Evaluation of PCR-Based Identification and Genotyping Methods for the iap Genes in Listeria monocytogenes and other Listeria spp.

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By using 181 Listeria spp. strains except for L. ivanovii, we evaluated the three distinct PCR methods targeting the iap genes encoding p60 proteins for genus or species identification, and the PCR-RFLP analysis for genotyping. Each of the two species-specific PCR methods yielded the expected amplicons from all of the 126 L. monocytogenes and 43 L. innocua strains tested. The genus-specific PCR method amplified the target regions (about 1.4 kb) from 178 Listeria strains other than 3 L. grayi strains tested. The PCR-RFLP analysis with either HhaI or HindIII showed distinct several patterns: 97 L. monocytogenes strains divided into 2 genotypes (types I and II) with HindIII and 5 genotypes (types A, B, C, D and E) with HhaI. On the other hand, 43 L. innocua strains showed an identical genotype (type I with HindIII and type F with Hhal). Both L. seeligeri and L. welshimeri strains showed a distinct genotype (type I with HindIII and type G with Hhal). The L. monocytogenes genotypes correlated with the serovars. All 4b strains exhibited one genotype (type I and type A). 1/2b strains exhibited type I and types A or B. In contrast, 1/2a strains exhibited type II and types C, D, or E. 1/2c strains exhibited only type II and type C. Thus, these results suggest that the PCR and PCR-RFLP methods can identify or estimate the genus and species of listerial strains rapidly and are useful tools to genotype Listeria spp. strains phylogenetically.

Key words: Listeria monocytogenes, iap gene, PCR-RFLP, Genotyping, Identification

Introduction

The genus Listeria contains six characterized species, L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri, L. welshimeri, and L. grayi, which are widespread in nature. Among these species, only L. monocytogenes is known to be a human pathogen, which can cause severe invasive diseases (septicemia, meningitis, and meningoencephalitis), especially in pregnant women, neonates, and immunocompromized individuals.

During the 1980s, the organism has been established as a food-borne pathogen, based on the occurrence of several large food-borne epidemic outbreaks in North America and Europe, due to milk, soft cheese, other dairy products, meat, meat products, vegetables, and seafoods. Therefore, to identify the species of Listeria isolates from humans, foods and food-processing environments is primarily important to assess the microbiological hazard. However, traditional culture method is laborious and often difficult to identify and distinguish L. monocytogenes from other Listeria species, in particular, L. innocua, avirulent species prevalent in foods and their environments.

On the other hand, subtyping of L. monocytogenes isolates is essential for studying the epidemiology of listeriosis and investigating
the incriminated foods in both sporadic and epidemic cases. By serotyping, the strains of serovar 4b have been found to be responsible for the majority of the reported food-borne outbreaks\(^8\,12\), and the predominant serovars of clinical *L. monocytogenes* isolates were 4b, 1/2b, and 1/2a\(^8\,12\). These serovars account for more than 90% of the sporadic cases\(^8\,12\). These epidemiological results suggest that there are differences in the pathogenic potential within *L. monocytogenes* serovars as well as species. However, it is not clear why these serovars are predominant in human listeriosis, because the precise virulence mechanisms and the ecology of this pathogen are not fully understood. Therefore, further investigations on both the virulence mechanisms and epidemiological characterization of *Listeria* species will be needed, and to elucidate human *L. monocytogenes* infection, the molecular subtyping techniques that can manipulate many isolates from various sources are crucial.

Several molecular techniques based on whole genomes (i.e. pulsed-field gel electrophoresis (PFGE)\(^1\,20\), ribotyping\(^20\), multilocus enzyme electrophoresis (MEE)\(^10\,19\)) have already been reported as high-resolution subtyping methods. However, these techniques have some demerits that they need specialized equipment and handling is time-consuming. On the other hand, PCR\(^2\,6\) and PCR-based typing techniques, PCR-RFLP\(^7\,9\,26\,30\,32\), random amplified polymorphism DNA analysis\(^13\,18\,20\) have excellent features that they are rapid and technically easy. Recently, several PCR-RFLP methods for the molecular epidemiological purpose have been reported so far\(^2\,6\,7\,26\,30\,32\). Interestingly, Bubert et al.\(^6\) reported PCR methods for the genus- and species-specific identification of *Listeria* spp. They were based on the fact that the *iap* genes\(^15\,16\) which encode the p60 proteins\(^4\,5\,11\,17\,34\) possess in all *Listeria* spp. Thus, the PCR methods would have more versatile applicability. This study reports the evaluation of the PCR-based identification methods and the epidemiological utility of the PCR-RFLP analysis of the amplified *iap* gene regions.

**Materials and Methods**

**Bacterial strains**

A total of 181 *Listeria* species strains (126 *L. monocytogenes*, 43 *L. innocua*, 3 *L. seeligeri*, 6 *L. welshimeri*, and 3 *L. grayi*) were used in this study. Twenty *L. monocytogenes* strains from sporadic human listeriosis in Japan were obtained from Dr. M. Terao, Niigata University. Remaining 158 *Listeria* strains were isolated from the retail foods and the rats, collected and captured in Tokyo and Hiroshima city. Identification of *Listeria* strains used was performed by a conventional culture method. Hemolytic activity was detected on Trypticase soy agar (TSA, Oxoid) supplemented with sheep blood (final concentration of 5%).

**Bacterial DNA extracts**

*Listeria* strains were grown on TSA overnight at 37°C. A loopful of bacteria was mixed with 0.5 ml of sterilized distilled water, vortexed, and boiled for 10 min in a water bath. The bacterial suspension was centrifuged at 12,000 rpm for 3 min, and the supernatants were removed to fresh microcentrifuge tubes and used as the source of template DNAs or stored at −30°C until required. In some cases, InstaGene™ (Bio-Rad) was used for preparing the template DNAs.

**PCR amplification**

In order to amplify the almost entire *iap* genes or portions of them from *L. monocytogenes* and other *Listeria* species strains, PCR amplification was performed with each of the oligonucleotide primers pairs previously reported by Bubert et al.\(^6\): UnilisA (5'-GCTACAGCTGGGATTGCGGT-3') and Lis1B (5'-TTATACGCGACCGAAGCCAA-3') specific for all *Listeria* species; MonoA (5'-CAAACTGCTAACACAGCTACT-3') and MonoB (5'-GCACTTGAATTGGCTGTTATTG-3') specific for *L. monocytogenes*; Ino2 (5'-ACTAGCACTCCAGTGCTGTTATTG-3') and Lis1B specific for *L. innocua*, in a Program Temperature Control System PC-700 (Astec Co., Ltd.). Amplification conditions and components reported by Bubert et al. were modified in the annealing and extension steps as follows: UnilisA-Lis1B, 30 cycles, each at 94°C for 45 sec, 58°C for 30 sec, and 72°C for 120 sec; MonoA-MonoB, 30 cycles, each at 94°C for 45 sec, 57°C for 60 sec, and 72°C for 120 sec; Ino2-Lis1B, 30 cycles, each at 94°C for 45 sec, 62°C for 55 sec, and 72°C for 90 sec.

Amplification was carried out in the reaction volume of 50 μl consisting 10 μl of boiled
PCR-RFLP analysis

A 17 µl sample of the PCR product was digested with 10 U of restriction enzyme HhaI, HindIII according to manufacturer’s instructions overnight at 37°C. The digest (17 µl) was mixed with loading buffer, and was analyzed by electrophoresis by using 3% NuSieve GTG agarose (FMC Bioproducts) in 0.5 × Tris–borate–EDTA (TBE) buffer containing 0.5 µg of ethidium bromide per ml. The gels were then examined by transillumination and photographed as described above. The patterns were classified as different genotypes (each type was denoted by a different letter [see Table 2 and Fig. 1]).

Serotyping

Serotyping of several L. monocytogenes strains was performed with a commercial antisera set (Denka Seiken Co., Ltd.).

Results

Evaluation of the three modified PCR methods

A set of primers, UnilisA and Lis1B, which are derived from the conserved 5’- and 3’-coding regions in the iap genes of Listeria species yielded the expected amplicons, approximately 1.4 kb, for all 178 Listeria species strains other than 3 L. grayi strains tested (Table 1). On the other hand, two sets of primers, one set specific for L. monocytogenes (MonoA and MonoB), and another specific for L. innocua (Ino2 and Lis1B) yielded the expected amplicons, about 0.4 kb and 1.0 kb from all of 126 L. monocytogenes and 43 L. innocua strains tested (Table 1). On the other hand, two sets of primers, one set specific for L. monocytogenes (MonoA and MonoB), and another specific for L. innocua (Ino2 and Lis1B) yielded the expected amplicons, about 0.4 kb and 1.0 kb from all of 126 L. monocytogenes and 43 L. innocua strains tested, respectively. These primer sets yielded no amplicons with Listeria spp. strains except for corresponding Listeria sp. (Table 1).

PCR-RFLP analysis of the iap genes of L. monocytogenes and Listeria spp. strains

In order to study the existence of RFLP within the iap genes of Listeria spp. except for L. ivanovii and L. grayi, the amplicons generated with UnilisA-Lis1B primers for L. monocytogenes, L. innocua, L. seeligeri and L. welshim
Table 1. PCR amplification of the iap genes from Listeria spp. strains by three PCR methods with different primer sets

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains tested</th>
<th>Unilisa-Lis1B</th>
<th>MonoA-MonoB</th>
<th>Ino2-Lis1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. monocytogenes</td>
<td>126</td>
<td>126</td>
<td>126</td>
<td>0</td>
</tr>
<tr>
<td>L. innocua</td>
<td>43</td>
<td>43</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>L. seeligeri</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>L. welshimeri</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L. grayi</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>181</td>
<td>178</td>
<td>126</td>
<td>43</td>
</tr>
</tbody>
</table>

* Unilisa-Lis1B primer set is for the amplification of genus Listeria.
* MonoA-MonoB primer set is for the amplification of L. monocytogenes.
* Ino2-Lis1B primer set is for the amplification of L. innocua.

Table 2. The iap genotypes of L. monocytogenes serovars and other Listeria spp. strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Serovar</th>
<th>No. of strains</th>
<th>HindIII</th>
<th>Hhal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>4b</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>1/2b</td>
<td>21</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>1/2a</td>
<td>30</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>1/2c</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>L. innocua</td>
<td>Unknown</td>
<td>43</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>L. seeligeri</td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>L. welshimeri</td>
<td>Unknown</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>145</td>
<td>99</td>
<td>46</td>
</tr>
</tbody>
</table>

* iap genotypes were determined by the electrophoretical patterns on a 2% agarose gel of the ampiclons digested with Hhal and HindIII.

As shown in Table 2 and in panel A of Fig. 1, with HindIII, 97 L. monocytogenes strains serotyped were divided into two distinct genotypes (designated as types I and II), and 43 L. innocua strains exhibited a type I. Two L. seeligeri and three L. welshimeri strains also exhibited a type I. On the other hand, in panel B of Fig. 1, with Hhal, L. monocytogenes were divided into 5 genotypes (types A, B, C, D, and E). L. innocua exhibited a distinct type F, further, L. seeligeri and L. welshimeri strains exhibited a same type G, distinguishable from those of L. monocytogenes and L. innocua.

By serovars, all of 30 serovar 4b strains tested exhibited only one genotype (type I, and type A). Twenty-one serovar 1/2b strains had two genotypes (type I, and types A or B). On the other hand, 30 serovar 1/2a strains had three genotypes (type II, and types C, D, or E) and 16 serovar 1/2c strains exhibited only one genotype (type II, and type C).

Discussion

Traditional microbiological culture methods require about 7 days to detect Listeria species and to identify L. monocytogenes. Among phenotypic characteristics, hemolysis by listeriolysin is known as an essential characteristics to differentiate L. monocytogenes from other avilurent Listeria species. However, L. monocytogenes generally exhibits weak hemolytic activity on a blood agar, and further, some L. monocytogenes exhibit no hemolytic activity. Moreover, atypical catalase-negative L. monocytogenes also were isolated. Thus, biochemical characteristics tests are not only time-consuming but also often not easy to identify such atypical isolates. Therefore, genetic detection methods for L. monocytogenes have mainly been developed to overcome the limitations above mentioned. Among them, Bubert et al. reported the three PCR methods, targeting iap(-related) genes for identification of genus Listeria, L. monocytogenes, and L. innocua. The merit of the PCR methods is that all Listeria species can be differentiated from each other.

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ria spp. possess the iap(-related) genes, each of which shares common and distinct sequence regions\(^6\). Therefore, the amplicons generated by the PCR methods would have the versatile applicability: genus- and species-identification, genotypic characterization, or phylogenetic study on \emph{L. monocytogenes} and other \emph{Listeria} spp.

In this study, we recognized that the modified genus-specific PCR method could amplify the main species of the genus \emph{Listeria} from humans and foods, \emph{L. monocytogenes}, \emph{L. innocua}, \emph{L. seeligeri}, and \emph{L. welshimeri}, although \emph{L. grayi} did not amplified. Further, both \emph{L. monocytogenes} and \emph{L. innocua} strains were also exactly identified by two species-specific PCR methods.

Listerial pathogenesis is a complex process involving a number of virulence factors expressed by virulence factor genes\(^1-23\). Several virulence factor genes of \emph{L. monocytogenes} have been identified: \emph{hly} (listeriolysin), \emph{plcA} (phosphatidylinositol-specific phospholipase C), \emph{mpl} (metalloprotease), \emph{actA} (protein involved in actin polymerization), \emph{plcB} (lecithinase)\(^23\). These virulence genes are located in a gene cluster and are regulated by the positive regulator factor PrfA\(^23\). The \emph{iap} gene encoding p60 is located in other chromosomal site of the gene cluster. The p60 primarily considered as a virulence factor protein involved in the invasion into nonphagocytic and phagocytic cells\(^4,11,15,17\), and it also acts as a hydrolase, which is essential for septum separation in a late step of cell division\(^34\). Recently, new aspects that the intact \emph{iap} deletion mutant of \emph{L. monocytogenes} has same level of invasion ability as that of wild-type strain, but has abnormal cell division and loss of actin-based motility were found\(^22\). Therefore, \emph{iap} is proposed to rename cwhA (cell wall hydrolase A) and the gene products could influence on the expression of other virulence genes and consequently reduce the pathogenicity\(^22\).

On the other hand, two phylogenetic lineage groups of \emph{L. monocytogenes} have been found from many studies by the genome-based techniques including MEE, PFGE, RAPD, and ribotyping, as well as by the gene-based techniques, PCR-RFLP, and sequencing of the virulence-associated genes\(^1, 7, 25, 30-32\). More recently, a third lineage was also described by using sequencing or RFLP of virulence-associated genes and ribotyping\(^24\). Through these study, the lineage groups have been known to correlate with the serovars. Serovars 4b and 1/2b consist of lineage I, and serovars 1/2a and 1/2c consist of lineage II, and further, serovars 4a and 4c consists of the third lineage\(^24, 31\).

The PCR-RFLP analysis with \emph{HindIII} in this study, were consistent with these studies: serovar 4b and 1/2b strains were type I, and serovar 1/2a and 1/2c were typeII. This result indicates that the PCR-RFLP analysis can use to rapidly differentiate between two lineages of \emph{L. monocytogenes} and to estimate possible serovars. Moreover, the PCR-RFLP analysis with \emph{HhaI} demonstrated the 5 genotypes of \emph{iap} genes in \emph{L. monocytogenes} and 2 genotypes of 3 other \emph{Listeria} spp. It is not apparent whether the genotypes of the \emph{iap} genes correlate with the level of pathogenicity. However, interestingly, serovar 4b \emph{L. monocytogenes} strains, most prevalent serovar in human cases, exhibited only genotype A. On the other hand, serovar 1/2b strains exhibited two genotypes A and B, and most 1/2b strains exhibited type B. Thus, the incidence of the genotypes possibly correlates with the differences in the prevalence of \emph{L. monocytogenes} serovars in human listeriosis.

\emph{p60} proteins have different repeated units, up to 21-fold, of the two amino acids threonine and asparagine (TN)\(^3, 4, 6, 26, 27\). Recently, several excellent DNA-based subtyping methods detecting the TN repeat portion have been reported\(^27, 31, 33\). These methods, however, need the expensive apparatus such as a high-resolution electrophoresis unit or a DNA sequencer to differentiate the accurate sizes of the amplified \emph{iap} genes or to sequence them. While our methods can not differentiate these minute differences, they can phylogenetically divide the genotypes of \emph{L. monocytogenes} with a simple apparatus. Furthermore, the PCR-RFLP analysis would also be useful for the differentiation of \emph{Listeria} spp., because \emph{L. innocua}, \emph{L. seeligeri}, and \emph{L. welshimeri} strains could differentiate from \emph{L. monocytogenes} by the PCR-RFLP patterns.

\emph{L. ivanovii} is a significant listerial species as a animal pathogen. We did not use \emph{L. ivanovii} strains. Therefore, we could not evaluate the PCR-RFLP patterns of this species in this study. However, Bubert \textit{et al.}\(^6\) reported that \emph{L. mono-
cytogenes-specific PCR gave no amplification from L. ivanovii. Hence, we would determine the necessity of the next procedure from the result.

In Japan, all listerial cases other than one outbreak in 2001 have occurred sporadically without verifying the incriminated foods, although the prevalence of L. monocytogenes in foods in Japan is similar with those of other countries. Therefore, further characterization of a number of L. monocytogenes isolated from various sources and origins in the country are needed to clarify the questions. Consequently, the methods reported here are considered useful for the rapid identification and phylogenetical characterization of L. monocytogenes and other Listeria spp. toward further researches.

Acknowledgements

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Listeria monocytogenes および Listeria spp. の iap 遺伝子に対する PCR 同定および遺伝子型別法の評価

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