A Mutant of *Listeria monocytogenes* Shows Decreased Virulence and Confers Protection Against Listeriosis in Mice

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Abstract

This research was performed to obtain a safe and highly immunogenic *Listeria* strain and evaluate the biological characteristics of the deletion mutant. Based on homologous recombination technology, we constructed a deletion mutant *Lm90-ΔinlB* of *L. monocytogenes*. Meanwhile, we characterized its safety and protective efficacy against listeriosis infection in mice. The results showed that the virulence of *Lm90-ΔinlB* could significantly decrease compared with the parental strain (*Lm90*). The deletion strain retained hemolytic activity and induced CD8⁺ T cell response comparable to that of *Lm90*. Mice immunized with *Lm90-ΔinlB* were capable of stimulating specific CD8⁺ T cells to the listerial epitopes LLO91-99 and P60217-225 at levels equivalent to *Lm90*. Importantly, immunization of mice with *Lm90-ΔinlB* displayed good protection against listeriosis. In conclusion, strain *Lm90-ΔinlB* is a vaccine candidate with the potential to be more immunogenic yet considerably less toxic than the parental strain.

**Keywords:** *Listeria monocytogenes*, *inlB* gene, CD8⁺ T cells, Protective efficacy, Vaccine

**How to Cite This Article**


**Article ID:** KVFD-2019-23466  Received: 09.10.2019  Accepted: 06.02.2020  Published Online: 06.02.2020

**INTRODUCTION**

*Listeria monocytogenes* is a Gram-positive pathogen that can cause listeriosis with gastroenteritis, meningitis and encephalitis. As a food-borne pathogen, *L. monocytogenes* can cross the intestinal barrier through intestinal epithelial cells or phagocytes and reach the liver and spleen via the lymph and bloodstream. *L. monocytogenes* multiplies rapidly and finally spreads to the brain through blood circulation. *L. monocytogenes* is known to affect pregnant women, immunocompromised individuals, the young, and the elderly via the oral route. *L. monocytogenes* has typical characteristics of intracellular parasitism and intercellular transmission, and could simultaneously cause MHC-I and MHC-II antigen delivery system and stimulate the host to produce strong cellular immune response. As cytotoxic T lymphocytes (CTLs) are thought to be an important defense against tumor, virus and intracellular bacterial pathogens, attenuated *L. monocytogenes* has the ability to stimulate this immune response, which has broad clinical relevance. At present, several attenuated *L. monocytogenes* have been successfully used in tumor, virus and other DNA vaccine vectors, and some vaccines have entered phase I and phase II clinical trials.
L. monocytogenes has abilities to infect not only phagocytes but also non-phagocytes. The infection process can be divided into four stages: invasion, escape phagocytosis, multiplication and transmission between cells. In the process of infection, each step needs to be done by a specific virulence factor. Internalins are the protein products of a family of virulence-associated genes found in pathogenic Listeria spp. Internalin A (InlA) and Internalin B (InlB) encoded by the inlAB operon were the first members of this family to be characterized. And the two proteins could play important role in L. monocytogenes invasion [10]. Specifically, InlA binds to E-cadherin receptor through its LRR region for invading intestinal epithelial cells and trophoblast cells. InlB not only binds to Met receptor through LRR region, but also binds to receptors of gC1qR and GAGs through GW region, thus invading hepatocytes, Vero cells and Hela cells [11,12]. Meanwhile, L. monocytogenes strongly induces cell-mediated immune responses. As a result of its cytoplasmic location during infection and its particular advantages as a neonatal vaccine vehicle, L. monocytogenes can facilitate a long-term cellular immune response, which makes attenuated vaccine strains a focus of attention in vaccine development [9,10]. Previous studies have shown that L. monocytogenes tropism into hepatocytes is mediated by the virulence factor InlB. Therefore, deletion of inlB is expected to limit liver toxicity. Undeniably, ΔinlB mutants display reduced hepatocyte entry during the infection of monocytes as efficiently as wild-type strains [11,12].

In this study, we successfully constructed a live-attenuated vaccine strain, Lm90-ΔinlB. The biological characteristics of the deletion mutant were evaluated at the molecular, cellular and preliminary animal levels, which lays a scientific foundation for the further study of the Listeria vaccine vector and vaccine.

**MATERIAL and METHODS**

**Bacterial Strains and Cell Lines**

Listeria monocytogenes (Lm90, serotype 4b), isolated from a sheep with encephalitis in Xinjiang, China, was used in this study. An inlB deletion mutant (called Lm90-ΔinlB) was constructed using Lm90 as the parental strain. To achieve stationary phase, all the strains were grown in brain-heart infusion (BHI) agar (Oxoid, Basingstoke, UK) or broth without antibiotics at 37°C. For solid media, agar was added at 1.5% (w/v), and chloramphenicol (Cm) from Sigma was added at a concentration of 10 μg/mL.

MBMEC cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂ for adhesion, invasion and intracellular growth assays. Cell culture media and reagents were all obtained from Gibco.

**Animals and Ethical Concerns**

BALB/c mice of 6-8 weeks of age were obtained from the Laboratory Animal Research Institute of the China Academy of Medical Sciences. All mice were housed in the facilities with the relatively constant temperature of 25±2°C and were treated in strict accordance with the Ethical Committee for animal use in Shihezi University. During the experiments, every effort was made to minimize animal suffering.

**Construction and Identification of Lm90-ΔinlB**

A splicing-by-overlap-extension (SOE) technique was utilized to construct the deletion mutant [13]. The sequences of primers (Table 1) were designed using Primer 5.0 software (Premier Inc., Canada) according to the sequence within the L. monocytogenes F2365 genome in the GenBank database (accession number: AE017262.2). L. monocytogenes genomic DNAs were extracted using the DNA extraction kit (Tiangen Bio, Co.). The 5’ upstream and 3’ downstream homologous arms were amplified by polymerase chain reaction (PCR) using primers P1/P2 and P3/P4, respectively. Then mixed the products in a 1:1 ratio and used SOE PCR technique to achieve the fusion fragment with the primer P1/P4. The fusion fragment was recovered and ligated to pMD19-T cloning vector (TaKaRa Bio, Inc.). The ΔinlB fragment and shuttle vector pKSV7 were digested with EcoRI and HindIII (Takara Bio, Inc., respectively). Then the ΔinlB fragment was cloned into pKSV7 to obtain pKSV7-ΔinlB, and then the recombinant plasmid was transformed into L. monocytogenes competent cells by electroporation [14]. Under the pressure

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of chloramphenicol and temperature, the recombinant strain was selected on the basis of methods described previously [15].

**Growth Curve and Growth Activity Assays of the Bacteria**

Bacterial growth was measured by direct optical density detection at 600 nm and enumeration of the colony-forming units (CFU) in serial dilutions plated on BHI agar.

Specifically, after cultured for almost 16 h, appropriate amount of *Lm* and *Lm*-Δ*inlB* was inoculated into fresh medium at the ratio of 1:100 and cultured at 37°C. Bacterial growth was determined every 2 h by the optical density and CFU.

**Cell Culture and Infection Experiments**

MBMEC cells were cultivated in 24-well plates in DMEM medium containing 10% heat-inactivated FBS at 37°C under a 5% CO₂ atmosphere. For adhesion, invasion and intracellular growth assays, 2×10⁴ cells were seeded in 24-well tissue culture plates 1 to 2 days before infection. Meanwhile, *L. monocytogenes* were cultured to an optical density about 0.3 at 600 nm. Cell layers were washed with PBS and infected in triplicate with 1 mL of bacteria suspended in medium without FBS for 1 h. Then the culture media were collected, diluted and cultured on BHI agar plates to enumerate the bacteria. During the invasion experiment, the medium was replaced with fresh media for 2 h and 100 µL of bacterial culture was added into each well. After 1 h of cultivation, the cells were washed and incubated for different time points with medium containing 100 µg/mL of gentamicin (Sigma) to kill the extracellular bacteria. Finally, the cells were lysed in 2 mL of DMEM containing 0.2% Triton X-100 (Amresco Inc., USA) to release intracellular bacteria and then the mixture was diluted and spread on BHI agar plates to count the colonies.

**Virulence of Listeria Strains In-vivo**

Mice were randomly divided into 2 groups and each group had 5 subgroups (n=5). *L. monocytogenes* were cultured overnight, collected by centrifugation and washed with PBS for 3 times. Then the bacteria were diluted by 10-fold gradient in PBS and the mice were intraperitoneally injected with 100 µL of the bacteria with different dilution to determine the median lethal dose (LD₅₀) of listerial strains. The mice were then observed for 14 days and the LD₅₀ was measured. The remaining bacteria were cultured on BHI agar plates for bacterial counts. On the 1st, 3rd, 5th and 7th days after intraperitoneal injection of 0.1 LD₅₀, the livers and spleens of mice were sampled quickly to assay the number of bacteria.

**Determination of Hemolytic Activity**

Hemolytic activity was assayed as described by Portnoy [16] with some modifications. Listerial strains were cultured for 12 h, then the supernatant was collected and the concentration was adjusted according to the OD600 value. Briefly, two-fold serial dilutions of bacterial culture supernatant were made in PBS (pH 6.0) and 70 µL was added per well. After a 30-min incubation at 37°C, 30 µL of 1% sheep red blood cells was added to the sample and mixed. After an additional 30-min incubation at 37°C, the bacterial hemolytic activity was observed and expressed as the reciprocal of the highest dilution. A negative control comprising PBS only was included in the experiment.

**Immunization Procedure**

Mice were separated into 3 groups of 15 each. On days 1 and 14, one group of mice was immunized intraperitoneally (i.p.) with 0.1 LD₅₀ of *Lm*-Δ*inlB* in a total volume of 200 µL. On the same days, mice in the negative control group were given 200 µL PBS, whereas mice immunized i.p. with a sublethal dose of *Lm* (2.0×10⁴ CFU per 200 µL) were considered as a positive control group. On day 35, five mice from each group were euthanized for ELISPOT assay and 10 mice were prepared for challenge assay.

**ELISPOT Assay**

The enzyme-linked immunospot (ELISPOT) assay was used to determine the levels of the major *L. monocytogenes* antigens, LLOₙ-₉₉ and P₆₀₂₁₇-₂₂₅. To determine the levels of protective immunity of *Lm*-Δ*inlB*, T cell responsiveness was analyzed using a standard ELISPOT approach, which was performed according to the protocol in the published papers [17-20].

**Challenge Assay**

According to the immunization procedure above, 10 mice from each group were challenged with 3.0×10⁴ CFU per 200 µL of *Lm* via the i.p. route on day 35. Three days later, all mice were euthanized, then the spleens and livers were collected, homogenized and cultured to determine the CFU of *L. monocytogenes*. Bacteria were enumerated by plating serial dilutions of organ homogenates on BHI agar and incubating 16 h at 37°C.

**Statistical Analysis**

One-way ANOVA with post hoc analysis by the Dunn’s method was used in this study. Differences with a P-value of <0.05 were considered statistically significant. Differences between groups were analyzed using the Statistical Package for Social Sciences software (SPSS 20.0). Graphs were prepared using GraphPad Prism 6.0 graphing software.

**RESULTS**

**Construction of the inlB Deletion Mutant Lm90-ΔinlB**

Based on the homologous recombination technology described in the Materials and Methods, we constructed deletion mutant *Lm*-Δ*inlB*. A single band of 750 bp was amplified from the *Lm*-Δ*inlB* strain and a good genetic
stability was observed during continuous passage to 20 generations in vitro (Fig. 1), which indicated that inlB gene had been deleted from the Lm90 genome. With sequencing techniques, we further confirmed that the inlB gene had been deleted and had genetic stability (Fig. 2).

InlB did not Affect the Growth of L. monocytogenes

In the study, we measured the growth of Lm90 and Lm90-ΔinlB to examine whether the inlB deletion affected the growth of L. monocytogenes. The results showed that there were no obvious differences between Lm90 and Lm90-ΔinlB (Fig. 3-A). In addition, the number of CFU between the two strains also showed no obvious difference (Fig. 3-B). The results in this section indicated that the inlB deletion did not affect the growth of L. monocytogenes.

Adhesion and Invasion Characteristics of the Deletion Mutant

In the MBMEC cell adhesion assay, Lm90 exhibited 4.63% adhesion compared with only 2.52% for Lm90-ΔinlB. Similarly, in the MBMEC cell invasion assay, Lm90 exhibited 0.37% invasion compared with only 0.23% for Lm90-ΔinlB (Fig. 4-A). In addition, the number of viable intracellular bacteria for Lm90-ΔinlB was significantly lower than that of Lm90 (P<0.05) (Fig. 4-B).

The Virulence of the Deletion Mutant was Reduced in Mice

A notable reduction in virulence was observed with the deletion mutant compared with the parental strain, as shown in Table 2. The LD50 values for Lm90 and Lm90-ΔinlB in experimental mice were 104.60 and 107.38 CFU, respectively. The LD50 value of Lm90-ΔinlB was increased by 2.78 orders of magnitude, indicating that L. monocytogenes virulence significantly (P<0.05) decreased with the lack of inlB gene. Furthermore, the number of viable bacteria in the liver and spleen of mice infected with Lm90-ΔinlB was significantly lower than that of mice infected with Lm90 (P<0.05) (Fig. 5). In summary, the survival and proliferation of L. monocytogenes on days 1-7 post-infection significantly decreased due to the deletion of the inlB gene and the virulence of Lm90-ΔinlB was decreased. Taken together, these results indicated a significant improvement in the safety of Lm90-ΔinlB.

The Deletion Mutant Retains Hemolytic Activity

The major L. monocytogenes antigen listeriolysin O (LLO) confers the unique adjuvant characteristics of L. monocytogenes of being able to generate a Th1 immune response. Hemolysis activity results of the L. monocytogenes strains were presented in Fig. 6 and the hemolysis titer of Lm90-ΔinlB reached 25, which was a little lower than that of Lm90 (26). Therefore, we concluded that Lm90-ΔinlB had good hemolytic activity.

The Deletion Mutant Induces an Antigen-specific CD8+ T Cell Response Similar to the Parental Strain

In the light of immunization procedure in the methods, all experimental mice were vaccinated with primary inoculation
on day 1 and a booster on day 14. Using a standard ELISPOT method, T-cell responsiveness to LLO91-99 or P60217-225 of mice were examined at day 35 and the results were shown in Fig. 7. Mice immunized with \( \text{Lm}_90-\Delta \text{inlB} \) elicited LLO 91-99-specific CD8+ T cells at levels equivalent to \( \text{Lm}_90 \). In the negative control group, no induction of LLO-specific CD8+ cells was observed. Correspondingly, compared with the negative control group, the induction of P60 217-225-specific CD8+ T cells showed a statistically significant (\( P<0.01 \)) in the mice immunized with \( \text{Lm}_90-\Delta \text{inlB} \) or \( \text{Lm}_90 \), respectively.

**IP Vaccination with Deletion Mutant (\( \text{Lm}_90-\Delta \text{inlB} \)) Provides Protection Against Subsequent Parental Strain (\( \text{Lm}_90 \)) Challenge in Mice**

According to immunization procedure as referred above,
the remaining 10 mice from each group were challenged with Lm90 on day 35 via the i.p. route. Then all mice were euthanized 3 days post-challenge and the spleens and livers were harvested for bacterial counts. By comparison with the PBS-treated group, the mice immunized with Lm90-ΔinlB showed a protection from listerial challenge and the result was similar to that of Lm90 group (P<0.01). And the bacterial loads in the organs showed no significant difference between the Lm90-ΔinlB and Lm90 groups (P>0.05). On the other hand, the organs of mice in the PBS-treated group had a great deal of bacterial loads after 3 days (Fig. 8).

**DISCUSSION**

*Listeria monocytogenes* is capable of provoking strong cell-mediated immune responses and has been widely studied as a model intracellular pathogen. Attenuated listerial strains are often used to convey vaccine antigens since *L. monocytogenes* can induct of major histocompatibility complex (MHC) class I-restricted immune responses [21,22].

In our study, we successfully constructed deletion mutant Lm90-ΔinlB of *L. monocytogenes*. A product of 750 bp was generated by PCR amplification using recombinant Lm90-ΔinlB with primers P5/P6. When cultured at 37°C, no significant difference in growth between Lm90 and Lm90-ΔinlB was observed (P>0.05, Fig. 3-A,B). It is known that *L. monocytogenes* infects both phagocytes and non-phagocytic cells. The research of Gaillard et al. [23] is valuable to our understanding of the function of internalins. In their study, *L. monocytogenes* was shown to enter into non-phagocytic cells mediated by InlA and InlB. During *L. monocytogenes* infection, InlA promotes listerial uptake into human intestinal epithelial cells (IECs) by means of binding to E-cad [23]. Comparably, InlB has the property to promote invasion into hepatocytes by means of binding to InlB receptor, c-Met. InlB activates c-Met route in the process of invasion and makes easy to enter into cells by inducing actin polymerization [24]. Furthermore, gC1qR and glycosaminoglycans also bind to InlB and promote invasion. Here we focused on InlB and an inlB gene deletion strain (Lm90-ΔinlB) was constructed in Lm90. The virulence of *L. monocytogenes* in mice significantly decreased with the absence of inlB and its proliferation in the organs of mice also decreased. In cell adhesion and invasion experiments, the virulence of *L. monocytogenes* significantly decreased with the absence of inlB and the
L. monocytogenes infection induces robust CD8⁺ T cell responses, which play a critical role in resolving L. monocytogenes during primary infection and provide protective immunity against re-infection. It has been shown that both LLO and P60 are major antigens in the protective response against L. monocytogenes [29-31]. According to the studies provided by Yamamoto et al. [32] and Kono et al. [33], the secreted protein LLO encoded by the hly gene is prominent in generating a Th1 immune response. LLO has been shown to be processed very efficiently into peptides that are presented by MHC class I molecules. LLO 91-99 is an immunodominant epitope that induces CD8⁺ CTLs, which protect in vivo against L. monocytogenes infection and confer significant anti-Listeria immunity on naive mice upon passive transfer [34]. The P60 protein is encoded by the iap (for invasion-associated protein) gene [31] and has a notable induction effects on CD8⁺ T cell response [7]. It is noteworthy that several studies have used live bacterial vectors, such as Salmonella typhimurium (S. typhimurium) and Lactococcus lactis, expressing LLO and/or P60 for vaccination against listeriosis [36,37]. Here we tested the bacterial hemolytic activity of Lm90ΔinlB and Lm90, and no significant differences were detected between the two strains (Fig. 6). We also showed that mice immunized with Lm90ΔinlB could stimulate specific CD8⁺ T cells against LLO 91-99 and P60 217-225 at levels equivalent to Lm90. These data indicated that Lm90ΔinlB was capable of inducing a powerful Listeria-specific T cell response and considerably higher than that of rice inoculated with PBS (Fig. 7).

In conclusion, our study suggested that L. monocytogenes virulence, adhesion, invasion and proliferation were significantly related to the absence of inlB. Moreover, Lm90ΔinlB retains potent immunogenicity and exhibits significantly decreased virulence compared with parental strain Lm90. Our data also demonstrated that the activity of CTLs induced by Lm90ΔinlB extremely increased. Importantly, immunization of mice with Lm90ΔinlB offered complete protection against listeriosis. This suggests that strain Lm90ΔinlB is a vaccine candidate that is more immunogenic yet considerably less toxic than the parental strain and may be used in future work.

**AUTHOR CONTRIBUTIONS**

GY and JJ designed the overall study especially the immunization procedure and challenge assay. JR and MY performed all the experiments with assistance and advice from GY, JJ and PW. Laboratory data analysis was performed by MY. The manuscript was written by JR and MY, and reviewed by the co-authors.

**CONFLICTS OF INTEREST**

The authors declare that they have no conflicts of interest concerning this work.

**FUNDING**

This work is supported by funding from the National Natural Science Foundation of China (Grant No. 31260606) to J. Jiang.

**ACKNOWLEDGMENTS**

We gratefully thank Hua Yin, Xinyu Wang, and Guangyu Feng for assistance with animal and lab work, as well as the staff of the Key Laboratory of Preventive Veterinary Medicine. We also thank Dr. Weihuan Fang for giftng us the plasmid pKSV7 used in this paper.

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