# 5′ Fluorogenic PCR Assay for the *iap* Gene of *Listeria* species.

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**Summary**

We developed a fluorogenic 5′ nuclease polymerase chain reaction (PCR) assay targeting the *iap* gene, which is highly conserved in all *Listeria* species. We used this assay to screen tissues obtained during a survey of cull sows. To date, we have detected *Listeria* species in in caecal contents (66%, 23 of 35 samples), tonsil homogenates (67%, 4 of 6 samples), and ileocaecal lymph nodes (75%, 6 of 8 samples), but infrequently in feces collected before slaughter (4.4%,1 of 23 samples). Taken together, these data indicate that second-generation 5′ nuclease PCR (TaqMan) assays accelerates detection and quantitation of *Listeria* during pork production. This may reduce product recalls as well as reduce human illness and deaths. The development of portable instruments for 5′ nuclease assays will expedite field surveys.

**Introduction**

*Listeria* is a nonspore forming, gram-positive, facultatively anaerobic bacillus, that is found in soil, water, vegetation, fish, birds, insects, and a variety of mammals (9). There are six species of *Listeria*: *L. monocytogenes, L. ivanovii, L. seelegeri, L. murrayi, L. welshimeri,* and *L. innocua* (9).

*L. monocytogenes* affects immunocompromised human hosts, including infants and neonates, the elderly, and pregnant women, resulting in meningitis, bacteremia, mild gastroenteritis, and spontaneous abortions (4,9). In the United States, ~2,500 listeriosis cases are reported annually at a medical cost of ~$0.25 billion. The case fatality rate of 30% is the highest of any foodborne bacterial agent (9). There is “zero tolerance” for *L. monocytogenes* in ready to eat products. From 1990 to 1997 10 of 12 class I (voluntary) recalls involved pork (4). In 1999, 50% of food recalls were due to *L. monocytogenes* contamination (4). *L. innocua*, is usually found in concert with *L. monocytogenes* and thus may be an indicator of its presence (4).

In France, one of the largest listeriosis outbreaks was traced to pickled pork tongue and involved 279 human cases (33% pregnancy related; 7). A second outbreak in France involved 38 patients (82% pregnancy related) and incriminated contaminated pork rillettes (pate). A third episode occurred in spring 2000. Healthy carrier hogs were thought to be the most probable source of *L. monocytogenes* contamination in the plant (5). Because of its public health significance, highly specific polymerase chain reaction (PCR)-based strategies to identify *L. monocytogenes* have been detailed (1).

The invasion-associated protein (*iap*) gene is conserved in all species of *Listeria* and encodes the p60 protein for the invasion of nonprofesisonal phagocytic cell (2). Thus, the *iap* gene is an ideal candidate for the detection of all *Listeria* species (6). Herein we report a fluorogenic 5′ nuclease PCR assay specific for the *iap* locus.

**Materials and Methods**

*5′ Nuclease Assay.* Sequences and concentrations for the primers and probe targeting the *iap* gene in the 5′ nuclease fluorogenic PCR assay are shown in Table 1.

*Bacterial Strain.* Strains used to determine specificity were obtained from the National Animal Disease Center (Table 2).

*Genomic DNA Extraction.* A loop of bacterial growth was harvested from the surface of BHI plates into 200 µl of sterile water and boiled (15 minutes in a heat block at 103.0°C). The boiled suspensions were pelleted (11,500 rpm) and the supernates were stored (-20°C) for PCR analysis.

*Fluorogenic PCR.* Each amplification reaction (50 µl) consisted of template DNA (5 µl) , 5 mM $MgCl_2$, 0.4 mM dNTPs, 1X AmpliTaq Gold Buffer + Rox, and 2.5 U AmpliTaq Gold DNA polymerase. Amplification conditions included initial denaturation (95°C for 10 minutes) and 35 cycles of denaturation (95°C for 15 seconds), primer annealing (55°C for 30 seconds), and primer extension (72°C for 45 seconds) followed by a final extension (72°C for 3 minutes) and an indefinite hold (25°C). PCR reactions were performed in the ABI Prism 7700 Sequence Detector System and analyzed using the Sequence Detector Software, version 1.6.3 (Applied Biosystems, Foster City, CA).

*Cull Sow Tissues:* The optimized assay was used to detect *Listeria* spp in tissues collected during a survey of cull sows (8). Fecal samples and tissues were collected from cull sows (n = 181) during five sampling points (January –March 2001). For this study, 72 samples, including feces, caecal contents, ileocaecal lymph nodes, and tonsil homogenates, were screened for *Listeria*.

## Results

*Fluorgenic 5′ Nuclease PCR Assay.* The optimized 5′ nuclease assay amplified *L. monocytogenes, L. innocua, L. seelegeri, L. ivanovi,* and *L. welshimeri* (Table 2). No amplification was seen with *L. murrayi.*

*Genomic DNA extraction.* Initially, 5′ nuclease assays were conducted using bacteria harvested from Palcam. However, because Palcam contains acriflavine, which interferes with the TaqMan assay, it was necessary to subculture to BHI and then proceed with the DNA extraction.

*Cull Sow Tissues.* Preliminary analysis of the samples screened to date indicate *Listeria* in caecal contents (66%, 23 of 35 samples), tonsil homogenates (67%, 4 of 6 samples), and ileocaecal lymph nodes (75%, 6 of 8 samples), but infrequently in feces collected before slaughter (4.4%,1 of 23 samples).

## Discussion

The *iap* gene, which encodes the P60 protein needed for penetration into nonphagocytic cells, exists in all species of *Listeria*, including *L. innocua*.

We have designed a TaqMan 5' nuclease assay, which targets the *iap* gene, which is conserved in all species of *Listeria* of public health significance. We used this assay to screen tissues obtained during a survey of cull sows. To date, we have detected *Listeria* species in in caecal contents (66%, 23 of 35 samples), tonsil homogenates (67%, 4 of 6 samples), and ileocaecal lymph nodes (75%, 6 of 8 samples), but infrequently in feces collected before slaughter (4.4%,1 of 23 samples).

Taken together, these data indicate that second generation 5′ nuclease PCR (TaqMan) assays accelerate detection and quantitation of *Listeria* during pork production. This may reduce product recalls as well as reduce human illness and deaths. The development of portable instruments for 5′ nuclease assays will expedite field surveys.

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**Table 1. Sequences and concentrations of primers and probe for amplification of the *iap* gene of *Listeria* species.**

| Primer/Probe | Annealing site | Sequence | Concentration (nM) |
|---|---|---|---|
| *Iap*-P-5 | 49 to 68 | 5′ CGG TAA CAG CAT TTG CTG CGC CAA C 3′ | 250 |
| *Iap*-F | 1 to 26 | 5′ ATG AAT ATG AAA AAA GCA ACT ATC GC 3′ | 300 |
| *Iap*-R | 1419 to 1437 | 5′ TTA TAC GCG ACC GAA GCC A 3′ | 50 |

**Table 2. Specificity of *iap* assay for various *Listeria* strains**.

| Bacteria | NADC strain | Amplification of *iap* |
|---|---|---|
| *L. monocytogenes* (4b) | 2045 | + |
| *L. monocytogenes* (1/2a) | 2052 | + |
| *L. monocytogenes* (4b) | 2840 | + |
| *L. innocua* | 2841 | + |
| *L. innocua* | 2842 | + |
| *L. murrayi* | 2843 | - |
| *L. seelegeri* | 2844 | + |
| *L. welshimeri* | 2845 | + |
| *L. ivanovii* | 6185 | + |
| *S. aureus* | 3649 | - |
| *S. aureus* | 3690 | - |
| *E. coli* | 8176 | - |
| *H. alvei* | 8526 | - |
| *P. vulgaris* | | - |
| *K. pneumoniae* | | - |
| *P. aeruginosa* | | - |
| *E. clocae* | | - |
| *C. coli* | 5095 | - |
| *E. coli* | 8252 | - |