Incidence of *Listeria* spp. and *Listeria monocytogenes* in a Poultry Processing Environment and in Poultry Products and Their Rapid Confirmation by Multiplex PCR

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The incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing plant and in raw and cooked poultry products was determined over a 6-month period. Within the raw and cooked poultry processing environments, 46% (36 of 79) and 29% (51 of 173) of the samples contained *Listeria* spp. while 26% (21 of 79) and 15% (27 of 173) contained *L. monocytogenes*, respectively. Various sites within the processing environment were found to be consistently positive for *L. monocytogenes* throughout the entire sampling period. Of the raw and cooked products tested, 91% (53 of 58) and 8% (8 of 96) were found to contain *Listeria* spp. while 59% (34 of 58) and 0% (0 of 96) contained *L. monocytogenes*, respectively. Although *L. monocytogenes* was not detected in the cooked products examined, the presence of other *Listeria* spp. highlights the potential which exists for postprocessing contamination. Multiplex PCR proved to be a convenient and time-saving technique for rapid confirmation of *Listeria* spp. and *L. monocytogenes* in a single reaction.

Listeria monocytogenes is associated with meningoencephalitis, septicemia, and abortion in humans. Pregnant women, neonates, and immunocompromised individuals are particularly susceptible to infection. In recent years, the organism has been established as a food-borne pathogen and has become a major concern to the food industry and health authorities. The association between the consumption of cooked and chilled ready-to-eat poultry products and several cases of listeriosis in England (1, 13, 14) and the United States (3), together with the finding that *L. monocytogenes* was present in 12 to 27% of such products at the retail level (11, 15, 21), indicates that ready-to-eat and inadequately reheated chilled foods may constitute a public health risk.

Implications of processed meats as a cause of listeriosis and concern regarding the growth of listeriae in processed foods at refrigeration temperatures resulted in the institution of a zero-tolerance policy for *L. monocytogenes* in ready-to-eat products by the U.S. Department of Agriculture in 1989 (22). However, the high prevalence of *L. monocytogenes* in food production plants (20), its ability to persist and grow in this environment, and its reintroduction on raw materials each contribute to the complexity of producing *Listeria*-free foods.

In the work reported here, the incidence of *Listeria* spp., in particular, *L. monocytogenes*, in the poultry processing environment and in raw and cooked poultry products at the various stages of processing was determined over a 6-month period to identify the critical stages at which contamination occurred. Since confirmation of *Listeria* spp. and *L. monocytogenes* by conventional means proved to be both time consuming and labor intensive, the use of a multiplex PCR technique, in which three separate regions of the bacterial genome are coamplified in a single PCR, was evaluated as an alternative to biochemical confirmation.

Primary processing of chickens was carried out off site, and

carcasses were transported under refrigeration (4°C) to the processing plant, where they were further processed, cooked, and packaged. This plant was part of a fully integrated operation with a supply of chickens from the slaughterhouse. Environmental and product sampling was carried out in the raw and cooked poultry areas within the processing plant each month for a total of 6 months from March to August 1992. To minimize the risk of contamination, sampling was always carried out in the cooked poultry area before proceeding to the raw poultry area.

Environmental sampling. Gauze swabs were premoistened with inactivating diluent (quarter-strength Ringer solution [Oxoid, Basingstoke, Hampshire, England] with 0.04% [wt/vol] lecithin [BDH Chemicals Ltd., Poole, England], 0.02% [vol/vol] Tween 80 surfactant [BDH], and 0.05% [wt/vol] sodium thiosulfate [May and Baker, Dagenham, England]) and rubbed firmly over the surface to be examined (1,000 cm² when possible) by using parallel strokes and rotation of the swab after each stroke. The top of the swab was broken off aseptically into a plastic screw-cap bottle (30-ml universal bottle; Sterilin) containing 12.5 ml of inactivating diluent, and the procedure was repeated by using strokes at right angles to the first set. The two swabs together with diluents were shaken by hand to release debris and cells attached to the gauze, and both swabs and diluents were then added to 225 ml of primary *Listeria* enrichment broth (LEB1; UVM formulation; Oxoid). To allow comparison of results, the same area for each surface was sampled on each sampling occasion.

Drains were examined by attaching a length of sterile ribbon gauze (25 by 15 cm) on polypropylene string to the drain cover. This attachment was inserted in each drain 2 days before sample collection. The gauze was suspended freely in the drain during this time. On the day of sample collection, this was removed by cutting the string and placing the gauze into a 300-ml, wide-mouth, sterile, plastic, screw-cap container (Sterilin) containing 25 ml of the inactivating diluent described above. The gauze was mixed with the diluent by gentle manual shaking to encourage inactivation of inhibitory compounds which may have been present on the gauze because of the

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cleaning agents used. Gauze, together with diluent, was then added to 225 ml of LEB1 as described above. For examination of liquids, 12.5 ml of each sample was collected aseptically and mixed with an equal volume of double-strength inactivating diluent and added, to 225 ml of LEB1 as described above.

Gloves and aprons were placed in sterile plastic bags. LEB1 (225 ml) was added, ensuring that only the outside of the gloves came into contact with the broth. The samples were hand massaged for 2 min, and the LEB1 was poured back into the flask.

In all cases, environmental samples were enriched within 2 h of collection.

Examination of air in each processing area within the plant and inside the holding chills was done with the Biotest RCS Centrifugal Air Sampler (Biotest-Folex Ltd., Moseley, Birmingham, England) and agar strips containing *Listeria* selective agar (LSA; Oxford formulation with *Listeria* selective supplement [Oxoid]). A fresh LSA strip was used for each location, and a test sampling time of 4 min was used. This equated with sampling of an air volume of 160 dm³.

Product sampling. Raw products (two samples of each type) were collected on line and placed in separate sterile plastic bags (Stomacher bags; 7 by 12 in.; Seward and Co. Ltd., London, England). Cooked products (cooked the previous day) were collected as retail packs (four samples of each type). All products were kept in insulated boxes containing ice packs, where the temperature was maintained at <7°C during transportation to the laboratory. Product samples were analyzed within 6 h of collection.

Microbiological analyses of raw and cooked chicken products. Raw and cooked whole birds were sampled by removing 25 g of skin from the neck flap, underneath the wing, and the breast. For raw and cooked portions, 25 g of skin and surface flesh was used. Samples were homogenized with 225 ml of LEB1 for 2 min in a Stomacher (Colworth Stomacher 400; Seward and Co. Ltd.). Homogenate was then poured back into the 500-ml conical flask for incubation. Duplicate samples were tested. Further duplicate samples of each cooked product were stored at 4°C and sampled as described above after 7 days of storage. This was the use-by date for the cooked product.

Isolation of *Listeria* spp. A modification of the U.S. Department of Agriculture Food Safety and Inspection Service procedure, as described by McClain and Lee (17), was used to isolate *Listeria* spp. from environmental and product samples. Although typical *Listeria* colonies on LSA are visible after 24 h at 30°C, incubation was continued for a further 24 h (a total of 48 h) to ensure detection of slowly growing strains. Up to five typical colonies were removed from each plate and transferred to nonselective media for purification. A single, well-isolated, *Listeria*-like colony on nonselective agar, Tryptone soya agar (Oxoid) with an additional 0.6% yeast extract (TSAYE), demonstrating blue coloration by the Henry illumination technique (25) was transferred to a vial of beads in cryopreservative fluid (Protect; Bury, Lancashire, England) and stored at -80°C for further identification.

Identification of *L. monocytogenes* by multiplex PCR. Presumptive *Listeria* isolates (93 strains) from the poultry processing environment and raw poultry products on the first sampling occasion were identified biochemically by using the criteria outlined by McLauchlin et al. (18). These strains were then analyzed by multiplex PCR by using a modification of the method described by Border et al. (4). Crude cell lysates were prepared by resuspending a loopful of cells (from the surface of a TSAYE plate) in 50 µl of sterile distilled water, heating them in a microprocessor-controlled thermal cycler (Perkin Elmer Cetus) at 100°C for 10 min, and then cooling them to

TABLE 1. Sequences of oligonucleotide primers used to identify *L. monocytogenes* and other *Listeria* spp. by PCR^a

Primer	Strand of origin ^b	Sequence ^c (5'-3')	Location on 16S rRNA ^d
U1	+	CAG CMG CCG CGG TAA TWC	519-536
U2	-	CCG TCA ATT CMT TTR AGT TT	927-908
LI1	-	CTC CAT AAA GGT GAC CCT	1457-1440
LM1	+	CCT AAG ACG CCA ATC GAA	
LM2	-	AAG CGC TTG CAA CTG CTC	

^a As described in reference 4.

^b +, positive; -, negative.

^c M denotes A or C, W denotes A or T, and R denotes A or G.

^d According to reference 6.

4°C. Oligonucleotide primers as described by Border et al. (4) were synthesized by Operon Technology, Alameda, Calif. These ranged from 18- to 20-mer and are shown in Table 1. Primers U1 and U2 are based on 16S rRNA sequences that are essentially conserved throughout all bacteria and yield a product of 408 bp. Primer LI1 is also based on 16S rRNA sequence data and is specific to the genus *Listeria*. When used in conjunction with the U1 primer, this yields a PCR product of 938 bp which is specific for *Listeria* spp. Primers LM1 and LM2 are based on listeriolysin O gene sequence data and give a characteristic PCR product of 702 bp.

A master mixture of the components used in the amplification reaction, except for the cell lysate, was prepared and dispensed into 49-µl aliquots each containing 5 µl of 10× PCR buffer (10 mM Tris-HCl [pH 8.3 at 25°C], 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin); each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) at 200 µM; primers U2, LI1, LM1, and LM2 at 1 µM each; 2 µM primer U1; and 0.5 µl of *Taq* DNA polymerase (all reagents were from Perkin Elmer Cetus). One microliter of the appropriate crude cell lysate was added to each tube except one (negative control). All tubes were overlaid with liquid paraffin (2 drops) to avoid evaporation during thermal cycling. Thermal cycling was done with a Perkin Elmer Cetus DNA thermal cycler. DNA was amplified by temperature cycling through the following temperature profile: 24 cycles of 94°C for 80 s (denaturation), 50°C for 90 s (annealing), and 72°C for 120 s (amplification), with a final cycle extending amplification conditions to 72°C for 10 min.

Amplified products were kept at 4°C and resolved by horizontal agarose gel electrophoresis in 3% (wt/vol) NuSieve Agarose (FMC Bioproducts, Rockland, Maine) and 1% (wt/vol) Agarose NA (Pharmacia Biotech Ltd.) in 1× Tris-borate buffer (0.089 M Tris-HCl, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) at 70 V for 4 h. φX174 replicative-form DNA-*Hae*III fragments (1 µg) were included as molecular weight markers. Gels were stained by immersion in ethidium bromide solution (0.5 µg ml⁻¹) for 30 min, washed briefly in running tap water, and photographed with a UV transilluminator (UV Products, San Gabriel, Calif.) at 254 nm. All strains were examined in duplicate.

All presumptive *Listeria* strains subsequently isolated from environmental and product samples were identified by the PCR procedure described above.

Enumeration of *Listeria* spp. in cooked poultry products after 7 days of storage at 4°C. If *Listeria*-like colonies were isolated by enrichment from cooked products on the day of collection, then enumeration of *Listeria* spp. by direct plating on LSA was done after 7 days of storage at 4°C. Ten grams of the product (skin and outer flesh) was homogenized with 90 ml of peptone saline maximum-recovery diluent (Oxoid CM 733)

TABLE 2. Number of samples positive for *L. monocytogenes* and other *Listeria* spp. from a poultry processing environment and poultry products tested from March to August 1992

Sample	No. of positive samples/total (% positive)		
	<i>L. monocytogenes</i> ^a	Other <i>Listeria</i> spp. ^b	Total ^c
Environment			
Raw	21/79 (26)	15/79 (19)	36/79 (46)
Cooked	26/173 (15)	25/173 (14)	51/173 (29)
Products			
Raw	34/58 (59)	19/58 (33)	53/58 (91)
Cooked	0/96 (0)	8/96 (8)	8/96 (8)
Total	81/406 (20)	67/406 (17)	148/406 (37)

^a Many of these samples also contained other *Listeria* spp.

^b Number of samples which did not contain *L. monocytogenes* but did contain other *Listeria* spp.

^c Total number of samples positive for any *Listeria* sp.

for 2 min in a Colworth Stomacher 400. Further decimal dilutions were prepared in maximum-recovery diluent up to 10^{-3} . Triplicate LSA plates were spread with 0.1 ml taken from each dilution (10^{-1} to 10^{-3}). Plates were incubated at 30°C for 48 h, after which colonies surrounded by a black zone were counted.

A total of 406 environmental and product samples collected over the 6-month period were analyzed by the U.S. Department of Agriculture enrichment technique for *L. monocytogenes* and other *Listeria* spp. The numbers of positive samples within each processing environment and within raw and cooked poultry products are shown in Table 2.

In general terms, the incidence of *L. monocytogenes* and other *Listeria* spp. in raw and cooked poultry processing environments was fairly consistent over the time period of the study (Fig. 1). Sites which were positive for *L. monocytogenes* included stainless steel surfaces of processing equipment, conveyor belts, door handles, floors, squeegees used for cleaning the floor, gloves from personnel, and drains. Other *Listeria* spp. were also frequently isolated from these sites, and in some cases they were present when no *L. monocytogenes* was found.

The consistent isolation of *L. monocytogenes* from various food contact surfaces within the processing environment demonstrates that either these sites become contaminated from within, e.g., by aerosol production during cleaning, that these organisms are not eliminated by cleaning-sanitization, or that these organisms have the potential to survive for extended periods under conditions found in many processing plants. The

FIG. 1. Incidence of *L. monocytogenes* and other *Listeria* spp. in a poultry processing environment and in raw and cooked poultry products from March to September 1992. (A) Raw (R) and cooked (C) areas within the processing environment. (B) Raw and cooked poultry products. ■, *L. monocytogenes*; □, *Listeria* spp. (not *L. monocytogenes*); □, no *Listeria* spp.

frequency with which *L. monocytogenes* was found within the cooked poultry processing environment (29%) was somewhat surprising considering that this area was treated as high risk. It was completely separated from raw poultry processing, had a rigorous cleaning-sanitization schedule, and employed personnel who were regularly screened for *Listeria* carriage. These results therefore demonstrate the difficulties involved with complete elimination of *L. monocytogenes*.

Attachment of bacterial cells to food contact surfaces may be one explanation for the survival and persistence of *L. monocytogenes* in the food processing environment. Mafu et al. (16) have shown that the organism can attach to various surfaces used in the food industry after a contact time of only 20 min. Attached bacteria may form a dynamic microcolony environment where growth and multiplication allow the development of a multicellular biofilm layer from which bacteria are continuously released into the environment, resulting in subsequent product contamination. Moreover, formation of a biofilm can confer protection on bacterial cells subject to sanitization and a biofilm can therefore become an important reservoir of *L. monocytogenes* in food processing plants (19).

Air was not considered to be a major source of contamination in either the raw or cooked poultry processing environment, since all of the air samples taken contained <1 CFU dm^{-3} .

An increase in the incidence of *Listeria* spp. and *L. monocytogenes* in raw products was correlated with increased processing. The lowest incidence was found in raw whole birds coming into the processing plant, where 4 of 12 birds were found to contain *Listeria* spp., 2 of which also contained *L. monocytogenes*, while the highest incidence was found in raw chicken breast fillets, which are manually removed from the bone and 12 of 12 samples of which were found to contain *Listeria* spp. (10 of the 12 samples also tested positive for *L. monocytogenes*). This trend is in agreement with previous studies (8, 9, 12, 24) and reflects environmental contamination and/or spread of organisms during further processing and packaging. However, the presence of *Listeria* spp. in 91% of raw products is substantially higher than that found previously.

The cooked products which were found to contain *Listeria* spp. (8%) included four cooked chicken breast portions collected in March and four portions of spiced wings collected in August. In both cases, the level of *Listeria* spp. did not exceed 100 CFU g^{-1} after 7 days of storage at 4°C. The most likely source of contamination for these products was postprocessing contamination, since cooked portions were cooked to an internal temperature of 82°C. This temperature has been shown to be effective in eliminating the risk that *L. monocytogenes* will survive the cooking process (5), and it is therefore likely that contamination of the cooked products originated from a food contact surface within the cooked poultry processing environment.

These results compared favorably to previous surveys of cooked and ready-to-eat poultry at the point of retail sale within the United Kingdom, where 12 to 27% of samples were found to contain *L. monocytogenes* (11, 15, 21), in some case at levels of up to 700 CFU g^{-1} (2). This emphasizes the need for adequate control measures, including prevention of postprocessing contamination and strict temperature control at all points of the food processing chain. Control measures for *L. monocytogenes* in foods are also influenced by differing worldwide government policies regarding the presence of the organism in food, a problem compounded by the lack of knowledge regarding the infective dose of the organism. Within the United Kingdom, provisional guidelines consider a cooked, ready-to-eat food as fairly satisfactory with *L. monocytogenes*

FIG. 2. Amplification products obtained when the genomic DNAs from presumptive *Listeria* spp. were subjected to multiplex PCR. Lanes: M, DNA molecular weight markers ϕ X174 replicative-form DNA-HaeIII fragments (1 μ g); 1 to 5, PCR profile 1, produced by *L. monocytogenes* (49 of 93 isolates); 6, 7, 8, and 11, PCR profile 2, produced by other *Listeria* spp. (not *L. monocytogenes*) (40 of 93 isolates); 9, 10, 12, and 13, PCR profile 3, produced by non-*Listeria* spp. (4 of 93 isolates).

levels of up to 100 CFU g^{-1} (10). However, whether or not worldwide food regulatory agencies should tolerate low levels of *L. monocytogenes* in cooked, ready-to-eat foods is a subject of some debate. Concern regarding growth of the organism in processed foods before consumption resulted in the institution of a zero-tolerance (absent in 25 g of a product) policy for *L. monocytogenes* in ready-to-eat products by the U.S. Department of Agriculture in 1989 (22). Although this would be the ideal situation, one has to question whether this level of safety is attainable in practice. The results of this study clearly demonstrate the difficulties encountered in producing a *Listeria*-free food processing environment and serve to highlight the potential which exists for postprocessing contamination of cooked, ready-to-eat products with listeriae from the processing environment.

Of the 93 isolates examined biochemically, 89 gave responses typical of *Listeria* spp. (gram-positive, regular, non-spore-forming rods, blue appearance on TSAYE by Henry illumination, catalase positive, oxidase negative, tumbling motility at room temperature, positive for methyl red and Voges-Proskauer tests, negative for production of urease and reduction of nitrates, acid but no gas from glucose, esculin, salicin, and α -methyl-D-mannoside). Of these 89 confirmed *Listeria* spp., 49 isolates also displayed beta-hemolysis on horse blood and gave a positive CAMP reaction with *Staphylococcus aureus* NCTC 1803, indicating *L. monocytogenes*. The most likely biochemical identification for the non-*Listeria* isolates was the genus *Carnobacterium* (catalase negative and rhamnose positive), which shares many properties with the genus *Listeria* and is frequently isolated from chicken meat (23).

Amplification products obtained when the genomic DNA from these strains were subjected to PCR by using a combination of primers U1, U2, LI1, LM1, and LM2 were of three types. An example of these is shown in Fig. 2 together with the total number of isolates which produced each PCR profile. All of the isolates tested displayed the characteristic U1-U2 PCR product at 408 bp. The 938-bp LI1-U1 product which is specific to members of the genus *Listeria* was present in 89 of the 93 isolates tested. These isolates were those which gave biochemical responses typical of *Listeria* spp. Forty-nine of these also showed the characteristic 702-bp LM1-LM2 product, indicating *L. monocytogenes*. These isolates were those which had been biochemically confirmed to be *L. monocytogenes*. Negative controls produced no detectable product.

The results of this investigation demonstrate that multiplex PCR is a rapid, specific, and reliable means for identification of

Listeria spp. and *L. monocytogenes* isolated from the poultry environment and poultry products. This technique is also deemed to be more reliable than conventional identification, since it is based on stable genotypic characteristics rather than relying on biochemical or physiological traits which can be genetically unstable. The use of multiplex PCR with three primer sets running in parallel maximizes the level of information over direct PCR. Since the listeriolysin O gene (*hlyA*) has been shown to be necessary for the full virulence of *L. monocytogenes* in mice (7), the 702-bp LM1-LM2 product provides a clinically relevant marker for identification purposes and overcomes problems encountered with atypical hemolytic responses. Moreover, the U1 and U2 universal 16S rRNA primers provide a useful positive control for the PCR. In the present study, the presence of this PCR product alone in four of the isolates confirmed that the target sequences had been successfully amplified and that these four isolates were not listeriae.

For this investigation, presumptive *Listeria* colonies were taken from TSAYE plates for confirmation and multiplex PCR offered a considerable time saving over conventional techniques, allowing a result to be obtained on the same day, compared with up to 7 days by traditional methodology. The added advantage of PCR confirmation at this stage is that the bacterial colony can then be stored at $-80^{\circ}C$ in glycerol-coated beads for further characterization and typing. Further characterization of the *L. monocytogenes* isolated during this study is ongoing to establish possible sources of contamination and to measure the persistence of individual strains within this poultry processing plant.

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