Observation of a New Pattern in Serogroup-Related PCR Typing of Listeria monocytogenes 4b Isolates

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Molecular serogroup-related PCR typing has made the determination of serotypes of Listeria monocytogenes isolates easy and rapid. Amplification of selected lineage- and serotype-related genes can produce serotype patterns reflecting the four major serotypes, 1/2a, 1/2b, 1/2c, and 4b. We found that four isolates in our routine testing had a pattern with the four hands lmo0737, ORF2110, ORF2189, and prs positive, a pattern which has not been previously reported in the literature. After testing with a lineage-specific PCR, hybridization, and conventional agglutination serotyping, the isolates with the new pattern were considered to be serotype 4b.

Listeria monocytogenes is the pathogen responsible for listeriosis in humans and animals, resulting in severe clinical symptoms and high fatality rates (20). Sporadic and large outbreaks are associated with the consumption of L. monocytogenes-contaminated food (16). To investigate the relationship between contaminated foods and clinical outbreaks, a number of molecular methods and conventional serotyping techniques have been developed and used (1, 4, 11–13). Due to the epidemiological and clinical relevance of serotypes of this pathogen, serotyping is still required as a first-level subtyping method to characterize suspected L. monocytogenes isolates. There are 13 identified serotypes of L. monocytogenes, 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (2). Various molecular analyses have revealed that L. monocytogenes is divided into three distinct lineages and that the lineages are associated with serotypes (24, 27). A PCR method has been developed to determine the lineages based on prfA virulence gene clusters (26).

The conventional serotyping scheme has the following disadvantages. It is time-consuming, it is inconvenient for dealing with a large number of samples, and the cost of commercially produced antisera is high (6). A number of serogroup-related PCR typing schemes have been developed to overcome the shortcomings of conventional serotyping (3, 5, 6, 10, 17, 22). The typing scheme developed by Doumith et al. (6) has been validated by interlaboratory comparisons, and we found this typing scheme easy to use and the interpretation of the PCR band patterns to be straightforward (7, 14). The scheme includes five genes in a multiplex PCR, producing five distinct band patterns, 1/2a (370 and 691 bp), 1/2b (370 and 471 bp), 1/2c (370, 691, and 906 bp), 4b (370, 471, and 597 bp), and 4a and 4c (370 bp) (6, 17). This scheme has limitations. The PCR pattern of 1/2a isolates is the same as that of 3a isolates. Similarly, the multiplex PCR does not differentiate 1/2b strains from 3b strains, 1/2c strains from 3c strains, or 4b strains from 4e and 4ab strains (6, 17). However, the serotypes identified in food and patient isolates are predominantly 1/2a, 1/2b, 1/2c, and 4b, accounting for >96% of the isolates. In addition, 3a, 3b, 3c, 4e, and 4ab are rarely encountered and seldom implicated in human listeriosis (11, 14, 15, 25). Therefore, in terms of epidemiological significance, this lack of precision may not have a great impact and only the main clinical strains will be referred to henceforth.

Recently, a new PCR typing pattern in three L. monocytogenes isolates among patient isolates submitted from New South Wales (NSW) was observed in this laboratory. The same new pattern was noted in a Victoria (VIC) patient sample as well. Here we report the PCR typing pattern, biochemical identification, lineage determination, hybridization, and conventional agglutination serotyping results of these four isolates. They were identified as 4b isolates.

Isolates. A total of four patient isolates with the new PCR typing pattern were analyzed, of which three were from NSW and one was from VIC. There was no epidemiological link identified among the cases, which had no unusual features. An additional four isolates PCR typed as 1/2a, 1/2b, 1/2c, and 4b and 4b type strain NCTC11994 were used as controls in this study (Table 1).

PCR serotyping and lineage determination. PCR typing was done as previously described by Doumith et al. (6). Lineage determination was carried out as described by Ward et al. (26).

Biochemical tests. The four isolates with the new PCR pattern (10M127, 10M130, 10M138A, and 10M198) and one 4b pattern isolate (10M123) were biochemically tested with the API Listeria kit (bioMérieux, Inc., Durham, NC) by following the manufacturer’s instructions.

Conventional serotyping. Samples were serotyped with a commercially available serotyping kit (Denka Seiken Co., Tokyo, Japan) by following the manufacturer’s instructions.

Hybridization. To verify the five expected amplified PCR bands in the PCR typing reactions, the Luminex xMAP system...
(Luminex Co., Austin, TX), a microsphere-based suspension array technique, was used (8). Among the five primer sets, each of the forward primers for \textit{lmo1118}, \textit{lmo0737}, ORF2110, ORF2819, and \textit{prs} was labeled with biotin. The capture probes corresponding to the five genes were modified with Amino Modifier C12 for coupling to carboxylated microspheres. The primers and probes were supplied by GeneWorks (Hindmarsh, SA, Australia). The multiplex PCR and detection were performed as described by Doumith et al. (6) and Dunbar et al. (8).

In the initial PCR, three isolates, 10M127, 10M130, and 10M138A, had four bands, \textit{lmo0737}, ORF2110, ORF2819, and \textit{prs}, positive in the gel. Soon, a similar observation was encountered in VIC (Fig. 1A). The isolate (10M198) was sent to the Queensland Public Health Microbiology Laboratory for further analysis along with the NSW isolates.

The first test done for these four isolates was to find out if they had aberrant biochemical reactions or not with the API \textit{Listeria} kit. Another 4b isolate from NSW was used as a control. The biochemical profiles of these five isolates were the same, and the API numerical profile was 6510 with beta-hemolysis. They were identified as \textit{L. monocytogenes}, suggesting that these four isolates were typical \textit{L. monocytogenes} isolates without aberrant biochemical reactions.

A lineage identification test was carried out, as a strong association between lineages and serotypes has been found in \textit{L. monocytogenes} isolates (26). The lineages can be identified

\begin{table}
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\begin{tabular}{|l|l|l|l|l|l|l|l|}
\hline
QHFSS' lab no. & Original lab no. & Origin & PCR typing & Conventional serotyping \hline
 & & & & O antigen & H antigen & Serotype \hline
10M127 & SSW0021290 & NSW & New pattern & V, VI & A, B, C & 4b \hline
10M130 & NSA0196319 & NSW & New pattern & V, VI & A, B, C & 4b \hline
10M138A & GWA0005589 & NSW & New pattern & V, VI & A, B, C & 4b \hline
10M198 & MDU1004756 & VIC & New pattern & V, VI & A, B, C & 4b \hline
10M123 & SSW0019069 & NSW & 4b & V, VI & A, B, C & 4b \hline
NCTC11994* & NCTC11994 & University of Queensland & 4b & V, VI & A, B, C & 4b \hline
10M131 & SSW0019638 & NSW & 1/2a & I, II & A, B & 1/2a \hline
10M134 & NSA0195546 & NSW & 1/2b & I, II & A, B, C & 1/2b \hline
09M3788 & SSW09M3788 & Queensland & 1/2c & I, II & B, D & 1/2c \hline
09M706 & MDU911366 & VIC & Otherb & V, VII & A, B, C & 4c \hline
\hline
\end{tabular}
\caption{The isolates used in this study and the conventional serotyping results of these isolates}
\end{table}

\textit{a} The type strain was from The Australian Collection of Microorganisms, curated in the University of Queensland.

\textit{b} “Other” means that the serotype might belong to lineage III of either 4a or 4c.

\textit{c} QHFSS, Queensland Health Forensic and Scientific Services.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{PCR typing patterns and lineage determination results obtained with the isolates used in this study. (A) PCR typing results. The sample order for lanes 1 to 10 is 10M127, 10M130, 10M138A, 10M198, 10M123, ACM98, 10M131, 10M134, 09M3788, and 09M707, respectively. Lanes M, DNA size marker GeneRuler (Fermentas). The amplified bands, from top to bottom, are \textit{lmo1118} (906 bp), \textit{lmo0737} (691 bp), ORF2110 (597 bp), ORF2819 (471 bp), and \textit{prs} (370 bp). (B) Lineage determination test. The sample order for lanes 1 to 10 is the same as that in panel A. Results are detailed in text.}
\end{figure}
by the specific virulence gene prfA PCR method. Lineage I contains serotype 1/2b and 4b isolates, producing a 373-bp PCR product with the actA1-t and actA1-r primers. Lineage II consists of serotypes 1/2a and 1/2c, producing a 564-bp PCR fragment with the plcB2-t and plcB2-r primers. Lineage III contains serotype 4c and part of 4b, producing a 277-bp PCR product with the actA3-t and plcB3-r primers (26). The lineage identification test in Fig. 1B shows that the four isolates, another two 4b isolates (10M123 and type strain ACM98), and a 1/2b isolate (10M134) were lineage I marker positive (lanes 1 to 6 and 8). The 1/2a (10M131, lane 7) and 1/2c (09M3788, lane 9) isolates were identified as belonging to lineage II. Isolate 09M706 (lane 10) was lineage III marker positive.

To verify that the amplified bands in these four isolates were not nonspecific bands, a hybridization test was preformed using the probes designed from the five intended amplified genes, lmo1118, lmo0737, ORF2110, ORF2819, and prs, using the Luminex suspension array method. The results showed that the bands amplified in the four isolates produced positive hybridization with the probes for lmo0737, ORF2110, ORF2819, and prs, suggesting that the bands were specific (data not shown). The putative lmo0737 fragment of the four 4b isolates was amplified and sequenced along with that of the control 1/2a and 1/2c isolates. The lmo0737 sequences from the four isolates were the same and matched the lmo0737 sequences of fully sequenced L. monocytogenes EGD-e (GenBank accession no. NC_003210.1) well, with identities of 687/691. The lmo0737 sequences of the control 1/2a and 1/2c isolates had alignment identities of 689/691 and 691/691, respectively, with that of L. monocytogenes EGD-e.

Because the new pattern contained a mixture of the bands from the 1/2a and 4b patterns, the real serotypes were hard to determine. A conventional agglutination serotyping was carried out. The O and H antigens of the isolates are presented in Table 1. The four isolates with the new pattern had the same O and H antigen profile as the typical PCR serotyping 4b strain (10M123) and 4b type strain NCTC11994. Therefore, these four isolates were considered to be 4b. The isolates PCR typed as 1/2a, 1/2b, and 1/2c matched the serotypes obtained by conventional serotyping. The isolate PCR typed as “other” was typed as 4c by the conventional method (Table 1).

A study of cultures from the new pattern isolates in two lineages, I and III. The serotype 4b isolates from lineage I and serotype 4b from lineage III have distinct molecular characteristics (9, 10, 19, 22). Lineage I 4b isolates are responsible for a significant proportion of both sporadic and epidemic human listeriosis cases (16). In this study, the four isolates belonged to the predominant lineage I 4b serotype as they were lineage I-specific gene positive (Fig. 1). It is interesting that the four lineage I 4b isolates produced an lmo0737-positive band, which is currently thought to be associated with serotypes 1/2a, 1/2c, 3a, and 3c (6). This observation might be a result of genetic exchange among the different serotypes of L. monocytogenes. For example, a supposed L. innocua species-specific gene, Lin0464, was detected in a small proportion of L. monocytogenes isolates (26 out of 230) tested in a Listeria species identification study (14, 18). These observations and others demonstrated that horizontal gene transfer and recombination may occur within and between Listeria species (21, 23).

In summary, four isolates with a new PCR profile were observed in samples from two states of Australia. This new profile had four bands, lmo0737, ORF2110, ORF2819, and prs, positive in PCR serotyping. They were identified as lineage I 4b serotype isolates.

Nucleotide sequence accession numbers. The lmo0737 sequences of the isolates used in this study have been deposited in GenBank with accession no. HQ221543 to HQ221548.

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