCl pH 8.4, 0.05 mol l^{-1} KCl, 0.1 g l^{-1} bovine serum-albumin (BSA, New England, Biolabs, Inc., Beverly, USA), 200 μmol l^{-1} of each deoxynucleotide triphosphate (dATP, dTTP, dCTP, dGTP, Boehringer GmbH, Mannheim, Germany), 1 μmol l^{-1} of primer, 1 U of Taq Polymerase (Boehringer GmbH, Mannheim, Germany) and 2 μl of DNA solution. A negative control was included in which DNA was replaced by 1 μl of sterile distilled water. For PB4 and Lis11, the mix contained 2.5 mmol l^{-1} $MgCl_2$, for HLWL74, Lis5 and UBC127 the concentration was increased to 3 mmol l^{-1}. The amplification reaction was performed on a 9600 GeneAmp thermocycler (Perkin Elmer Instruments, Norwalk, CT, USA). A first cycle of 95°C for 2 min was followed by a program composed of 3 cycles of 94°C for 1 min, 36°C for 2 min and 72°C for 2.5 min, and then 40 cycles of 94°C for 15 s, hybridisation temperature for 45 s and 72°C for 1 min. After optimisation, the final hybridisation temperatures

were 40°C for HLWL74 and 52°C for Lis5. The final cycle was of 72°C for 10 min. Amplified products were resolved by electrophoresis for 5 h at 2.5 Vcm^{-1} on 1.5% agarose (Agarose standard, Eurobio, France) in 1 × TBE–buffer (89 mmol l^{-1} Tris–HCl, 89 mmol l^{-1} boric acid, 2.5 mmol l^{-1} EDTA, pH 8.3) gel stained with 0.5 mg l^{-1} ethidium bromide. Raoul (Appligene Oncor, France) and ΦX174 DNA cleaved with *Hae*III (Boehringer GmbH, Mannheim, Germany) were included on gels as molecular weight standards. Banding patterns were visualized on a shortwave UV transilluminator and photographed. RAPD analysis was conducted at least twice for each *Listeria monocytogenes* isolate with the repetitions being carried out with cells grown and harvested on different days.

2.5. Macrorestriction and PFGE analysis

2.5.1. DNA Macrorestriction

Before cleavage with *Apa*I, each 25-μl mini-plug was incubated overnight at 4°C in a 100 μl solution containing A buffer (Boehringer GmbH, Mannheim Germany). DNA within each agarose plug was cleaved with 60 U of *Apa*I (Boehringer GmbH, Mannheim, Germany) for 5 h at 25°C, according to the manufacturer's instructions.

2.5.2. PFGE

Macrorestriction fragments were resolved by the PFGE technique of contour clamped homogeneous electric field (CHEF-DRII Bio-Rad Laboratories, Richmond, USA). *Apa*I macrorestricted samples were loaded in the wells of 1.2% agarose (Agarose standard, Eurobio, France) gel in 0.5 × TBE–buffer (44.5 mmol l^{-1} Tris–HCl, 44.5 mmol l^{-1} boric acid, 1.25 mmol l^{-1} EDTA, pH 8.3). Macrorestriction fragments were resolved at 200 V with pulse times from 5 to 40 s over 15 h and 4 to 12 s over 9 h in 0.5 × TBE. The buffer temperature was maintained at 14°C. The lambda DNA concatamers (Boehringer GmbH, Mannheim, Germany) were used as molecular weight size standards.

2.5.3. Analysis of PFGE patterns

Gels were stained with ethidium bromide and images were captured under U.V. illumination by the Gel Doc 1000 video system (Bio-Rad Laboratories, Richmond, USA). Pulsed-field electrophoretic patterns were compared by means of Molecular Analyst software fingerprinting (Bio-Rad Laboratories, Richmond, USA). Similarities between profiles, based on bands positions, were derived from the Dice correlation coefficient with a maximum position tolerance of 1.2%. *Listeria monocytogenes* strains were clustered by the technique of the unweighted pair group method using arithmetic averages (UPGMA) and a dendrogram was constructed to reflect the similarities between them.

2.6. PCR–restriction enzyme analysis

The PCR–REA technique described by Ericsson et al. (1995) consists of the amplification of a 2.916 pb fragment containing parts of *inl*A and *inl*B genes, associated with *Listeria monocytogenes* virulence, with the couple of primers Lip32 and Lip23 (Gaillard et al., 1991), and on the further restriction of this fragment with the endonuclease *Alu*I. The original procedure was modified as described in Section 2.7.

2.7. PCR analysis

PCR was performed using the hot start procedure as described in the manufacturer's instructions of the r*Tth* DNA Polymerase (XL PCR Kit, GeneAmp, Perkin Elmer, USA). The PCR mixture of 50 μl contained 1 mmol l^{-1} of $Mg(OAC)_2$, 200 μmol l^{-1} of each deoxynucleotide triphosphate (dNTP Ultrapure, Clontech, Ozyme, France), 1 × XL Buffer II, 20 μmol l^{-1} of each primer and 2.0 U of r*Tth* DNA Polymerase. A template of 25 ng of DNA was added. PCR was carried out in a 9600 GeneAmp thermocycler (Perkin Elmer Instruments, Norwalk, CT, USA). Prior to the hot start, PCR mixture was submitted to a denaturation cycle of 95°C for 1 min. Following the hot start, the amplication reaction consisted of a program composed of 14 cycles of 94°C for 15 s, 52°C for 30s, a ramp of 3 min from 52°C to 68°C and 68°C for 35s. This was followed by 14 cycles of 94°C for 15s, 52°C for 30s, a ramp of 4 min from 52°C to 68°C and 68°C for 35s. The final cycle devoted to elongation was of 72°C for 10 min. Prior to restriction with *Alu*I, amplified products were resolved by electrophoresis on 1% agarose (Agarose standard, Eurobio, France) in 1 × TBE–buffer (89 mmol l^{-1} Tris–HCl, 89 mmol l^{-1} boric acid, 2.5

mmol l^{-1} EDTA, pH 8.3) and gel stained with 0.5 mg l^{-1} ethidium bromide in order to check the size of the amplified product. Raoul (Appligene Oncor, France) was included on gel as molecular weight standard. Banding patterns were visualized on a shortware UV transilluminator and photographed.

2.8. Restriction endonuclease analysis of the PCR product with AluI

Aliquots of 7.5 μl of the PCR product were cleaved with 8 U of the restriction enzyme *Alu*I (New England, Biolabs, Inc., Beverly, USA) according to the manufacturer's instructions. Each sample was then mixed with 3 μl of loading buffer and separated on a 2.5% agarose gel (Agarose standard, Eurobio, France), stained with ethidium bromide in 1 × TBE–buffer (89 mmol l^{-1} Tris–HCl, 89 mmol l^{-1} boric acid, 2.5 mmol l^{-1} EDTA, pH 8.3). The molecular weight standard Raoul (Appligene Oncor, France) and ΦX174 DNA cleaved with *Hae*III (Boehringer GmbH, Mannheim, Germany) were included on gels. Banding patterns were visualized on a shortware UV transilluminator and photographed.

2.9. Serotyping

Serotyping was applied to 33 *Listeria monocytogenes* isolates. One to four *Listeria monocytogenes* isolates were randomly chosen from each genotype as determined by the three genotyping methods. Serotyping was achieved according to the manufacturer's instructions using O and H sera (Eurobio, France).

3. Results

3.1. RAPD analysis

From 287 *Listeria monocytogenes* isolates, a unique RAPD pattern was obtained from PB4, 5 from Lis5, 6 from Lis11, 12 from UBC127 and 13 from HLWL74 (data not shown). Approximately, 7–12 bands were observed with PB4, Lis11 and Lis5. UBC127 and HLWL74 produced a slightly higher number of bands, some of them being very faint. The bands obtained with each primer ranged in size from approximately 200 to 1800 pb. The five primers did not classify isolates in the same way and the combination of patterns allowed to gather the isolates into 17 RAPD types, named *r*1–*r*17. The RAPD types were composed of very heterogeneous numbers of isolates (Table 2). A total of 186 *Listeria monocytogenes* isolates were grouped into the same RAPD type *r*1. RAPD types *r*4, *r*10, *r*11, *r*9 and *r*8 were composed of more than 10 isolates, which were, respectively, 16, 15, 15, 12 and 11 isolates, and RAPD types *r*13, *r*2, *r*16, *r*12 and *r*17 gathered less than 10 isolates, respectively, 8, 7, 6, 3 and 2 isolates. On the opposite, 6 of the RAPD types, *r*3, *r*5, *r*6, *r*7, *r*14 and *r*15, were composed of a single isolate.

3.2. PFGE analysis

For the 287 tested *Listeria monocytogenes* isolates, the total number of PFGE types was 17, named

Table 2
Listeria monocytogenes genotypes (*1–19*) as determined by results obtained via genomic macrorestriction using *Apa*I with PFGE (*a*1–*a*17), RAPD (*r*1–*r*17) and PCR–REA (Ericsson et al., 1995) (*p*1 and *p*2) and serotypes performed on 33 randomly chosen *Listeria monocytogenes* isolates representative of each genotype

Genotype	PFGE type	RAPD type	PCR–REA type	Serotype[a]	*N*[b]
1	*a*1	*r*1	*p*1	1/2a (3)	186
2	*a*2	*r*2	*p*2	1/2a (2)	3
3	*a*3	*r*2	*p*2	1/2a (1)	4
4	*a*3	*r*3	*p*2	1/2a (1)	1
5	*a*4	*r*4	*p*1	1/2a (5)	16
6	*a*1	*r*5	*p*1	1/2a (1)	1
7	*a*5	*r*6	*p*2	1/2a (1)	1
8	*a*6	*r*7	*p*2	1/2a (1)	1
9	*a*7	*r*8	*p*1	1/2a (2)	11
10	*a*8	*r*9	*p*1	1/2a (2)	12
11	*a*9	*r*10	*p*1	1/2c (2)	15
12	*a*10	*r*11	*p*1	3a (2)	15
13	*a*11	*r*12	*p*1	1/2a (2)	3
14	*a*12	*r*13	*p*1	1/2a (1)	8
15	*a*13	*r*14	*p*1	1/2a (1)	1
16	*a*14	*r*15	*p*1	1/2a (1)	1
17	*a*15	*r*16	*p*1	1/2c (1)	1
18	*a*16	*r*16	*p*1	1/2c (2) and 3c (1)	5
19	*a*17	*r*17	*p*1	1/2a (1)	2

[a] Figures in brackets indicate the number of isolates performed for serotyping.
[b] The total number of *Listeria monocytogenes* isolates of a given genotype among the 287 ones.

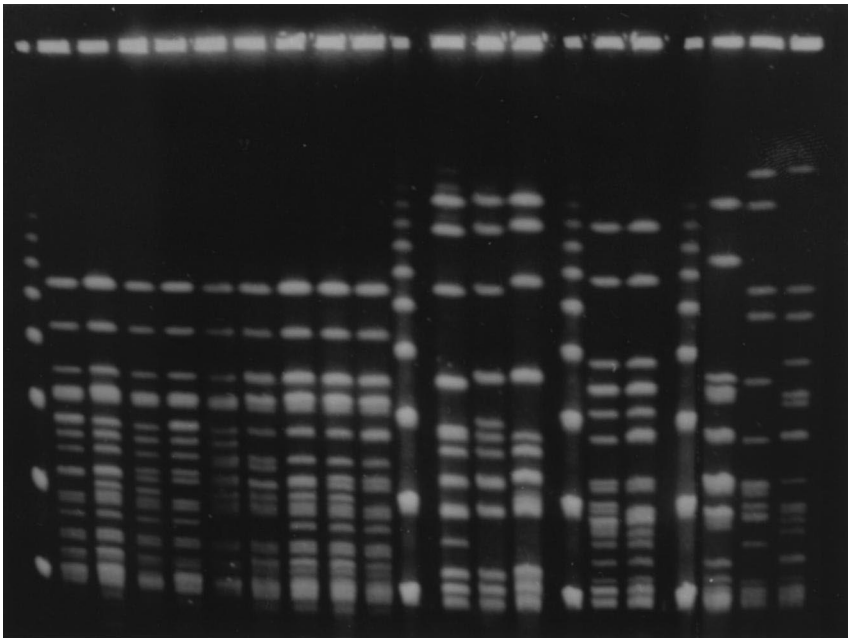

Fig. 1. PFGE resolving *Apa*I macrorestriction fragments representative of the 17 profiles (*a*1–*a*17) obtained from 287 *Listeria monocytogenes* isolates collected from five pork slaughtering and cutting plants. Lanes M are lambda ladder concatamers.

*a*1–*a*17. The different macrorestriction patterns observed with *Apa*I are depicted in Fig. 1.

For each isolate, macrorestriction fragments of sizes longer or equal to 48.5 kb were recorded for analysis with the Molecular Analyst software fingerprinting (Bio-Rad Laboratories, Richmond, USA). Agglomerative hierarchical cluster analysis led to the discrimination of *Listeria monocytogenes* isolates into four main clusters (Fig. 2). There were 255 *Listeria monocytogenes* isolates that grouped into the major cluster (cluster A), in which strains presented at least 85% similarity. Within this cluster, isolates belonging to PFGE type *a*1 were composed of 187 isolates. Profiles were composed of 16–18 fragments ranging from 48.5 to 270 kb. Differences in patterns within cluster A were mainly observed between 48.5 and 145.5 kb. Cluster B, composed of PFGE types *a*9, *a*15 and *a*16, showing more than 70% similarity among them, grouped 22 *Listeria monocytogenes* isolates. Profiles were composed of 12–13 bands ranging from 48.5 kb to more than 436.5 kb. Cluster C grouped 8 isolates of PFGE types *a*2 and *a*3 linked with 95% similarity. Those profiles showed 17 and 16 bands respectively, ranging from 48.5 to 436.5 kb. PFGE types *a*5 and *a*6, grouped in cluster D with 72% similarity, were both composed of a single isolate. The corresponding profiles showed 13 bands ranging from 48.5 kb to more than 485 kb. PFGE type *a*13 was composed of a single isolate which showed low similarity coefficient with all other isolates and whose profile was of composed of 15 bands ranging from 48.5 to more than 485 kb.

Results obtained from RAPD and PFGE were closely related (Table 2). Both techniques allowed to gather the same isolates into the same groups with a few exceptions. An isolate from plant I belonging to PFGE type *a*1 was not of RAPD type *r*1, as all the other ones, but *r*5. Isolates belonging to PFGE types *a*2 and *a*3 gathered into the RAPD type *r*2, except one isolate of PFGE type *a*3 from plant I, which exhibited an original RAPD type, *r*3. Finally, isolates of PFGE types *a*15 and *a*16 gathered into a unique RAPD type *r*16.

3.3. PCR–REA

By using *Alu*I for the restriction of the PCR product obtained from the amplification of the *inl*A and *inl*B regions, two different cleavage profiles were obtained, named *p*1 and *p*2 (Fig. 3). Isolates of

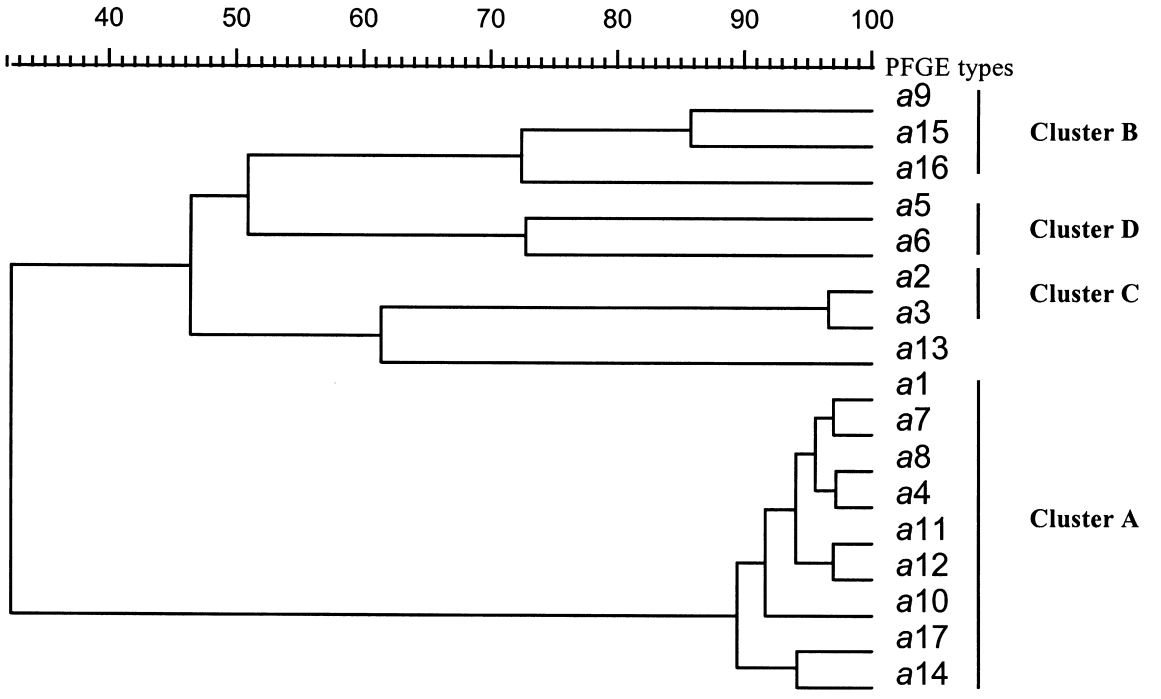

Fig. 2. Dendrogram demonstrating the genetic relationships of the 17 PFGE types (*a*1–*a*17) observed from 287 *Listeria monocytogenes* isolates collected from five pork slaughtering and cutting plants. The scale corresponds to the percentage of similarity.

PFGE types *a*2, *a*3, *a*5 and *a*6 yielded the same PCR–REA *p*2 profile. All other isolates yielded the *p*1 profile (Table 2).

3.4. Genotyping combined results

Combined results obtained via genomic macrorestriction using *Apa*I with PFGE, RAPD and PCR–REA lead to 19 genotypes being distinguished (*1–19*) among the 287 *Listeria monocytogenes* isolates (Table 2).

3.5. Serotyping

Results obtained from serotyping, performed on 33 *Listeria monocytogenes* isolates representative of the 19 genotypes observed in this study are given in Table 2. On 20 isolates belonging to cluster A according to PFGE typing, all displayed the a somatic antigen type. More precisely, isolates of PFGE types *a*1, *a*4, *a*7, *a*8, *a*11, *a*12, *a*14 and *a*17 grouped in cluster A were of serotype 1/2a, which was also the case of 7 isolates belonging to PFGE types *a*2, *a*3, *a*5, *a*6 and *a*13. Two isolates of PFGE type *a*10 belonging to cluster A were of serotype 3a. Six isolates of PFGE types *a*9, *a*15 and *a*16 belonging to cluster B displayed the c somatic antigen. More precisely, they were of serotype 1/2c, except one isolate of PFGE type *a*16 which was of serotype 3c.

3.6. Distribution of Listeria monocytogenes genotypes within the five pork slaughter and cut processing environments

The distribution of *Listeria monocytogenes* genotypes within plants I–V, in relation to their sources and dates of isolation, is described in Table 1. Genotyping allowed to trace different *Listeria monocytogenes* types both within the investigated plants and also from a plant to another one.

In 1995, *Listeria monocytogenes* strain of genotype *1* was isolated from four of the five investigated plants. In plants I and V, this strain was found in the abattoir, after cleaning and disinfection procedures, just before the slaughtering process started in the

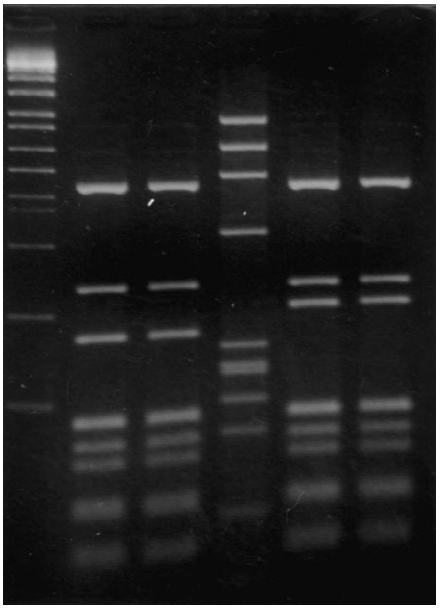

Fig. 3. PCR–REA profiles *p*1 and *p*2 produced by the amplification of the *inl*A and *inl*B regions followed by cleavage with *Alu*I, obtained from 287 *Listeria monocytogenes* isolates collected from five pork slaughtering and cutting plants. Lane M1: Raoul (Appligene Oncor, France) marker; lane 1: *p*2 profile obtained form an isolate belonging to PFGE type *a*2 and of serotype 1/2a; lane 2: *p*2 profile obtained form an isolate belonging to PFGE type *a*5 and of serotype 1/2a; lane M2: ΦX174 DNA, cleaved with *Hae*II, maraker (Boehringer GmbH, Mannheim, Germany); lane 3: *p*1 profile obtained from an isolate belonging to PFGE type *a*1 and of serotype 1/2a; lane 4: *p*1 profile obtained from an isolate belonging to PFGE type *a*9 and of serotype 1/2c.

morning. It was isolated from the live pigs entering plants I and II. It was collected, in plants I, II and V, from the abattoir environment during activity and from pork carcasses, just after slaughtering and/or chilling. This strain was also found in the cutting rooms of plants I and V, in the environment as well as on various pork cuts, 2 months after the checking of the abattoir in each case. Besides strain of genotype *1*, some closely related strains were isolated in these plants. In plant I, strains of genotypes *5* and *6* were found on pork cuts. In plant V, strains of genotypes *9* and *5* were isolated from the environment of the cutting room both after cleaning and disinfection and during activity. At the same time, strain of genotype *10* was isolated there from pork cuts. These very closely related strains, belonging to cluster A if we refer to PFGE typing, were still present in 1996 in the environment of the chilling and cutting rooms of plant V after cleaning and disinfection procedures. It is noticeable that strains of cluster A were isolated, whether in 1995 or 1996, both from pork contact surfaces and in the wider environment of plant V, including ceilings, conveyor rails and ventilators.

Besides the strain of combined type *1*, other different types of *Listeria monocytogenes* were found on live pigs. One isolate of genotype *2* was collected from live pigs entering plant I. Isolates belonging to this type were collected at the same time from the cutting room part, respectively both in the environment after cleaning and disinfection and on pork cuts in plant V. Furthermore, isolates of genotypes *3* and *4*, yielding 95% similarity according to PFGE typing with the strain of genotype *2*, were isolated on a conveyor belt in the cutting room of plant I and on carcasses after chilling in plant V. On the contrary, one isolate of genotype *8* was isolated once on live pigs in plant V but was not isolated anywhere else. In the same way, an isolate of genotype *7* was found once in the slaughterhouse of plant V during activity.

In plant II, one isolate of genotype *16*, closely related to genotype *1*, according to PFGE typing, and one isolate of genotype *15*, were isolated from carcasses after chilling. Isolates of genotypes *5*, *10* and *19*, also closely related to genotype *1*, were isolated in the cutting room 2 weeks after the sampling in the abattoir part. Besides, strains of genotypes *11*, *17* and *18*, corresponding to strains belonging to cluster B according to PFGE typing, were found in the environment of the slaughterhouse and in the cutting room, both in the environment and on pork cuts. Unfortunately, some isolates collected from the cutting room of plant II, after cleaning and disinfection procedures, were not preserved for molecular characterization.

In plant III, *Listeria monocytogenes* isolates were only collected from the slaughterhouse and the chilling room. In 1995, the environment after cleaning and disinfection, during activity and the carcasses after slaughtering were contaminated by very closely related strains, which were of genotypes *5*, *10*, *12*, *13* and *14* (cluster A). These strains were always present in 1996 in the slaughterhouse and chilling room environment (walls, ventilators, etc.) after cleaning and disinfection and a strain of type *9*, also closely related to the others, was also present.

The case of plant IV, from which a few isolates were preserved and characterized is noticeable since strains of combined types *1* and type *11* were isolated there from pork cuts and that this plant is far away from the others.

4. Discussion

In this study, the application of three molecular typing methods, i.e., RAPD with five different primers, genomic macrorestriction using *Apa*I with PFGE and a PCR–REA technique according to Ericsson et al. (1995), based on the analysis of fragments obtained by cleavage with the enzyme *Alu*I of the PCR product amplified from the *inl*A and *inl*B regions, to 287 *Listeria monocytogenes* isolates collected from five pork slaughterhouses and cutting plants were used to identify 19 *Listeria monocytogenes* genotypes (*1* to *19*). Either PFGE or RAPD yielded, respectively, 17 different types, whereas the PCR–REA analysis only revealed two profiles among the 287 *Listeria monocytogenes* isolates. Serotyping performed on 33 representative isolates of the 19 genotypes of *Listeria monocytogenes* revealed the presence of at least four serotypes: 1/2a, 3a, 1/2c and 3c. Previous findings have shown that *Listeria monocytogenes* strains isolated from meat processing environments are frequently of serotypes 1/2a, 1/2b and 1/2c (Jay, 1996). The majority of all sporadic cases as well as for all outbreaks reported so far are associated with the serotypes 4b, 1/2b and 1/2a (Rocourt and Bille, 1997). Only two profiles were generated by PCR–REA among the 287 isolates. This indicates the low degree of polymorphism in the *inl*A and *inl*B regions, as was already shown by Ericsson et al. (1995). This was also observed about other *Listeria monocytogenes* virulence associated gene regions like the *hly* gene (Rasmussen et al., 1991) and the *prf*A and, to a lesser extent, *iap* and *mpl* genes (Vines et al., 1992). Isolates of serotype 1/2a which gathered into cluster A, according to PFGE typing, and all those of serotypes 3a, 1/2c and 3c displayed the same profile PCR–REA *p*1. Ericsson et al. (1995) also observed that strains of serotypes 1/2a and 1/2c may yield the same PCR–REA profiles. Generally, strains of serotypes 1/2a and 1/2c group together, using polymorphism of different *Listeria monocytogenes* virulence associated genes (Vines et al., 1992), and polymorphism of the *ltrB* gene essential for growth at low temperatures (Zheng and Kathariou, 1995). This is also the case for multilocus enzyme electrophoresis at genetic loci encoding metabolic enzymes (Piffaretti et al., 1989). These comments of Vines et al. (1992), quoted in Ericsson et al. (1995), can be reported here, that ''structural diversity in virulence associated genes occured at about the same rate as changes in genes coding for essential cytoplasmic enzymes and the immunogenic surface antigens that are utilized in serotyping''. Nevertheless, in this study, *Listeria monocytogenes* isolates of serotype 1/2a divided into the two PCR–REA profiles, *p*1 and *p*2. In fact, all *Listeria monocytogenes* isolates of serotype 1/2a which did not group in cluster A, according to their corresponding PFGE types, displayed the second PCR–REA profile *p*2. In our study, this is noticeable that isolates displaying identical PFGE pattern belonged to the same serotype, except isolates within the PFGE type *a*16 which showed at least two serotypes: 1/2c and 3c (Table 2). Loncarevic et al. (1997) also stated that *Listeria monocytogenes* strains with identical PFGE patterns generally belong to the same serotype, with a few exceptions. However, it must be kept in mind that Brosch et al. (1994) have shown that *Listeria monocytogenes* isolates with different serotypes may display identical PFGE patterns obtained with *Apa*I or *Asc*I, particularly for some isolates belonging to the same somatic serogroup. Hence, the greatest care must be taken in attempting to allocate a *Listeria monocytogenes* isolate to a given serotype on the basis of its PFGE pattern shared by other strains of known serotype.

Both RAPD (Mazurier and Wernars, 1992; Boerlin et al., 1995) and genomic macrorestriction using *Apa*I with PFGE (Brosch et al. 1991; Jacquet et al., 1995) are considered valuable tools with high discriminatory power for typing epidemiologically related *Listeria monocytogenes* isolates. In this study, RAPD performed with PB4 showed a very poor discriminatory power since a unique profile was obtained from 287 isolates. RAPD carried out with the other four different primers, i.e., Lis 11, Lis5, HLWL74 and UBC 127, or *Apa*I macrorestriction with PFGE yielded respectively 17 *Listeria monocytogenes* types. Results obtained from PFGE and RAPD typing were very closely related. They al-

lowed us to gather the same isolates into the same genomic types, except for a few exceptions (Table 2): PFGE type *a*1 was divided into RAPD types *r*1 and *r*5 while RAPD type *r*16 was divided into PFGE types *a*15 and *a*16. Consequently, the use of both genotypic methods (RAPD and PFGE) increased the discriminatory ability to detect differences among isolates of *Listeria monocytogenes* as was also observed by Destro et al. (1996) using RAPD and PFGE to trace *Listeria monocytogenes* strains in a shrimp processing environment. However, some difficulties relative to RAPD profiles reproducibility were encountered. When RAPD reactions were performed using the more discriminating primers (HLWL74, UBC127, and, to a lesser extent, Lis5) on separate DNA extractions for some isolates, we have observed minor bands which were not reproducibly demonstrable. Those pattern differences from a trial to another were mainly observed as variations in band intensity. It was shown, within other purposes, that DNA concentration greatly influences patterns obtained with RAPD, which may be due to variable efficiency of cell lysis from one bacterial culture to another one (Meunier and Grimont, 1993; Boerlin et al., 1995). PFGE patterns obtained with *Apa*I, were composed of 12–18 fragments readily dicernible (Fig. 1), and compared to RAPD ones were consistently reproducible and easy to analyze. For this reason and considering the closeness of results obtained with both techniques, only PFGE patterns were used to study the hierarchical links between *Listeria monocytogenes* isolates.

From an epidemiological point of view, the application of three molecular typing methods to 287 *Listeria monocytogenes* isolates, collected from five pork slaughtering and cutting plants in 1995 and 1996, identified the presence of some very closely related strains in each investigated plant. In fact, 9 of the 19 genotypes obtained from the 287 isolates (*1, 5, 9, 10, 12, 13, 14, 16* and *19*), belonging to cluster A, according to PFGE typing, represented nearly 90% of the investigated isolates. Isolates belonging to these nine genotypes were each isolated in one or more of the five investigated plants (Table 1). These strains were isolated from live pigs, pork meat and rind, as well as from diverse environmental surfaces, either in contact with pork or in the wider environment (ceilings, ventilators, walls, conveyor rails), representing various ecological conditions in terms of temperature, relative humidity and composition of the soils. Thus, strains belonging to cluster A appeared to be particularly well adapted to the pork processing environments investigated in this study. The high level of similarity of the strains within the cluster A suggests that these strains probably belong to the same clonal line. Especially, *Listeria monocytogenes* strain of genotype *1* (PFGE type *a*1) was spread throughout four of the five plants investigated in 1995. Those strains belonging to the eight other PFGE types grouped in cluster A may derive from the strain of PFGE type *a*1 by some genomic mutations. The same conclusion may be done for the strains of PFGE types *a*2 and *a*3 (genotypes *2, 3* and *4*) which showed patterns similarities and may derive from a common ancestor.

In this study, we have shown that *Listeria monocytogenes* strains of genotypes *1* and *2* could either be found on live pigs or in the pork slaughtering and cutting processing environment, even after cleaning and disinfection procedures. This leads to the hypothesis that these strains, primarily isolated from animals, are able to become established in pork processing environments. The observation of identical *Listeria monocytogenes* contamination of the live pigs and the pork process contradicts the findings of Boerlin and Piffaretti (1991) who showed, using multilocus enzyme electrophoresis, that *Listeria monocytogenes* types dominating in slaughter pork, fresh meat, meat products were different from those found on live animals. Our results would strengthen the findings of Lawrence and Gilmour (1995) and Ojeniyi et al. (1996) who found identical epidemiological *Listeria monocytogenes* types on live animals and in poultry processing environments. The presence of strains originating from live animals on pork and on various meat contact surfaces indicates either that a continuous source of inoculation by live pigs or the persistence of these strains. However, the known low prevalence of *Listeria monocytogenes* among pigs (Skovgaard and Nfrrung, 1989; Nesbakken et al., 1994; Jay, 1996) and the consistent finding of the strain of genotype *1* and closely related ones in environmental samples, e.g. walls, ceilings and ventilators, which are not directly in contact with pork carcasses, support the latter hypothesis. Several studies have also shown the implication of the processing environment, including hte chilling room, in keeping with the psychrotrophic properties of the

bacterium (Luchansky and Doyle, 1991; Borch et al., 1996; Nesbakken et al., 1996; Sammarco et al., 1997).

This study allowed the identification of the presence of very closely related strains, belonging to cluster A, over a 1-year period in plants III and V. This has already been shown by Lawrence and Gilmour (1995) in a poultry processing environment for at least 6 months and by Unnerstad et al. (1996) in a dairy industry environment for 7 years The presence of these *Listeria monocytogenes* types over a 1-year period in the same plants, even after cleaning and disinfection procedures, suggests the establishment and the persistence of these strains within the wider processing environment. Some types or clones of *Listeria monocytogenes* may be adapted to specific niches. This may be enhanced by the ability of *Listeria monocytogenes* to attach to surfaces and form biofilms, giving them added protection against biocidal agents (Mafu et al., 1990b). The colonisation of specific niches by some *Listeria monocytogenes* types may also be illustrated by the nature of the contamination by *Listeria monocytogenes* in plant II, where closely related strains of cluster B were found both in the cutting room environment, including surfaces in close contact with pork meat, and on pork cuts.

5. Conclusion

The application of RAPD, PFGE and a PCR–REA allowed the identification of 19 genotypes and the observation of a high polymorphism among the 287 *Listeria monocytogenes* isolates collected from five pork slaughtering and cutting plants, in 1995 and 1996. This may reflect the molecular diversity of *Listeria monocytogenes* either in the investigated environments or, more generally, in meat environments.

This work shows that very closely related strains, belonging to cluster A, were present over a 1-year period in two plants. The detection of endemic *Listeria monocytogenes* strains within pork processing environments over an extended period has not been reported until now, although previous studies have demonstrated systematic contamination of pork processing environments by this bacterium. This study highlights the ability for some *Listeria monocytogenes* strains (especially cluster A) to become established in pork processing environments. It emphasizes the problem of the efficiency of cleaning and disinfection procedures used in pork slaughterhouses, chilling and cutting rooms. Moreover, some equipment and machines are difficult to clean because of their conception and state of wear. Actually, some areas or equipment, such as chilling room walls, especially above a height of 2 m, ceilings, conveyor rails or ventilators, are very rarely submitted to sanitizing operations. Hence, the existence of areas contaminated by *Listerai monocytogenes* represents a potential source of contamination of pork products. From these observations, the sources of the contamination of pork carcasses and pork cuts by *Listeria monocytogenes* could originate from either improper cleaning and disinfection or introduction of contaminated live pigs.

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