Virulence of *Listeria monocytogenes* Isolates from Humans and Smoked Salmon, Peeled Shrimp, and Their Processing Environments

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**ABSTRACT**

The virulence of 82 *Listeria monocytogenes* isolates from human cases and cold-smoked salmon, cooked peeled shrimp, and their production environments was assessed using the plaque-forming assay and a subcutaneous inoculation test in mice. These isolates were previously typed using serotyping and pulsed-field gel electrophoresis. The isolates from food-production environments were collected in several surveys over the period of 5 years. Sixty-eight (99.8%) of 69 isolates tested from food and food-processing environments were considered virulent while only one was avirulent. All clinical isolates (13) were highly virulent. The isolates were from raw materials, final products, and the production environment. This stresses the importance of hygiene in the processing environment as well as among personnel to avoid contamination of the final product.

The relative ease in finding *Listeria monocytogenes* in food and food-production environments has created interest in developing tests to identify virulent strains (14). Many qualitative and quantitative tests to study the virulence of *L. monocytogenes* strains have been developed, including tissue culture assays and experimental infection models of immunocompromised and immunocompetent laboratory animals, particularly mice (2, 14, 15).

In previous publications, we have characterized isolates from cold-smoked salmon (CSS) (7), cooked peeled shrimp (CPS) (6), processing environments, and patients (8). We have demonstrated that although the raw material and the production environments are contaminated with *Listeria* sp., *L. monocytogenes* is an infrequent contaminant of Icelandic CSS and CPS products. As both CSS and CPS are ready-to-eat (RTE) items, evaluation of potential virulence of *L. monocytogenes* isolates at the end of the production line is critical to evaluate food safety.

The aim of this project was to assess the virulence of *L. monocytogenes* isolated from processing environments and end products of shrimp and salmon, and to compare it with the virulence of isolates from cases of human listeriosis using a plaque-forming assay (PFA) and inoculation in mice. The information gained in this study is a valuable input for risk assessment.

**MATERIALS AND METHODS**

**Isolates.** Eighty-two *L. monocytogenes* isolates from CSS and its processing environment (n = 42), CPS and its processing environment (n = 27), and human clinical isolates (n = 13) were included in the study. All isolates have been characterized by pulsed-field gel electrophoresis (PFGE) and serotyping (6–8). To serve as negative controls, eight *L. innocua* and two *L. seeligeri* isolates were included. The *L. monocytogenes* isolates represented 33 pulsotypes (20 from CSS and 13 from CPS) and between 1 and 14 isolates from each pulsotype were examined. The number of isolates analyzed from a particular pulsotype reflected the number of isolates in the respective pulsotype.

**Cell line.** The human adenocarcinoma cell line HT-29 (ECACC no. 85061109, Salisbury, UK) was used between passages 30 and 45. Cells were grown in 75-cm² plastic tissue culture flasks (NUNC A/S, Roskilde, Denmark) using standard culture medium (Dulbecco’s modified Eagle medium [DMEM], 4.5 g liter⁻¹ glucose DMEM; Invitrogen, Carlsbad, Calif.) supplemented with 10% (vol/vol) fetal calf serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). Antibiotics (100 IU ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin; Sigma, St. Louis, Mo.) were added to the culture medium except for the medium used for the virulence assay. Cells were maintained in an incubator (Forma Scientific division of Malinckrodt Inc., Marietta, Ohio) at 37°C under 5% (vol/vol) CO₂ (14).

**PFA.** The plaque-forming assay (PFA) was performed according to Roche et al. (14). Trypsinized HT-29 cells (3 × 10⁴) were deposited in 96-well tissue culture plates (NUNC A/S) and cultured to obtain a constant proliferation rate. Cells were incubated in media containing antibiotics for 3 days to obtain confluent monolayers followed by incubation in media without antibiotics for an additional 24 h. The *Listeria* strains were grown overnight on brain heart infusion agar slopes (BHIA; Becton Dickinson, Sparks, Md.) and diluted to a concentration of 2 × 10⁸ bacterial cells per ml in DMEM using photospectrometer (NovaspecII, Cambridge, UK). Cell monolayers were infected with 2 to 7 log *Listeria* per well for 2 h at 37°C, followed by an incubation for 1.5 h in culture media containing 10 μg ml⁻¹ gentamicin (Sigma). Each well was then overlayed with medium containing 0.48% indubiose (BioSepra A.S., Cercy-Saint-Chris-
tophe, France) in gentamicin-supplemented (10 μg ml\(^{-1}\)) culture media, the same medium without indubiose was then added to prevent cell starvation. Using an inverted microscope, the plaques were counted after 24 h of incubation and again after 48 h if the plaques were too small. \(L.\) \(monocytogenes\) EGD (well-characterized strain of serotype 1/2a) was used for each experiment as a reference strain. The PFA test was performed in duplicate, i.e., twice for each isolate. All \(L.\) \(monocytogenes\) isolates were stored at \(-70°C\) in tryptic soy broth (Becton Dickinson) with 0.6% yeast extract (TSB+YE; Becton Dickinson) and 20% glycerol (Norsk Medisinaldepot, Oslo, Norway).

Enumeration of viable \(Listeria\) strains deposited was performed on tryptic soy agar (TSA; Becton Dickinson) plates with 0.6% yeast extract. The results were calculated as the number of plaques obtained for 7 log \(Listeria\) deposited per well. Roche et al. (14) reported a cut-off level of 3.34 log, in this study we had to establish a new cut-off level at 1.9 log. This was done because the PFA value of the EGD reference strain, which was 6.32 ± 0.12 in the work of Roche et al., was significantly lower in our study, being 4.88 ± 0.38 (measured 69 times). This was confirmed with the mouse virulence assay.

**Hemolysis.** The hemolytic activity was evaluated by streaking isolates onto horse blood agar plates (4). Following overnight incubation at 37°C, the zone of clearing around a single colony was measured with a transparent ruler. Results were expressed in millimeter of clearance measured from the edge of a colony to the end of the clearing. If isolates were nonhemolytic it was repeated using titration of hemolytic activity (14).

**Statistical analysis.** Data analysis was carried out using Number Cruncher Statistical Software (NCSS, 329, North 1000 East, Kaysville, Utah). The Student's \(t\) test was carried out to study the difference between serovars 1/2 and 4 at 95% level.

**Mouse virulence assays.** Seven-week-old conventional Swiss female mice were used (Iffa-Credo, Saint-Germain-sur-l’Arbresle, France). They were maintained on sterilized wood shavings with free access to water and sterilized food. Groups of five mice were inoculated subcutaneously (s.c.) into the left hind footpad for each strain. Bacteria were grown on BHIA slopes (Difco, Becton Dickinson) for 17 h at 37°C. The culture was standardized turbidimetrically and diluted appropriately in phosphate-buffered saline (PBS, pH 7.3). The inocula were resuspended to obtain 4 log CFU in 50 μl for s.c. injection (1). Each inoculum was verified by determining viable counts on TSA plates (bio-Mérieux, Marcy l’Etoile, France). Mice were sacrificed by cervical dislocation 3 days after s.c. injection. Spleens were removed aseptically. Samples were ground and homogenates were appropriately diluted in PBS and plated onto TSA plates. Viable numbers of bacteria were assessed after incubation at 37°C for 48 h. The results are expressed as the number (log) of bacteria per organ. The average number of CFU per sample was calculated only from positive samples.

**RESULTS**

Of 69 isolates tested from food and food-processing environment, 68 (98.5%) were considered virulent and 1 was avirulent. The results are shown in Table 1. The mean number of plaques are grouped according to isolate source and serotype. The clinical isolates were all found to be highly virulent (mean number of plaques for 7 log equals 5.36 ± 0.4). The reference strain, \(L.\) \(monocytogenes\) EGD, gave a mean 4.88 ± 0.38 plaques for 7 log (measured 69 times). All \(L.\) \(nocua\) and \(L.\) \(seeligeri\) isolates tested were avirulent (data not shown).

One isolate of serotype 4b was found to be avirulent; it was nonhemolytic on horse blood agar, but when the hemolytic activity was titrated, it showed a titer of 4 (EGD has titer of 16). All other isolates demonstrated hemolytic activity on blood agar with a 2- to 3-mm diameter clearance zone in no correlation with the PFA value.

Isolates of serovar 1/2 showed lower mean PFA value (3.92 ± 0.94) than serovar 4 (5.16 ± 1.11, excluding nonhemolytic strain) \((P < 0.05)\). This difference was not noted between clinical isolates (serovar 1/2, 5.38 ± 0.18; serovar 4, 5.33 ± 0.65) \((P > 0.05)\).

Isolates that gave a PFA value below 3.34, the cut-off limit that Roche et al. (14) reported, were tested using the mouse virulence assay. The mouse bioassay virulence assay was performed on 20 isolates. Only one isolate tested in the mouse virulence assay was avirulent.

**DISCUSSION**

All isolates were found to be virulent, except one which was avirulent. This finding is more or less in agreement with other publications where the proportion of virulent \(L.\) \(monocytogenes\) isolates from food and environment ranged from 20 to 97%. However, none of these reports could detect any systematic differences in virulence between clinical and food isolates of \(L.\) \(monocytogenes\) (2, 3, 5, 12, 15–17). Some studies have shown that all nonvirulent isolates tested were derived from cheeses (12, 16).

No correlation between the PFA assay and the size of hemolysis zones produced by \(L.\) \(monocytogenes\) on blood agar was observed. All isolates showed hemolysis zones on blood agar, except the avirulent isolate that showed hemolysis when it was titrated, which is in agreement with the work of others (2, 9, 16, 19). Hemolytic activity of \(L.\) \(monocytogenes\) has no direct relation with the virulence, and thus the hemolytic activity cannot be a reliable criteria for the virulence assessment of \(L.\) \(monocytogenes\) (19).

The study revealed that food isolates of serovar 4 showed a higher number of plaques than isolates of serovar 1/2; however, this was not the case for the clinical isolates. Although the key virulence factors known to date (e.g., listeriolysin O, phospholipases, ActA, and internalins A and B) are present in all serotypes, their regulation of expression may differ among serotypes.

In this study, the isolates belonged to 33 pulsotypes (6, 7). It was noticed that one pulsotype that was found to dominate in the CSS production environment (7) had a significantly lower PFA value (2.95 ± 0.95) than other pulsotypes. Several researchers have studied the connection between molecular types and virulence. \(L.\) \(monocytogenes\) has been divided into three distinct genotypic lineages using ribotypes and virulence gene alleles for actA, hly, and inlA. Lineage I contained all strains isolated during epidemic outbreaks of listeriosis, lineage II strains were most common among food isolates, and lineage III represented about 15% of animal clinical isolates (18). Lineage I contains all the strains belonging to serotypes 4b and 1/2b, lineage II contains all the strains belonging to serotypes 1/2a and 1/2c,
TABLE 1. Mean number of plaques according to the source of strains

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>Serotype</th>
<th>n</th>
<th>PFA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hemolysis&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical isolates</td>
<td>4b</td>
<td>5</td>
<td>5.33 ± 0.65</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2a</td>
<td>4</td>
<td>5.28 ± 0.20</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2b</td>
<td>4</td>
<td>5.48 ± 0.12</td>
<td>+</td>
</tr>
<tr>
<td>Cold-smoked salmon processing</td>
<td>1/2a</td>
<td>5</td>
<td>5.32 ± 0.41</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2b</td>
<td>2</td>
<td>5.11 ± 0.52</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2c</td>
<td>1</td>
<td>4.62 ± 0.14</td>
<td>+</td>
</tr>
<tr>
<td>Processing environment</td>
<td>1/2a</td>
<td>14</td>
<td>3.69 ± 1.04</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2b</td>
<td>6</td>
<td>4.61 ± 0.39</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2c</td>
<td>2</td>
<td>4.05 ± 1.00</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>1</td>
<td>0</td>
<td>— d</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>1</td>
<td>5.76 ± 0.19</td>
<td>+</td>
</tr>
<tr>
<td>Final product</td>
<td>1/2a</td>
<td>10</td>
<td>3.1 ± 0.50</td>
<td>+</td>
</tr>
<tr>
<td>Cooked peeled shrimp processing</td>
<td>1/2a</td>
<td>1</td>
<td>4.14 ± 0.12</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2c</td>
<td>1</td>
<td>3.45 ± 0.28</td>
<td>+</td>
</tr>
<tr>
<td>Shrimp shell</td>
<td>1/2a</td>
<td>1</td>
<td>4.09 ± 0.39</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>1</td>
<td>4.77 ± 0.83</td>
<td>+</td>
</tr>
<tr>
<td>Processing environment</td>
<td>1/2a</td>
<td>1</td>
<td>3.81 ± 0.32</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2c</td>
<td>8</td>
<td>3.52 ± 0.37</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/2c</td>
<td>4</td>
<td>3.98 ± 1.17</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>6</td>
<td>5.47 ± 0.23</td>
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</tr>
<tr>
<td></td>
<td>4ab</td>
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<td>5.31 ± 0.06</td>
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</tr>
<tr>
<td></td>
<td>4c</td>
<td>3</td>
<td>4.42 ± 2.30</td>
<td>+</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>82</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The values are presented as the mean (log) ± standard deviation.

<sup>b</sup> The zone of clearance for hemolytic strains ranged from 2 to 3 mm. Hemolytic activity is expressed as positive or negative.

<sup>c</sup> Not typeable using the commercial H antisera.

<sup>d</sup> This isolate was positive in hemolytic titration.

and lineage III contains serotypes 4a and 4c strains (10). This division has not been made according to pulsotypes. Nørrung and Andersen (12) correlated electrophoretic types (ETs) to virulence and found that all the nonvirulent strains belonged to 2 of 33 ETs. Norton et al. (11) found 3 of 18 ribotypes among isolates from the smoked fish industry with attenuated virulence.

Norton et al. (11) suggested that the smoked fish industry represents a good model system to investigate the hypothesis that some of the *L. monocytogenes* subtypes present in food and food-processing environments may have limited human-pathogenic potential. A better understanding of the characteristics of virulence-attenuated and avirulent *L. monocytogenes* subtypes and the ability to rapidly identify them could prevent product recalls due to subtypes that do not present a public health risk (11).

In conclusion, this study shows that the majority of the isolates from seafood production environments are virulent. This stresses the importance of hygienic processes in the production environments and among personnel striving to prevent contamination of the final product. These information will be useful for risk assessment. The fact that virulent strains predominate in the production environment stresses the importance of limiting the shelf life of products like CSS to minimize the risk of harm in the consumer.

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REFERENCES


6. Gudmundsdóttir, S., B. Gadbjørnsdóttir, H. Einarsson, K. G. Kris-


