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Production of Recombinant Heat Shock Protein 60 (HSP60) From *Salmonella enterica serovar* Typhimurium ATCC 19585 and Its Evaluation as a Vaccine Candidate in BALB/c Mice

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Abstract

The gene for heat shock protein 60 (HSP60) was amplified from the DNA extract of *Salmonella enterica serovar* Typhimurium strain (LT2), ATCC 19585, using Polymerase Chain Reaction (PCR). The HSP60 gene was partially sequenced, inserted into expression vector and cloned into a competent *Escherichia coli*. The expressed recombinant HSP60 protein was purified by Ni-NTA affinity chromatography. Immunization of BALB/c mice with the purified recombinant HSP60 protein resulted in a significant anti-HSP60 antibody titers. The groups of the immunized and control mice were challenged with lethal doses of *S. Typhimurium* (LT2) ATCC 19585. Immunized mice had a higher survival rate than control mice suggesting the protective value of the recombinant HSP60 protein used for vaccination.

Keywords: *Salmonella Typhimurium*; Heat shock protein 60 (HSP60); Immunogenicity; Challenge testing

Introduction

Salmonella serovars are responsible for diseases ranging from mild gastroenteritis to life-threatening systemic infections [1]. Typhoid fever is an acute systemic infection caused by the bacterium *Salmonella enterica serovar* Typhi. *Salmonella enterica serovars* Paratyphi A, B and C cause the clinically similar condition, paratyphoid fever with symptoms, which are milder with lower mortality rate. Typhoid and paratyphoid fevers are collectively referred to as enteric fevers [2]. Non-typhoidal salmonellosis or enterocolitis is caused by at least 150 Salmonella serotype with *Salmonella Typhimurium* and *Salmonella Enteritidis* being the most common serotypes causing gastroenteritis of worldwide distribution [3].Numbers of Typhoid cases are relatively low in developed countries. However, non-typhoidal salmonellosis has increased during the last three decades. Non-typhoidal Salmonella infections account for 1.3 billion cases with 3 million deaths [4,5]. The emergence of multidrug-resistant strains of Salmonella with increased virulence was associated with increased morbidity and mortality and has further complicated the management of the cases among the infected humans and animals. Currently, there are no efficient vaccines against Salmonella infections and henceforth the continuing efforts for the development of suitable vaccines [6]. Heat shock proteins (HSPs) are highly conserved and abundant proteins in most bacterial species. Although there are several families of molecular sizes of HSP, we decided to choose HSP60 due to its immunogenicity as HSP with smaller molecular sizes tend to be less immunogenic. HSP are often produced in response to a variety of physiological insults and serves to enhance the survival of the bacterial cells under stressful conditions. As a novel vaccination approach, Heat Shock Proteins (HSPs) based-vaccines have become an attractive strategy for disease prevention [7] and they can be early targets in the immune response against a variety of pathogens, they are being exploited as antigens for vaccine development [8]. There is a significant difference between the typhoid fever that is caused by *Salmonella Typhi* and the non-typhoidal salmonella gastroenteritis that is caused by *Salmonella Typhimurium* and other non-typhoidal Salmonella serotypes. There is no current effective vaccine for the non-typhoidal *Salmonella* infections. Therefore the scientific effort for the development of a vaccine against non-typhoidal salmonellosis should be active and continuous to alleviate the millions of cases that impact the human populations worldwide. However, the production of heat shock protein from *Salmonella Typhimurium* implies the growth and handling of large volumes of the Salmonella culture with the risk of massive contamination of the facility and accidental infection of the operators. Therefore, the objectives of the current study were the production of a recombinant Heat Shock Protein 60 (HSP60) from *Salmonella Typhimurium* through the cloning of the gene for heat shock protein into a carrier *E. coli* that is safe to handle during culture. The final objective was to evaluate the recombinant heat shock protein as a vaccine candidate in BALB/c mice.

Materials and Methods

Bacterial strains

Salmonella enterica subspecies *enterica serovar* Typhimurium (LT2) ATCC 19585 was obtained from American Type Culture Collection (USA). The isolate was used for the extraction of genomic DNA. *Escherichia coli* One Shot TOP10 chemically competent cells were obtained from Invitrogen Company (USA). All reagents used were of analytical grade.

Bacterial cultivation and DNA techniques

All bacterial strains were grown in Luria Bertani (LB) medium at 37˚C. Genomic and plasmid DNA was isolated by using the Gentra Puregen cell kit (Qiagen/USA) and QIAGEN plasmid Maxi Kit respectively, as per the manufacturer's instructions. The concentration

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of the eluted genomic DNA was determined spectrophotometrically using Nanodrop ND-1000 (RocLand/USA). Purification of plasmid and PCR product were carried out by QIAquick Gel extraction kit (Qiagen/USA).

Molecular cloning and sequencing

The full-length of coding region of groEL genes of *S. Typhimurium* (LT2) ATCC 19585 was amplified by Polymerase Chain Reaction (PCR) using the following sets of primers–Forward primer 5'-ACC-ATG-GCA-GCT-AAA-GAC-GTA-AAA-TTC-3', which was designed to include a unique NcoI site and the–Reverse primer was 5'-CAT-CAT-GCC-GCC-CAT-ACC-AC-3', which was designed to remove the native stop codon in the gene of interest and preserve the reading frame through the C-terminal tag (6-His tag). PCR was performed in a PTC-100 programmable Thermal Controller (MJ Research.Inc) using initial activation step at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by final extension step at 72°C for 10 min. PCR amplified product was resolved in 1% agarose gel by electrophoresis and visualized by UV-transillumenator. To verify the sizes of the generated fragments, 1 kb DNA ladder was used. The amplified fragments were cloned into pBAD-TOPO TA expression vector (Invitrogen/USA) in frame with 6-Histidin tag sequence at C-terminal end by the TA cloning techniques, and the derivative was transformed into *E. coli* One Shot TOP10 (Invitrogen/USA) chemically competent cells. The presence of right gene insert and orientation was confirmed by double digestion of the plasmid with NcoI and PmeI enzymes, plasmid PCR, followed by DNA sequencing at Research Technology Support Facility (RTSF) at Michigan State University.

Expression and purification of recombinant HSP60

Expression kit (pBAD TOPO TA expression kit/Invitrogen/USA) was used for the expression and purification of recombinant HSP60. Recombinant Heat Shock Protein 60 (HSP60) was purified by (Ni-NTA) affinity chromatography, which was obtained from Qiagen Company as "QIA expressionist" kit. The purity of the expressed proteins was verified by analysis of the product on 10% SDS-PAGE along with protein molecular mass marker. Proteins were visualized by staining with Coomassie blue G-250. The eluted protein was dialyzed against distilled water overnight at 4°C to remove the urea before lyophalization. The protein concentration was measured by Bradford method [9].

Experimental animals

After approval from the institutional animal care and use committee at Michigan State University (IACUC), twenty BALB/c mice were purchased from Charles River Laboratories (Boston, MA, USA). Mice were between 6-8 weeks of age and were all females for better handling. Mice were kept in cages and provided with balanced pelleted mouse diet food (Ralston Purina) which meet or exceed the NRC (National Research Council) requirements. Food and water were provided ad libitum. Before the challenge experiment, the immunized and control mice were moved to the University infectious disease containment facility. Michigan State University laboratory animal housing and management are approved by National Institute of Health (NIH) and the United State Department of Agriculture (USDA) and are subject to periodical inspection by these federal agencies.

Immunization of mice: Ten mice were randomly selected for immunization with HSP60 whereas 10 other mice were left as control. A volume of the HSP60 emulsified in Complete Freud's Adjuvant (CFA) was adjusted to contain 30 µg of the HSP60 in 100 µl of the emulsion before it was injected subcutaneously into each mouse. For the 2nd and 3rd doses, Incomplete Freund's Adjuvant (ICFA) was used for the emulsification of the same concentrations of HSP60. Immunization was performed at 3 weeks interval between the primary immunizing dose and the first and second booster doses. Control mice were injected with the same material but without the HSP60 using the same schedule.

Determination of antibody titer by ELISA

The mice were bled prior to the immunization for a baseline measurement of the HSP60 antibody and 7 days after the primary immunization and each booster from the saphenous vein. Blood was allowed to coagulate for several hours at 37°C before placing the tubes at 4°C at a walking cold room to allow effective shrinkage of the clot before centrifugation at 3000 rpm for 3 min to collect the serum. Sera were kept at -20°C till used for ELISA measurement of the heat shock protein 60 (HSP60)-specific antibody.

ELISA protocol: ELISA plate wells were coated with 2 µg HSP60 was dissolved in carbonate-bicarbonate buffer (0.05 M), pH 9.6. After usual ELISA steps, plates were read at a 405 nm in a Molecural devices ThermoMax® plate reader. ELISA titers for sera obtained from the 10 mice prior to immunization had an average OD of 0.013 when measured spectrophotometrically at 405 nM.

Such baseline titer may be attributed to exposure of the mice to Salmonella or other gram negative bacteria with shared antigenic constitution to Salmonella.

Challenge experiment

Salmonella Typhimurium ATCC 19585, the same strain that used to produce the recombinant HSP60 was used as the Live-challenge Salmonella strain. The strain was grown overnight in tryptic soy broth at 37°C in an incubator shaker set at 200 rpm. It was counted that the overnight culture had 108 CFU/ml of the broth. Dilutions were made to produce levels of the LD50 (median Lethal Dose) before Intraperitonial Injection (IP) of the vaccinated and control mice. After 7 days of the last vaccination dose, five vaccinated mice and five unvaccinated controls were challenged IP with 100,000 CFU of the live parent strain of the ATCC 19585 *Salmonella Typhimurium* which is approximately equals to 100 LD50 for this serotype. Another 5 vaccinated mice and 5 unvaccinated mice were challenged with 1000,000 CFU of the strain, which is approximately 1000 LD50 [10,11]. Only 5 mice per LD50 dose were used due to the difficulty in securing approval to use larger number of mice for this experiment. For this number of animals, Fishers exact test was used to reveal the significance of differences in the survival of the immunized and control mice after the challenge. Challenged mice were placed in specially designed cages that minimize environmental contamination at the Infectious Disease Containment facility, Michigan State University. The caged mice were observed 3 times/day for the duration of the experiment. Dead mice were removed from the cages as soon as they were detected.

Results

The full-length of the Heat Shock Protein 60 (HSP60) of *S. Typhimurium* was amplified from genomic DNA of *S. Typhimurium* ATCC (LT2) 19585. Specific primers were used to amplify the entire gene, which yielded PCR product of approximately 1650 bp (Figure 1). The optimum time of the recombinant protein expression after induction with 0.02% L-arabinose was determined, and the result was revealed on SDS-PAGE of the crude and purified GroEL protein,

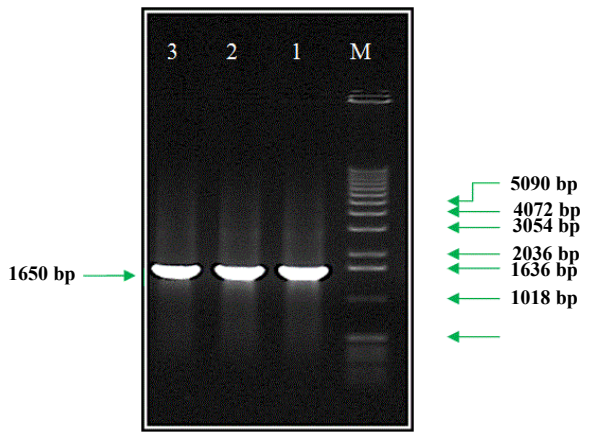

Figure 1: Agarose gel electrophoresis of the amplified shock protein 60 (HSP60) genes using the primer set, on 1% agarose gel, at 100 V. for 1 hour. M: DNA marker. Lane 1, 2 and 3: amplified shock protein 60 (HSP60) gene from *S. Typhimurium* ATCC 19585 the fragments have size of around 1650 bp.

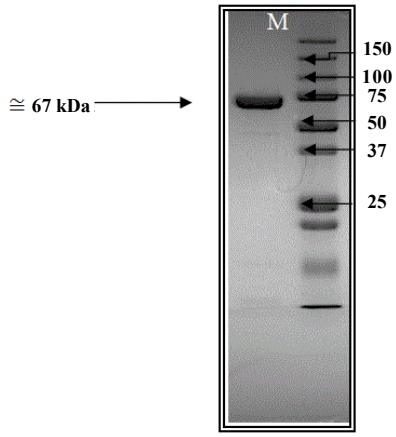

Figure 2: SDS-PAGE analysis of recombinant Heat Shock Protein (HSP60) purified by purification by using Ni-NTA super flow column (affinity chromatography), at 200 V. for 40 minutes, M: Protein marker, 1: Purified Heat Shock Protein (HSP60).

which indicated that the optimum time of protein expression was after 5 and 6 hours of induction with 0.02% L-arabinose, while the Bradford method that was used to determine the concentration of the purified GroEL protein (By using Ni-NTA spin columns) indicated that the protein concentration was increased with increasing the time of induction, with no significant difference between the protein concentration at the 4, 5 and 6 hour times of induction. The protein concentrations were 102, 85, 100 µg/ml. Therefore, the 4 hours' time was considered as the optimum time of induction in this study to avoid long time of incubation of the recombinant protein because it leads to increase the protein degradation. ATCC (3-1) clone was chosen to be used in the next experiments, because it showed high protein expression after determination of the GroEL concentration from the purification step of protein at the optimum time. The purified PCR products of was submitted to the Research Technology Support Facility (RTSF) at Michigan State University for sequencing and the sequence was compared with groEL sequence of *S. Typhimurium* LT2 from the GenBank library. The sequenced part of the gene by RTSF was found to be identical to the sequence of GenBank. The gene that was inserted into pBAD TOPO expression vector was then cloned into *E. coli* and the expression of the recombinant protein was induced by 0.02% of L-arabinose in the growth medium. The protein was predominantly expressed in insoluble form and was purified by (Ni-NTA) affinity chromatography under denaturing conditions of SDS-PAGE (10%) electrophoresis. Analysis of the purified protein revealed a single band of approximately 65-67 kDa (Figure 2). The eluted protein was dialyzed against distilled water overnight at 4°C to remove the urea, and then the protein was filter-sterilized before using for immunization of BALB/c mice.

ELISA Measurement of amplified Heat Shock Protein 60 (HSP60)-specific Antibody in Sera of Vaccinated Mice

Result of ELISA testing of sera from the vaccinated mice during the 9 weeks of vaccination is depicted in (Figure 3). The 3 points represent the means of triplicate values from the 10 vaccinated mice, which were 0.226 for the first immunization dose (30 µg GroEL+CFA) and 0.276 and 0.616 for the two booster doses with (30 µg HSP60+ICFA) respectively, with 3 weeks intervals between each dose. The trend suggests a progressive rise in the titer of the anti-HSP60 antibody to a significant level by the end of the 9 weeks. This suggests that the adjuvanted HSP60 injected subcutaneously into the BALB/c mice has a good immunogenic property. Sera obtained from the control mice did not reveal any significant level of the anti-HSP60 antibody.

Challenge experiment

To demonstrate the effectiveness of the protective effect of the heat shock protein 60, the survival of the vaccinated and control mice, after challenge, was measured (Figure 4). It is noteworthy that all the control mice that received the 1000 LD50 of the challenge Salmonella died after 72 hours whereas the vaccinated mice survived the same challenge dose of the organism for that period of observation. Due to the small number of animals Fishers exact test was used (Fisher exact test, P-value=0.0039) [12].

However one mouse of the vaccinated group that was challenged by the 100 LD50 died after 72 hours. The overall survival of the vaccinated

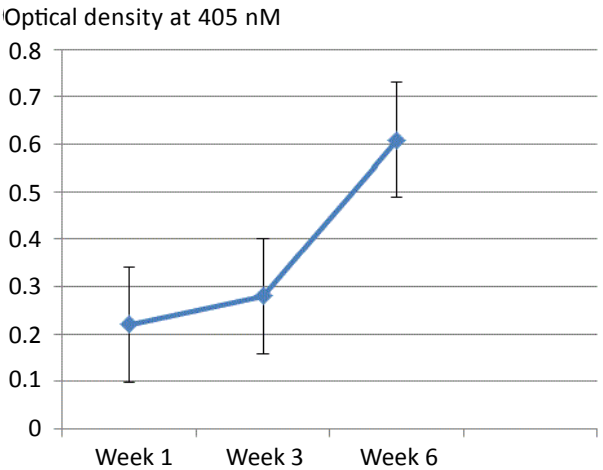

Figure 3: Time course study of the immune response of BALB/c mice immunized with 30 µg heat shock protein 60 at 3 weeks intervals. Each point represents the mean and standard errors of serum IgG from 10 mice measured by ELISA. Mean OD titers for the mice before immunization was "0.013).

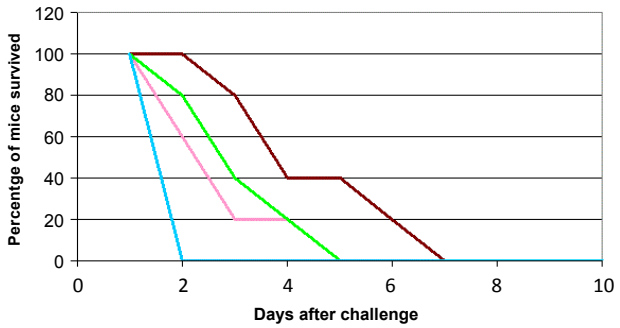

Figure 4: Survival curve of BALB/c mice against lethal dose of *S. Typhimurium* ATCC 19585. Red line: vaccinated mice challenged with 100 LD50 dose, Green line: Vaccinated mice challenged with 1000 LD50 dose, Pink line: Control mice challenged with 100 LD50 dose, Sky blue line: control mice challenged with 1000 LD50 dose.

mice compared to control mice throughout the challenge experiment suggests the protective value of the HSP60 among the vaccinated mice in comparison to the control mice. Most of the reported challenge experiments used a significantly lower challenge LD50. However, due the limited number of mice approved by IACUC, we had to design the challenge experiment using a higher LD50 to capture the differences in resistance between the vaccinated and the control mice to the challenge.

Discussion

Purification is vital for the characterization of the function, structure, and interactions of any protein targeted for study. Regarding Heat Shock Proteins (HSP), different traditional methods of purification of the native forms of these proteins have been reported [13]. Adopted the Fast Protein Liquid Chromatography (FPLC) using Mono Q HR (5/5) ion-exchange column to purify the 66-kDa HSPs of *Salmonella Typhimurium*, and the purified protein was found to be free of any other protein contaminants after analyzing by SDSPAGE. As for the purification of recombinant HSP60 protein from different bacterial isolates, series of purification procedures including column chromatography on DEAE-cellulose, hydroxyapatite and Sephacryl S-200 were used [14]. With the advent of genetic engineering, it has become relatively easy to clone the cDNA encoding a given protein. It is then possible to construct an overproducing strain of *E. coli* that can be induced to produce large amounts of a desired gene product. Affinity chromatography is one of the most important and powerful method since it can offer high selectivity, high resolution, and high capacity for target proteins [15]. Significant number of studies has been conducted on *S. Typhimurium* because it is the leading cause of human gastroenteritis a foodborne disease in humans and a significant pathogen in domestic animals. This serovar also causes a typhoid-like disease in mice which is found a very good model in order to understand typhoid fever, a disease affecting millions of people worldwide annually and caused by *S. Typhi* [6,16]. Heat shock proteins are highly conserved as the encoding gene for which is also known as groEL, is known to be among the most conserved components in evolution. The groEL gene has become as a target gene in typing and identification of Salmonella, *Campylobacter jejuni*, Staphylococcus and *Bacillus cereus* [17-20].

In this study, we report the design and production of a recombinant Heat Shock Protein 60 (HSP60) from *S. Typhimurium* and the evaluation of its immunogenic property in mice. The fact that HSPs are targets of the immune response in a broad spectrum of infections may be related to the abundance of these proteins under stress conditions. For example, GroEL is one of the most abundant proteins expressed by salmonellae within infected macrophages, which are known to be critical antigen-presenting cells in the immune system. Given the potential importance of HSPs, it was of interest to identify and characterize immunogenic epitopes within this protein, as has been done with some pathogens and they indicated shared epitopes between them; e.g. *Escherichia coli* HSP60 (GroEL), GroEL of *S. Typhi*, HSP60 of *Chlamydia trachomatis* and HSP65 *Mycobacterium tuberculosis* [21,22]. Microbial HSPs have been reported to be dominant antigens for the host immune response to a variety of pathogens and the immune recognition of these HSPs serves as a first line of defense and a number of studies reported significant protection by using HSPs as vaccines in various infectious disease models [23].

The relative resistance of the HSP-immunized mice can be explained by the fact that during microbial infections, HSP determinants are expressed on the cell surface and can be recognized by antibodies induced by the immunization of mice with HSP60. Tobian *et al.* [24] reported that recombinant HSP fusion proteins (with antigenic sequences fused to the N or C terminus of the HSP60were associated with eliciting CD8+ T cell and Ab responses. The observed antibody-mediated protection in the present study could be attributed to surface localization of the HSP60 (GroEL). Our challenge results agree with other results of GroEL immunization and challenge studies on BALB/c mice. Khan *et al.* [25] demonstrated that the onset of death of immunized mice with GroEL was insignificantly delayed and all the mice died by the seventh day post-infection after they were challenged with 105 CFU of *S. pneumoniae*. As well as, Pinho and his co-worker showed that the rate of survival of the animals immunized with recombinant HSP60 was slightly higher than that of mice injected with PBS; however, all the animals died within two weeks after challenged with 106 CFU of the virulent *C. pseudotuberculosis* [26]. Therefore, our results can give strong evidence that immunization of animals with GroEL elicited a good humoral response. Antibodies response has been reported to play an important role in protection against Salmonella infection in mice [27,28].

Conclusion

It was possible to clone and express the gene for Heat shock protein 60 (GroEL) of *S. Typhimurium* (LT2) ATCC 19585 104 into a competent *E. coli*. Purified recombinant HSP60 (GroEL) has good immunogenic properties based on the immunization of BALB/c mice. Furthermore, Immunization of mice with the recombinant GroEL induced measurable protection against a challenge lethal dose of the parent *S. Typhimurium* (LT2) ATCC 19585 serotype and therefore suggests the potential value of recombinant HSP60 as a possible vaccine candidate for preventing salmonellosis in humans and animals.

Acknowledgment

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Conflict of Interest

All authors declare that they have no financial and/or personal relationship with any organization that may inappropriately influence their work.

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