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Short Communication

# Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes

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*Listeria monocytogenes* is an opportunistic bacterial pathogen that is an important cause of human food-borne illness worldwide. However, *L. monocytogenes* strains demonstrate considerable variation in pathogenic potential. In this report, virulent and avirulent *L. monocytogenes* isolates were compared by using a comparative screening strategy. Two clones were identified that contained DNA that was only present in virulent *L. monocytogenes* strains. PCR primers were designed for three genes from these clones and for five other selected *L. monocytogenes* genes. All eight primer sets predominantly detected virulent *L. monocytogenes* isolates, as determined by a mouse virulence assay; one of the putative internalin genes, *lmo2821*, was detected in all strains that were considered to be virulent. Primers from these eight genes were then tested by PCR against a larger panel of bacterial strains; each of the genes was detected predominantly in clinical or food *L. monocytogenes* isolates, rather than environmental isolates. The findings from this study suggest that virulent *L. monocytogenes* strains may possess genes that are not present in avirulent isolates, which could serve as markers for PCR assessment of *L. monocytogenes* virulence.

## Introduction

*Listeria monocytogenes* is an important food-borne pathogen that can cause septicaemia, encephalitis, meningitis and gastroenteritis, particularly in children, the elderly and immunosuppressed individuals; it also causes miscarriage in pregnant women (Robinson *et al.*, 2000). Despite being pathogenic at the species level, *L. monocytogenes* in fact comprises a diversity of strains with varying virulence. Whilst many *L. monocytogenes* strains have pathogenic potential and can result in disease and/or mortality, others have limited capability to establish infection and are relatively avirulent (Barbour *et al.*, 1996, 2001; Erdenlig *et al.*, 2000; Olier *et al.*, 2002; Roche *et al.*, 2003). The ability to distinguish virulent from avirulent strains of *L. monocytogenes* could potentially reduce unnecessary recalls of food products and help to prevent disease outbreaks.

Methods that have been developed for *L. monocytogenes* virulence assessment include mouse virulence assays and *in vitro* culture techniques. The mouse virulence assay provides an *in vivo* measurement of virulence and often serves as a reference standard for other methods (Pine *et al.*, 1991; Nishibori *et al.*, 1995; Erdenlig *et al.*, 2000; Roche *et al.*, 2001). *In vitro* culture techniques measure the ability of *L. monocytogenes* to cause cytopathogenic effects in the enterocyte-like cell line Caco-2 (Pine *et al.*, 1991), to form plaques in the human adenocarcinoma cell line HT-29 (Roche *et al.*, 2001) or to cause death in chicken embryos (Olier *et al.*, 2002). Although they are somewhat slow and technically demanding, these methods have good predictive value.

Unfortunately, no reliable molecular method for prediction of *L. monocytogenes* virulence has been developed. PCR detection of known *L. monocytogenes* virulence genes, such as *inlA*, *inlB*, *actA*, *hlyA*, *plcA* and *plcB*, has not proven suitable for differentiation of virulent and avirulent isolates (Nishibori *et al.*, 1995), as these genes are consistently found in *L. monocytogenes* (Jaradat *et al.*, 2002). Genetic lineage analysis has some predictive value for *L. monocytogenes* pathogenicity; however, the correlation between genetic lineages and pathogenicity is not consistent for all strains (Piffaretti *et al.*, 1989; Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997).

In the current study, we tested PCR primers that were derived from a panel of eight *L. monocytogenes* genes on 12 strains with known virulence and on a larger panel that included clinical, food and environmental isolates. We report evidence

Abbreviations: ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures.

**Table 1.** Bacterial strains used in this study

| Strain | Serovar | Source |
|---|---|---|
| *Listeria monocytogenes*: | | |
| ATCC 19111 | 1 | Poultry |
| ATCC 19112 | 2 | Human |
| ATCC 19113 | 3 | Human |
| ATCC 19114 | 4a | Human |
| ATCC 19115 | 4b | Human |
| ATCC 19116 | 4c | Chicken |
| ATCC 19117 | 4d | Sheep |
| ATCC 19118 | 4e | Chicken |
| ATCC 15313$^T$ | 1 | Rabbit |
| EGD (NCTC 7973) | 1/2a | Guinea pig |
| HCC7 | 1 | Catfish brain |
| HCC8 | 1 | Catfish brain |
| HCC12 | 4 | Catfish brain |
| HCC13 | 4 | Catfish kidney |
| HCC16 | 4 | Catfish brain |
| HCC17 | 4 | Catfish brain |
| HCC18 | 4 | Catfish spleen |
| HCC19 | 4 | Catfish spleen |
| HCC23 | 4 | Catfish brain |
| HCC24 | 4 | Catfish spleen |
| HCC25 | 4 | Catfish kidney |
| 168 | | Aborted calf fetus |
| 180 | | Human outbreak |
| 418 | | Freezer study |
| 742 | | Ground beef |
| 874 | | Cow brain |
| 1002 | | Pork sausage |
| 1084 | | Chicken |
| 1400 | | Jalisco outbreak |
| *Listeria innocua*: | | |
| ATCC 33090$^T$ | 6a | Cow brain |
| 415 | | Turkey burger |
| 416 | | Veal/beef patty |
| 417 | | Beef steak |
| 662 | | Raw milk |
| 1419 | | Ground cheese |
| 1425 | | Pecorino romano |
| 1720 | | Chicken |
| 1944 | | Ground turkey |
| *Listeria grayi*: | | |
| ATCC 19120$^T$ | | Chinchilla faeces |
| ATCC 25400 | | Corn leaves/stalks |
| ATCC 25401 | | Corn leaves/stalks |
| *Listeria ivanovii*: | | |
| ATCC 19119$^T$ | | Sheep |
| 3325 | | Cheese |
| *Listeria seeligeri*: | | |
| ATCC 35967$^T$ | | Soil |
| 3008 | | Soil |
| 3321 | | Cheese |
| *Listeria welshimeri*: | | |
| ATCC 35897$^T$ | | Plant |
| ATCC 43550 | 1/2b | Soil |
| ATCC 43551 | 6a | Soil |
| CCF4 | | Catfish brain |
| 1471 | | Environment |
| *Aeromonas hydrophila* ATCC 35654 | | |
| *Clostridium perfringens* | | |
| *Enterococcus faecalis* ATCC 29212 | | |
| *Escherichia coli* ATCC 25922 | | |
| *Flavobacterium indologenes* | | |
| *Klebsiella pneumoniae* ATCC 13883$^T$ | | |
| *Proteus vulgaris* ATCC 13315$^T$ | | |
| *Pseudomonas aeruginosa* ATCC 27853 | | |
| *Salmonella typhimurium* ATCC 14028 | | |
| *Serratia marcescens* ATCC 8100 | | |
| *Staphylococcus aureus* ATCC 25923 | | |
| *Streptococcus pneumoniae* | | |
| *Streptococcus pyogenes* ATCC 19615 | | |
| *Vibrio cholerae* | | |
| *Yersinia pseudotuberculosis* | | |

that virulent *L. monocytogenes* isolates contain genes that could potentially be used to differentiate virulent and avirulent isolates.

## Methods

**Bacterial strains and genomic DNA extraction.** In total, 52 *Listeria* strains and 15 other common Gram-positive and -negative species were examined (Table 1). *Listeria* strains were cultivated on tryptic soy agar plates with 5 % sheep blood or brain heart infusion (BHI) agar plates. Batch cultures were cultivated in BHI broth at 37 °C with rotary aeration. Genomic DNA was isolated from *L. monocytogenes* and other species by using a previously described technique (Liu *et al.*, 2003).

**Dot-blot hybridization.** A recombinant genomic DNA library of *L. monocytogenes* strain EGD was constructed in pGEM-3Zf(+) (Promega) as described previously (Liu & Yong, 1993; Liu, 1994). Cloned inserts were retrieved from the plasmid vector by using appropriate restriction enzymes (e.g. *Eco*RI and *Pst*I or *Sma*I and *Hin*dIII) for use as probes. Each insert was screened against a panel of six *L. monocytogenes* strains, which consisted of three virulent strains (ATCC 19115, EGD and HCC7) and three avirulent strains (ATCC 15313$^T$, HCC23 and HCC25) by dot-blot hybridization (Liu, 1994; Liu & Yong, 1993). Approximately 300 clones were screened and clones that showed preferential binding to *L. monocytogenes* virulent strains were selected. Plasmid DNA from the selected clones was purified by using a QIAprep Spin Miniprep kit (Qiagen); DNA sequences of the inserts were determined by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

**Mouse virulence assay.** The virulence of 12 *L. monocytogenes* strains was assessed by using a mouse virulence assay (Erdenlig *et al.*, 2000). Female, 6–8-week-old A/J mice (Jackson Laboratory) were housed (five mice per cage) and allowed to acclimatize for 1 week. For each *L. monocytogenes* strain, four groups of mice were inoculated intraperitoneally with 0·1 ml aliquots of appropriate dilutions of bacteria. One group of five mice was injected with 0·1 ml sterile saline and one group of three mice was not injected. On the fifteenth day after inoculation, all surviving mice were euthanized and one mouse per group was necropsied and cultured from the spleen for *L. monocytogenes*. The $LD_{50}$ for each strain was then calculated (Reed & Muench, 1938).

**PCR.** Each reaction was performed in a 25 µl volume of reaction mixture that consisted of 0·5 U *Taq* DNA polymerase (Fisher Scientific), 50 µM dNTPs, 25 pmol each primer and 10 ng DNA. Cycling conditions consisted of 94 °C for 2 min, 25 cycles of 94 °C for 20 s, 60 °C for 20 s and 72 °C for 45 s, and finally 72 °C for 2 min.

## Results and Discussion

### Identification of genes present in virulent *L. monocytogenes*

As a result of comparative screening against a panel of six *L. monocytogenes* strains of known virulence, two clones (lmo2–28 and lmo2–432) that hybridized predominantly with virulent isolates were selected from the recombinant DNA library of *L. monocytogenes* EGD (data not shown).

Clone lmo2–28 contained an insert of approximately 600 bp. DNA sequencing revealed that the first 458 bp corresponded to the genome sequence of *L. monocytogenes* EGD-e (serovar 1/2a) (GenBank accession no. AL591976) at positions 224 432–224 889. Therefore, the clone contained portions of two genes from the *L. monocytogenes* EGD-e chromosome: one encodes a protein similar to transcriptional regulators (*lmo0833*; bp 223 784–224 730) and the other encodes a protein of unknown function (*lmo0834*; bp 224 810–225 537) (Glaser *et al.*, 2001) (Table 2). Neither of these genes has an orthologue in the genome of *Listeria innocua* strain CLIP 11262 (serovar 6a) (Glaser *et al.*, 2001). PCR primers were designed to both genes.

The insert from clone lmo2–432 was approximately 900 bp in length. The first 420 bp of this insert was sequenced; it correlated to the genome sequence of *L. monocytogenes* EGD-e (GenBank accession no. AL591978) at positions 54 835–55 247. Therefore, this clone contained portions of a gene that encodes an unknown protein (*lmo1188*; bp 53 636–55 087) and a gene that encodes a putative transcriptional regulator (*lmo1189*; bp 55 085–55 679). *lmo1188* has no orthologue in the *L. innocua* CLIP 11262 genome; on the other hand, *lmo1189* does possess such an orthologue (Glaser *et al.*, 2001). Therefore, primers were designed to *lmo1188*.

These findings suggest that transcriptional regulator genes might serve as useful virulence markers for *L. monocytogenes*. Therefore, nucleotide–nucleotide BLAST searches were conducted to compare the gene sequences of *L. monocytogenes* transcriptional regulators against the genome sequence of *L. innocua* (Glaser *et al.*, 2001); three genes (*lmo2672*, *lmo1116* and *lmo1134*), which had the lowest nucleotide identity with any sequences from the *L. innocua* genome, were selected from the published *L. monocytogenes* EGD-e transcriptional regulator list. In addition, as internalins play important roles in listerial internalization and virulence (Gaillard *et al.*, 1991; Lingnau *et al.*, 1995; Parida *et al.*, 1998), we selected two genes (*lmo2470* and *lmo2821*) that encode internalin-like proteins from the *L. monocytogenes* EGD-e genome sequence, which do not have orthologues in the *L. innocua* genome (Glaser *et al.*, 2001).

**Table 2.** List of *L. monocytogenes* genes and primers used in the PCR assay

| Gene | Genome segment/location | Putative function | Size (aa) | Primer sequences (5′→3′) | Primer position | Size of PCR product (bp) |
|---|---|---|---|---|---|---|
| *lmo0833* | 4/223 784–224 730 | Transcriptional regulator | 296 | GGCTATTCTTTAGCGGAGGA | 223 996–224 015 | 638 |
| | | | | AGTAGCGCGAGGGATTTGTA | 224 633–224 613 | |
| *lmo1188* | 6/53 621–55 087 | Unknown protein | 483 | TTTCGCCGTTAGAAAATACGA | 54 027–54 047 | 663 |
| | | | | TTCGGACAAAAATTTGAATGG | 54 689–54 668 | |
| *lmo0834* | 4/224 810–225 537 | Unknown protein | 237 | AACTTCGCATTTGTTATGTGTTAC | 224 940–224 963 | 594 |
| | | | | TCACTGACCATTCCTCCAAA | 225 533–225 513 | |
| *lmo1116* | 5/262 997–263 783 | Transcriptional regulator | 257 | GGGAACGATGAAAACGAAGA | 263 006–263 025 | 591 |
| | | | | TGGCTTATCGCACAAGCTAAT | 263 593–263 573 | |
| *lmo2672* | 12/25 985–26 804 | Transcriptional regulator | 268 | CGGCACACTTGGATTCTCAT | 26 117–26 136 | 481 |
| | | | | AGGGCTAGTGACGGATGCTA | 26 597–26 578 | |
| *lmo1134* | 6/8 009–8 368 | Transcriptional regulator | 115 | ACCCGATAGCAAGGAGGAAC | 7 998–8 017 | 367 |
| | | | | AACTTCTCTCGATACCCATCCA | 8 364–8 343 | |
| *lmo2470* | 11/149 254–150 433 | Internalin | 388 | TGATTCCATGCAATTACTAGAACG | 149 527–149 550 | 545 |
| | | | | AGGATTCTAAACTAGGTAAGTTGGTG | 150 071–150 046 | |
| *lmo2821* | 12/188 153–190 708 | Internalin | 851 | TGTAACCCCGCTTACACAGTT | 188 989–189 009 | 611 |
| | | | | TTACGGCTGGATTGTCTGTG | 189 599–189 580 | |

## Mouse virulence assay

Previous mouse virulence testing had shown that strains EGD, ATCC 19115 and HCC7 are virulent, but HCC23 and ATCC 15313$^T$ are avirulent (Erdenlig *et al.*, 2000). In the current study, three of these strains were tested as controls (EGD, ATCC 19115 and ATCC 15313$^T$) and nine additional strains were assessed (Table 3). Strains EGD and 874 were the most virulent of the strains tested ($LD_{50} < 10^8$); ATCC 19115, ATCC 19116, ATCC 19117, HCC8 and 1002 were the next most virulent strains ($LD_{50} < 10^9$). Strains ATCC 19112 and ATCC 19118 had intermediate virulence ($LD_{50} < 10^{10}$) and strains ATCC 19114, HCC25 and ATCC 15313$^T$ were considered to be avirulent ($LD_{50} > 10^{10}$).

Spleens from mice that died during the challenge contained viable *L. monocytogenes* that was recovered on BHI agar and confirmed by PCR. On the other hand, spleens from mice that survived the *L. monocytogenes* challenge by day 15 had no viable *L. monocytogenes* detectable on BHI agar (data not shown).

## PCR

Results of PCR with oligonucleotide primers that were designed from the assembled panel of eight potential virulence genes (Table 2) correlated well with the mouse virulence results: avirulent strains ATCC 19114 and HCC25 ($LD_{50} > 10^{10}$) were non-reactive by PCR, whereas the other strains ($LD_{50} < 10^{10}$) were all PCR-positive for at least two of the primer sets tested (Table 4). In particular, primers derived from *lmo2821* permitted identification of all virulent *L. monocytogenes* strains in our panel. PCR results also correlated with two previously tested strains (Erdenlig *et al.*, 2000): HCC7, which is virulent by mouse assay, was PCR-positive for all primer sets tested, and HCC23, which is avirulent by mouse assay, was PCR-negative for all primer sets tested.

One notable exception was ATCC 15313$^T$, which was positive for seven of the eight genes tested by PCR, but was avirulent by mouse virulence assay. Strain ATCC 15313$^T$ was originally isolated from an infected rabbit during an outbreak of listeriosis, but it later became avirulent after successive laboratory subculturing (Kathariou & Pine, 1991). It has been demonstrated that ATCC 15313$^T$ does not express listeriolysin, a well-known virulence factor (Gaillard *et al.*, 1986), and it has been proposed that this occurred spontaneously after its original isolation (Kathariou & Pine, 1991). Therefore, it would be logical that ATCC 15313$^T$, which is derived from a virulent *L. monocytogenes* isolate, would retain many of its virulence properties. In support of this, ATCC 15313$^T$ has maintained its ability to cause cytopathogenic effects in Caco-2 monolayers (Kathariou & Pine, 1991).

**Table 3.** Mouse virulence trial results

| *L. monocytogenes* strain | Serovar | $LD_{50}$ |
|---|---|---|
| ATCC 19112 | 2 | $1{\cdot}6 \times 10^9$ |
| ATCC 19114 | 4a | $1{\cdot}9 \times 10^{10}$ |
| ATCC 19115 | 4b | $6{\cdot}0 \times 10^8$ |
| ATCC 19116 | 4c | $2{\cdot}6 \times 10^8$ |
| ATCC 19117 | 4d | $8{\cdot}8 \times 10^8$ |
| ATCC 19118 | 4e | $7{\cdot}8 \times 10^9$ |
| ATCC 15313$^T$ | 1 | $> 1{\cdot}2 \times 10^{11}$ |
| EGD | 1/2a | $< 1{\cdot}1 \times 10^7$ |
| HCC8 | 1 | $< 7{\cdot}0 \times 10^8$ |
| HCC25 | 4 | $3{\cdot}5 \times 10^{10}$ |
| 874 | ND | $< 8{\cdot}0 \times 10^7$ |
| 1002 | ND | $5{\cdot}2 \times 10^8$ |

ND, Not determined.

The eight primer sets were then evaluated by PCR against a larger collection of bacterial strains (52 *Listeria* strains and 15 common Gram-positive and -negative species; Table 4). At least two PCR primer sets were positive for 19 of the 29 *L. monocytogenes* strains tested. The remaining 10 *L. monocytogenes* strains were not detected by any of the primers tested. The 19 isolates that were recognized were primarily clinical or food isolates, whereas the negative strains were primarily catfish isolates. The catfish isolates were obtained from healthy channel catfish during routine screening for potential food-borne pathogens. Therefore, they are considered to be environmental isolates that could be isolated from normal catfish as they enter the processing plant. The one PCR-negative strain that was not isolated from catfish was ATCC 19114, which is a human isolate. However, mouse assay confirmed that this strain is avirulent. All eight primer sets generated no PCR products with genomic DNA from other *Listeria* species or the other 15 species tested.

Putative internalin genes were detected in most *L. monocytogenes* strains in our panel, with *lmo2821* being detected in 19 of 29 strains tested and *lmo2470* being present in 18 of 29 strains. In particular, strain 874 was highly virulent in mice, yet it was PCR-positive for the internalin genes and negative for all transcriptional regulator genes in our panel. The putative transcriptional regulator genes *lmo2672* and *lmo1134* were detected in 17 of 29 strains. Genes *lmo1116*, *lmo0834*, *lmo0833* and *lmo1188* were detected in 15, 15, 14 and 13 of 29 strains, respectively.

In summary, our results provide evidence that virulent *L. monocytogenes* isolates contain genes that are not present in avirulent *L. monocytogenes*, and that detection of these genes has the potential to provide an alternative method for distinguishing virulent from avirulent isolates. Further testing on a larger panel of *L. monocytogenes* strains with known virulence will determine whether this technique has the potential to serve as a diagnostic assay.

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**Table 4.** PCR results using primers derived from eight putative *L. monocytogenes* transcriptional regulator and internalin genes

Results shown are for *L. monocytogenes* strains only; all other *Listeria* strains and other bacterial species were negative for all eight primer pairs.

| Strain | *lmo0833/lmo1188** | *lmo0834* | *lmo1116* | *lmo2672/lmo1134*† | *lmo2470* | *lmo2821* |
|---|---|---|---|---|---|---|
| ATCC 19111 | + | + | + | + | + | + |
| ATCC 19112 | + | + | + | + | + | + |
| ATCC 19113 | + | + | + | + | + | + |
| ATCC 19114 | − | − | − | − | − | − |
| ATCC 19115 | − | + | − | + | + | + |
| ATCC 19116 | − | − | + | − | − | + |
| ATCC 19117 | − | + | + | + | + | + |
| ATCC 19118 | − | − | + | + | + | + |
| ATCC 15313$^T$ | + | − | + | + | + | + |
| EGD (NCTC 7973) | + | + | + | + | + | + |
| HCC7 | + | + | + | + | + | + |
| HCC8 | + | + | + | + | + | + |
| HCC12 | − | − | − | − | − | − |
| HCC13 | − | − | − | − | − | − |
| HCC16 | − | − | − | − | − | − |
| HCC17 | − | − | − | − | − | − |
| HCC18 | − | − | − | − | − | − |
| HCC19 | − | − | − | − | − | − |
| HCC23 | − | − | − | − | − | − |
| HCC24 | − | − | − | − | − | − |
| HCC25 | − | − | − | − | − | − |
| 168 | + | + | + | + | + | + |
| 180 | +/− | + | − | + | + | + |
| 418 | + | + | − | + | + | + |
| 742 | + | + | + | + | + | + |
| 874 | − | − | − | − | + | + |
| 1002 | + | + | + | + | + | + |
| 1084 | + | + | + | + | + | + |
| 1400 | + | + | + | + | + | + |

**lmo0833* and *lmo1188* yielded identical PCR results except for *L. monocytogenes* 168, which was positive for *lmo0833* and negative for *lmo1188*.

†*lmo2672* and *lmo1134* yielded identical PCR results.

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