Research Note

Low Potential Virulence Associated with Mutations in the \textit{inlA} and \textit{prfA} Genes in \textit{Listeria monocytogenes} Isolated from Raw Retail Poultry Meat

VICTORIA LÓPEZ,\textsuperscript{1,2} JAIME NAVAS,\textsuperscript{1} AND JOAQUÍN V. MARTÍNEZ-SUÁREZ\textsuperscript{1*}

\textsuperscript{1}Departamento de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Autopista A-6 km 7.5, 28040 Madrid, Spain; and \textsuperscript{2}Unidad de Bioinformática y Salud Pública, Instituto de Salud Carlos III, Carretera de Majadahonda a Pozuelo km 2, 28220 Majadahonda, Madrid, Spain

MS 12-304: Received 12 July 2012/Accepted 9 September 2012

\textbf{ABSTRACT}

Packaged raw foods can represent a potential source of \textit{Listeria monocytogenes} contamination when opened at home, and listeriosis is associated with the consumption of undercooked raw foods. The aim of this study was to characterize a group of \textit{L. monocytogenes} strains isolated from 56 packages of raw chicken meat from a single brand in order to determine the diversity of the strains that dominate in a particular food over time, as well as their pathogenic potential. Forty (71\%) samples were found to be positive for \textit{L. monocytogenes}, and three isolates per sample were subjected to PCR molecular serotyping. Subtyping of 45 isolates from different manufacturing dates (\(n = 40\)) or different molecular serotype within the same sample (\(n = 5\)) identified 11 different \textit{L. monocytogenes} subtypes as defined by pulsed-field gel electrophoresis and sequencing of virulence genes \textit{actA} and \textit{inlA}. Two of the subtypes accounted for 51\% of the isolates. About 40\% of isolates (three subtypes) were found to potentially present attenuated virulence because of the presence of mutations in the \textit{prfA} and \textit{inlA} genes.

\textit{Listeria monocytogenes} is an important foodborne pathogen, and virtually all human listeriosis cases are caused by ready-to-eat (RTE) foods that permit \textit{L. monocytogenes} growth. The relevance of \textit{L. monocytogenes} is primarily because of the severity of disseminated listeriosis, which is associated with high mortality rates (17\% in the European Union in 2010) (3). The prevalence of this pathogen is particularly high in poultry meat samples (15, 16, 25). Overall incidence rates of 25\% for \textit{L. monocytogenes} in fresh poultry have been reported, although contamination rates as high as 70\% have also been found during the latter stages of production and at the retail level (25). Raw chicken, as such, does not constitute a serious foodborne illness problem, but sporadic listeriosis can be associated with the consumption of undercooked chicken (26). In addition, cross-contamination of RTE foods with any raw meat or poultry product can occur within the retail (10) or home setting (21).

The actual rate of human listeriosis is low, and the incidence of the disease depends on various factors, including the virulence of the specific strain involved (7). \textit{L. monocytogenes} is subdivided into at least four evolutionary lineages (I through IV), which have different levels of virulence (19). The virulence attenuation presented by a large number of lineage II isolates is because of mutations that give rise to premature stop codons (PMSC) in the \textit{inlA} gene (which encodes internalin A) (8, 9), along with mutations in the \textit{prfA} gene (which encodes the central virulence regulator PrfA) (11, 23). Nevertheless, multiple distinct genetic mechanisms could be responsible for natural virulence attenuation in \textit{L. monocytogenes}. For example, naturally occurring, low-virulence \textit{L. monocytogenes} strains might also have causal mutations in other genes such as \textit{plcA} and \textit{inlB} (24). Virulence could therefore vary among the different strains of \textit{L. monocytogenes}, although no single marker is available that allows the virulence of field isolates from this species to be determined, so the actual incidence of avirulent strains of \textit{L. monocytogenes} in raw foods is not accurately known (24). The presence of varying levels of virulence in \textit{L. monocytogenes} has important implications for the detection of \textit{Listeria} strains and risk analysis.

The aim of this study was to characterize a group of \textit{L. monocytogenes} strains isolated from raw chicken meat in order to determine the diversity of the strains that dominate in a particular food over time, as well as analyze their pathogenic potential.

\textbf{MATERIALS AND METHODS}

\textbf{Sampling procedure and \textit{Listeria} analysis.} Fifty-six packages of raw ground chicken meat from a single brand were obtained from a local retailer over a 2-month period. The samples were processed and analyzed as described previously (16, 17), according to the procedure of the U.S. Department of Agriculture for detecting \textit{L. monocytogenes} (28).
Molecular typing and virulence gene sequencing. Three colonies from each positive sample were selected for PCR confirmation (17). The confirmed isolates were typed as described previously (12), by: (i) multiplex-PCR serotyping (2), an assay that differentiates isolates into major molecular serotypes (1/2a, 1/2b, 1/2c, and 4b), each of which represents more than one serotype, (ii) pulsed-field gel electrophoresis (PFGE), according to the protocol standardized and optimized by PulseNet for L. monocytogenes (5); and (iii) partial sequencing of virulence gene actA, as well as sequencing of the entire inlA gene, by using primers and PCR conditions described previously by Revazishvili et al. (22) and by Ragon et al. (20). In two of the isolates of the nonhemolytic strain R6 (Table 1), sequencing of prfA gene was performed on 295-bp DNA fragments generated by PCR with the primers prfAF (5’-AACGGGAAGCTTGGCTCTAT-3’) and prfAR (5’-TTCCCCAAGTAGCAGGACAT-3’).

Potential virulence phenotypes. The activity of virulence-associated proteins (listeriolysin O [LLO], phosphatidylinositol–phospholipase C [PC-PLC], and phosphatidylglycerol–phospholipase C [PI-PLC]) was determined semiquantitatively, based on the width of the halos formed by strains inoculated into the relevant differential solid media (sheep blood agar [bioMérieux, Inc., Marcy l’Étoile, France], Brilliance Listeria agar [Oxoid, Ltd., Basingstoke, UK], and CHROMagar Listeria [CHROMagar, Paris, France], respectively), after incubation for 48 h at 37°C. Titrations to determine the hemolytic activity were likewise performed with the supernatant of 6-h cultures (1). In addition to the sequencing of the inlA gene, we also performed a Western blot analysis of InlA by using a previously characterized monoclonal antibody against InlA (L7.7) (14), as described previously in detail (13).

In vivo mouse bioassay. Nonhemolytic strains were also analyzed by means of a bioassay to test their pathogenicity in artificially immunocompromised mice (6, 27). The bioassays were performed at the Vigilancia Sanitaria Veterinaria facilities of the Veterinary Department of the Complutense University, Madrid, Spain, in accordance with a protocol approved by their Animal Care Committee. Each strain was inoculated intraperitoneally into five mice at 10⁴ CFU per mouse.

### Table 1. Characteristics of Listeria strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>PFGE type</th>
<th>Molecular serotype</th>
<th>No. of isolates</th>
<th>Hemolytic activity titer</th>
<th>inlA PMSC type</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>7C</td>
<td>1/2a</td>
<td>6</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>R2</td>
<td>5D</td>
<td>1/2b</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>R3</td>
<td>5F</td>
<td>1/2b</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>R4</td>
<td>6E</td>
<td>1/2a</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>R5</td>
<td>8H</td>
<td>1/2a</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>R6</td>
<td>9G</td>
<td>1/2a</td>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>R7</td>
<td>10B</td>
<td>1/2b</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>R8</td>
<td>1A</td>
<td>1/2c</td>
<td>14</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>R9</td>
<td>2B</td>
<td>1/2b</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>R10</td>
<td>4B</td>
<td>1/2b</td>
<td>9</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>R11</td>
<td>3C</td>
<td>1/2a</td>
<td>3</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>ATCC BAA-679 (EGDe)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>L. innocua ATCC 33090T</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* L. monocytogenes unless otherwise stated.

* Numbers and letters represent the different AscI and ApaI PFGE profiles, respectively.

* Reciprocal of the highest dilution of culture supernatants at which 100% hemolysis took place (1).

* Identification of the L. monocytogenes inlA PMSC mutation types according to Van Stellen et al. (29). 0, full-length inlA sequence.

* NA, not applicable.

Reference strains. The reference strains used in the virulence assays were Listeria innocua ATCC 33090T and L. monocytogenes ATCC BAA-679 (EGDe) (as negative and positive controls, respectively).

### RESULTS AND DISCUSSION

Samples and L. monocytogenes isolates. All culture-positive samples had less than 100 CFU/g of L. monocytogenes before enrichment. After enrichment, 40 (71%) samples were found to be positive for L. monocytogenes, and a total of 120 isolates were identified and confirmed to be L. monocytogenes. A high prevalence of L. monocytogenes in raw chicken meat, especially after grinding, is commonly reported (15, 16, 25).

Molecular subtypes. Serotyping by PCR revealed that 45% of the isolates belonged to molecular serotype 1/2b, 32.5% to 1/2c, and 22.5% to 1/2a (Table 1). Eleven different PFGE types or pulsotypes (R1-R11) (Table 1) were identified from a selection of 45 isolates from different manufacturing dates (n = 40) or different molecular serotype within the same sample (n = 5). The two predominant pulsotypes, R8 (1/2c, 31% of isolates) and R10 (1/2b, 20% of isolates), accounted for more than half of all isolates.

From previous studies on the diversity of L. monocytogenes in populations of different origins (4), it has been observed that PFGE with AscI and ApaI has a high discriminatory power (Simpson’s index of diversity = 0.995). Using the same enzymes, the diversity of the pulsotypes in our sample was estimated at 0.846. This result can be interpreted as showing a relatively low genetic diversity in this group of 45 L. monocytogenes isolates, obtained over a 2-month period. The same subtypes found in raw chicken meat at the retail level could also be found in the poultry processing environments (13, 15). Thus, the presence of a limited number of specific subtypes of L. monocytogenes in retail food taken from a single brand over...
time could be subsequently used to identify persistent strains and determine their transmission in the corresponding processing plant.

**Pathogenic potential.** On the different solid media plates, all strains displayed similar LLO, PI-PLC, and PC-PLC activity, except for strain R6, in which no LLO or PC-PLC activity was detected, and PI-PLC activity was low but clearly detectable in CHROMagar *Listeria* after incubation for 48 h at 37 °C. These strains were in fact isolated from another differential medium for PI-PLC (Agar *Listeria* according to Ottaviani and Agosti) (17). Titration of LLO confirmed that the only nonhemolytic strain in this group was R6 (Table 1).

Sequencing of the virulence genes revealed that there were 10 different actA sequences and 11 different inlA sequences in the 11 different pulsotypes. The DNA sequences were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers EF558924 to EF558933 (actA) and EF558934 to EF558944 (inlA). The sequences of both genes were grouped by genetic similarity analysis according to their lineage. Three previously described PMSC in the inlA sequence (29) were detected in subtypes R4, R6, and R8 (Table 1). Western blot analysis of InlA confirmed that all three strains with PMSC in their inlA gene sequences (R4, R6, and R8) did not express the entire protein. These three pulsotypes represented 18 of the 45 isolates studied (Table 1), meaning 40% of the isolates from this group would present attenuated virulence (8, 18).

**Mouse bioassay.** No mouse death was observed in the case of the R6 strain or that of *L. innocua*, whereas all mice inoculated with *L. monocytogenes* EGDe died. Therefore, the R6 strain, which showed no hemolysin or lecithinase activity, was avirulent in immunocompromised mice. Sequencing of the prfA gene of this R6 strain revealed that it had a seven-nucleotide insertion at codon 171, which would give rise to a truncated PrfA protein (PrfAΔ174-237) (23). This mutation can revert when using high infective doses in certain animal models, giving rise to a fully virulent phenotype (11).

All of the results published on avirulent strains of *L. monocytogenes* suggest that their prevalence might have been underestimated in the past. According to Roche et al. (24), if one includes all of the strains that are nonhemolytic, have either a truncated InlA (approximately 45% of the *L. monocytogenes* isolates from RTE foods (29)) or prfA mutations (about 25% of the low-virulence strains (23)), or present any other defects in potential virulence, avirulent or low virulence strains could easily represent 50% of all *L. monocytogenes* field strains. Thus, *inlA* PMSC appear the most common virulence attenuation mechanism at the population level (29), although prfA mutants could be common at specific food environments (11). One possibility for the underestimation of avirulent strains of *L. monocytogenes* is that most agar media currently used for the isolation of *Listeria* are based on the detection of virulence-associated proteins (LLO, PI-PLC, and PC-PLC).

In conclusion, this study identified three strains of *L. monocytogenes* with potentially reduced virulence because of the presence of mutations in the *inlA* gene (and one of the three also in the prfA gene), and these accounted for 40% of the isolates obtained from 56 samples of raw chicken meat. As a large number of *L. monocytogenes* isolates from foods may be avirulent, their study and genetic characterization are essential to develop more accurate risk assessments supported by scientific data (8, 18, 19). This is usually studied in isolates from RTE foods including different poultry products (29), but packaged raw foods, especially chicken meat, can represent a potential source of *L. monocytogenes* contamination when opened at home (21).

**ACKNOWLEDGMENTS**

The research work was supported by the projects RTA2008-00080-C02 and RTA2011-00098-C02 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria–Fonds européen de développement régional) of the Spanish Ministry of Economy and Competitiveness. We thank Sagrario Ortiz and Pilar López for her assistance.

**REFERENCES**


17. Navas, J., S. Ortiz, P. López, V. López, and J. V. Martínez-Suárez. 2007. Different enrichment procedures for recovery of Listeria monocytogenes from raw chicken samples can affect the results of detection (by chromogenic plating or real-time PCR) and lineage or strain identification. J. Food Prot. 70:2851–2854.