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Original Research Article

Title: Expression and Purification of Histidine-Tagged Salmonella typhi Cell invasion protein (SipC) and its diagnostic utility

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ABSTRACT

Background: Salmonella enters non-phagocytic cells, initiates intracellular replication, and causes enteric fever in humans. Engulfment of bacteria happens through macropinocytosis by nucleation of host cell actin polymerization and condensation of actin filaments. This macropinocytosis is induced by contact-stimulated delivery of a series of effector proteins, which includes Salmonella cell-invasion-proteins (SipC). Owing to function, the SipC is considered as an attractive target for the detection of salmonella.
Objectives: SipC protein purification was not scaled-up previously; hence, its diagnostic usefulness are not therefore thoroughly elucidated. The SipC gene was therefore cloned into a pET-21b vector to enhance protein expression and purification to detect its diagnostic viability.
Methodology: The SipC gene was amplified from genomic DNA of Salmonella and cloned into the pET-21b expression vector. The cloned plasmid containing SipC was transformed into Escherichia coli(E. coli) BL21 (DE3) strain, grown and expressed by Isopropyl-β-d-1-thiogalactopyranoside (IPTG) induction. The enzyme-linked immunosorbent assay (ELISA) ELISA was performed to understand the clinical utility of recombinant SipC.
Results: The histidine-tagged SipC protein was expressed and purified using Ni-NTA affinity chromatography. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis has shown approximately 44 kDa molecular weight protein, also confirmed by immunoblot with the anti-His antibody. Protein purification was completed within a day, and 1 litre of E. coli cell culture produced approximately 27 mg of viable recombinant SipC protein. ELISA results showed that the Anti-SipC antibody was binding specifically with greater sensitivity, to the His-rSipC protein.
Conclusion: The developed protocol for the expression and purification of SipC provides a high concentration of protein. The ELISA results had demonstrated that SipC can be used for the evaluation of both IgM and IgG shows the clinical usability of SipC. This may have utility as intermediates for the development of point-of-care tools for the diagnosis of Salmonella infections

Running Title: Salmonella typhi SipC protein uses in diagnostic

Introduction:

Enteric fever caused by Salmonella typhi is a major public health issue [1-3]. Approximately 21 million patients and 2,22000 deaths/year are caused globally by Salmonella typhi [4]. Fluoroquinolones, ampicillin, cotrimoxazole, chloramphenicol and other 3rd generation cephalosporin are the antibiotics utilized for the treatment of enteric fever[5, 6]. However, Salmonella species are developing resistant to these traditional antibiotics especially in developing countries [6, 7]. If this situation continues, the death rate due to enteric fever might increase by 30% [8]. The accurate diagnostic is paramount in resistance control, which is dependent on the availability of the target protein for the development and testing of bioassay [9, 10].

Salmonella cell invasion proteins (SipC) are an actin-binding protein that interferes with the host the cytoskeleton of the cell [11, 12]. SipC nucleates actin polymerization and condensate actin filaments into cables (bundling)[13]. SipA potentiates SipC activity and both are required for an efficient bacterial internalization by the host cell. SipC encodes a polypeptide of 409 amino acid residues and has three domains, Domain 1, 2, and 3 [11, 12, 14]. Domain 1 starts from amino acid 1 119 and works in actin-bundling. Domain 2 is 180 amino acid long and stretches from 120-200 and bacteria inserts this domain in the host plasma membrane. The function of domain-3 is actin polymerization and it is from 201-409 amino acids. All three domains combine and result in a non-numeric protein of a molecular weight of approximately44 kDa [11, 12].

Purification of protein is a prerequisite to study the structure, mechanism of its action, and development of bioassay [15].Owing to the difference in solubility, size, and charge, one single method can be used to isolate all proteins [16]. To isolate one particular protein from a pool of proteins in a cell is a daunting task and quantity is also limited,

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therefore, recombinant DNA technology is utilized and the protein is cloned and expressed in a prokaryotic cell and then isolated. This ensures a high quantity production of protein and reproducibility. Here, we report the cloning of the Salmonella SipC gene into the pET-21b plasmid expression vector next to the His-tag sequence and transformed into the E. coli strain BL21(DE3), which is an ideal platform for protein expression. Also, we attempted to decipher the uses for the expressed recombinantly expressed SipC protein.

Materials and Methods:

Enzymes for DNA cloning and amplification were purchased from New England Biolabs (NEB, MA USA). The plasmid pET-21b was from Novagen (Madison, WI USA). Bacterial strain E. coliBL21(DE3) was from Invitrogen and manipulated and maintained using standard techniques. The Ni-NTA affinity matrix was purchased from Qiagen (Valencia, CA USA). Buffers and all other chemicals were purchased from SIGMA (St. Louis, MOUSA). Primers for the cloning of the SipC gene were synthesized by IDT. IPTG was purchased from Gold Biotechnology (St. Louis, MO USA).

Plasmid Construction

The Genomic DNA from enterobacterial strain *Salmonella enterica* subsp. *enterica* serovar *typhi* [17, 18]were used for SipC gene amplification by PCR, using specific primers {S. typhi SipC(GenBank: AL513382); primer pairs: (Forward, 5'GCATCATATGATGTTAATTA GTAATGTGGG3', 5' GCATCTCGAGTTAAGCGCGAATATT GCC3'}. PCR amplified fragments were digested with Neisseria denitrificansI(NdeI)/ Xanthomonas holcicolaI (XhoI), and cloned into respective sites in the multiple cloning site (MCS) of pET-21b vector Novagen (Madison, WI) to construct plasmid pET-21b-SipC(409 AA), for the expression of *S. typhi* recombinant SipC protein. Plasmid constructs made were sequenced, which showed 100% sequence homology with their respective SipC gene. The pET-21b-SipC plasmid was transformed into the bacterial host E. coliDh5αF' cells using standard procedures. The transformed cells were selected on LB plates containing ampicillin (100µgmL-1) and incubated at 37°C, overnight for positive clone screening. Each plate contained about 20 colonies. Ten colonies were picked from one of the plates and used to inoculate 5 mL of autoclaved LB broth containing 100 µg mL-1 ampicillin. The culture plates with the remaining colonies were stored at 4°C. The cultures were grown overnight at 37°C with shaking at 250 rpm. The pET-21b-SipC plasmids were isolated using a Qiagen DNA Mini-Prep kit. Each plasmid sample was subjected to colony PCR with gene-specific primers and double digestion by both NdeI and XhoI. Agarose gel electrophoresis was carried out on the digested products to confirm a 1254bp insert, the expected size of the SipC gene along with vector sequence coding His-tag.

Expression and purification of protein

Protein was expressed in E. coliBl21 (DE3) cells containing the pET-21b-SipC plasmid. Briefly, the overnight culture was inoculated in 1 L of fresh 2X Tryptone yeast (TY) media (1:100) supplemented with 100 µg mL-1 of ampicillin, 50 µg mL-1 chloramphenicol, and allowed to grow until the optical density at 600 nm reached ~0.4. The culture was then induced with 0.5 mM IPTG and allowed to grow overnight at 37°C. Cells were harvested by centrifugation at 6000 × g for 10 min and checked for expression of His-tagged recombinant SipC (His-rSipC) by SDSPAGE. Most of the target protein was present in the pellet as inclusion bodies (IBs).

Isolation of Inclusion Bodies (IBs)

E. coli cell pellet (~4 g wet weight) obtained from 1 L culture was resuspended in 50 mL of Buffer [50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM PMSF] and sonicated on the ice at amplitude of 50 for 10 cycles with 1 min gap between each cycle [19]. Each cycle of 1 m comprised of alternate on and off pulses of 10 s (Q 700 sonicator, Qsonica, USA). The lysed bacterial suspension was centrifuged at 15,000 × g for 20 min at 4°C (Sorvall RC 6+, USA). The pellet obtained was washed in Buffer [50 mM Tris-HCl, pH 8.0] and centrifuged at 15,000 × g for 20 min at 4°C. The washing process was carried out two times. The washed IBs were finally resuspended in 2 mL of MQ water.

Solubilization of IBs, refolding and purification of His-rSipC

Purified His-rSipC IBs (1 mL) were solubilized in 9 mL buffer [50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol, 3 M Urea] and incubated at room temperature for 1 h, followed by centrifugation at 15,000 × g for 20 m at 10°C. The denatured target protein was refolded by diluting it in a pulsatile manner in 100 mL refolding buffer [100 mM Phosphate buffer, pH 8.0, 300 mM NaCl, 1 mM β-mercaptoethanol] at 4°C with constant stirring. The refolded sample was centrifuged at 24,000 × g for 30 min at 4°C and supernatant obtained post-centrifugation was concentrated. Purification of refolded recombinant His-rSipC protein, by immobilized metal ion affinity chromatography (IMAC) using HisTrap FF column (GE Healthcare Buckinghamshire, UK), was performed using native conditions. Protein was eluted using buffer [100 mM Phosphate buffer, pH 8.0, 300 mM NaCl, 1 mM β-mercaptoethanol, 50-250 mM Imidazole]. The purified His-rSipC was dialyzed against buffer [100 mM Phosphate buffer, pH 8.0, 300 mM NaCl, 1 mM β-mercaptoethanol] withthree times exchange of buffer. The final buffer exchange of protein to buffer [100 mM Phosphate buffer, pH 8.0, 300 mM NaCl, 10% glycerol] was performed by the PD10 desalting column (GE Healthcare Buckinghamshire, UK), as per manufacturer's protocol. The protein was quantitated by bicinchoninic acid assay (Thermo Scientific Pierce, Rockford, IL, USA) and analyzed with SDS-PAGE, and confirmed by Western blotting, using an anti-His monoclonal antibody (Cell Signaling Technology, Inc., MA, USA).

Western blot

The 40 µg of total protein was mixed with 3× Laemmli sample buffer, boiled at 95°C, and cooled on ice. Purified His-rSipC were resolved on a 15% reducing Tris-glycine, SDS-PAGE, and transferred to nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). Membranes were incubated in blocking solution for overnight 5% non-fat milk (Difco, NJ USA) in PBST buffer (PBS + 0.1% Tween-20). Then membranes were washed and incubated with primary rabbit Anti-His (Cell Signalling Technology, Inc., MA, USA) monoclonal antibodies. Membranes were washed and incubated with HRP-conjugated secondary antibody (1:10000 dilution anti-rabbit) (Cell Signalling Technology, Inc., MA, USA) for 1 h. Finally, membranes were washed with PBST and then developed using Amersham ECL Prime Western Blotting detection reagent (GE Healthcare, Buckinghamshire, UK).

Diagnostic utility

Indirect enzyme-linked immunosorbent assay (ELISA) was performed to demonstrate the detection of His-rSipC specific antibodies in the serum as a potential diagnostic candidate for S. typhi infections [20].All the experimental procedure associated to use of specimen from human subjects is in compliance with ethical standards as per current legislation for human studies at the Institute Ethics Committee. Therefore, for this experiment the optimum concentration of the His-rSipC (1 µg mL-1), were used, for coating 96 well plate (Nunc Maxisorb), by adding 100 µl bicarbonate buffer (pH 9.5) per well and incubated overnight at 4°C. Plates were blocked with 5% non-fat milk in PBS for 2 h at 37°C. The blocked wells were washed thrice with 1X PBS, 0.1% Tween-20 (Wash Buffer; PBS-T). Serum dilutions (1:100) in blocking buffer and 100 µL was added to each well and the plate incubated for 1 h at 37 °C. The plate was washed three times with wash buffer and incubated with anti-human IgM-HRP (signalling technology, MA, USA) at a dilution 1:80,000 in blocking buffer for 1 h at 37°C. The plate was washed five times with wash buffer and then 100 µL/well of the chromogenic substrate (3,3,5,5′ tetramethylbenzidine (TMB), BD biosciences) was added and the plate was incubated in dark for 20 min. The reaction was stopped with 50 µl/well of 2 NH2SO4 and the plate was read at 450 nm with an automated microplate reader (Biorad, USA). The cut-off was determined by using the mean optical density (O.D.) for the

blood culture-negative control group plus three standard deviations (S.D.).

Results and Discussion:

The SipC gene was successfully isolated from one of the indigenous clinical isolate of Salmonella typhi(data for strain has been characterization reported previously, [21, 22])available with the groupand cloned into the pET-21b expression vector for His-tagged fusion construct (Figure 1).

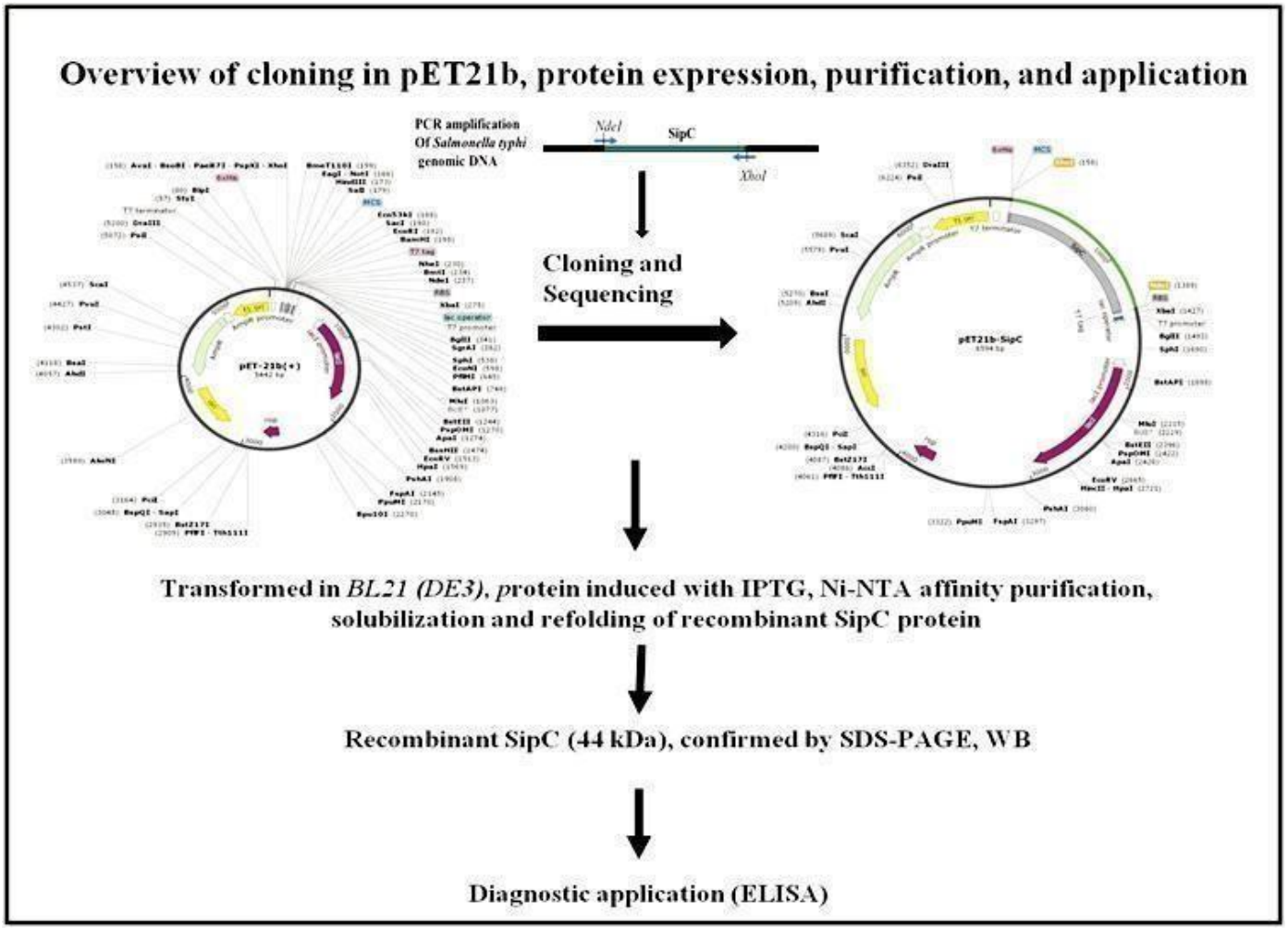
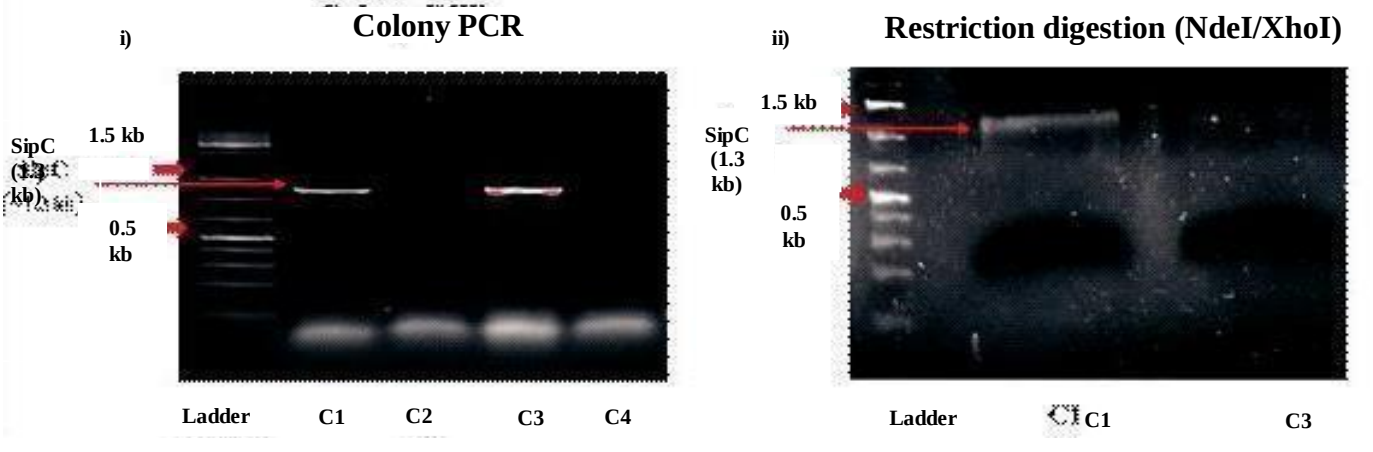
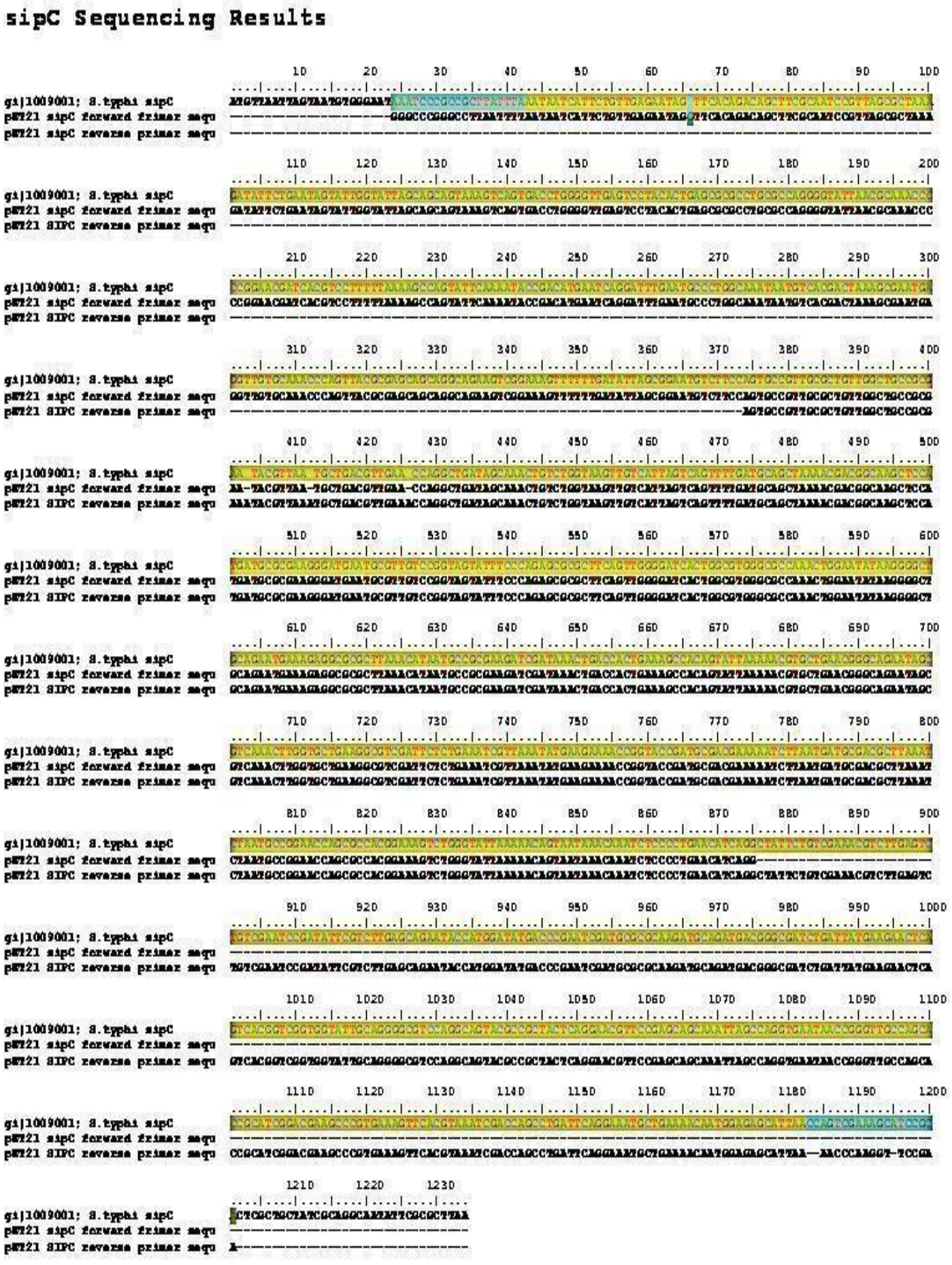


Figure 1: Overview of cloning in pET21b, protein expression, purification, and application.

Out of five ampicillin selected positive clones, only two clones showed with intact SipC genes as confirmed by all the three, colony PCR, restriction digestion with NdeI and XhoI, and DNA sequencing, methods for initial characterization of viable plasmid construct for protein expression (Figure 2).



iii)


sipC Sequencing Results
gi|1009001; S.typhi sipC
pET21 sipC forward primer sequ
pET21 SIPC reverse primer sequ

iv) Translation of SipC

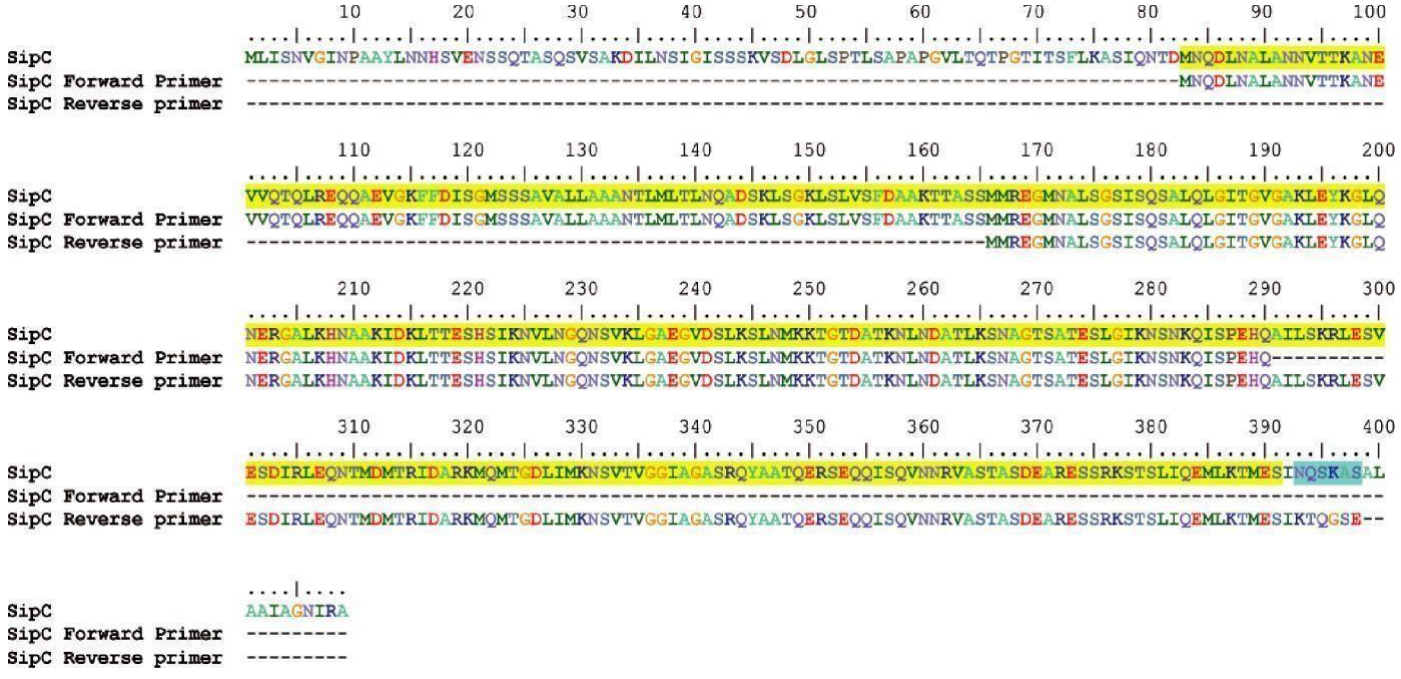


Figure 2: Analysis of cloned colonies by i) colony-PCR (C1, C2, C3, and C4 are ampicillin-resistant colonies), ii) restriction digestion (C1 and C3 are ampicillin-resistant colonies), iii) results of sequencing with BLAST analysis, and iv) translation of the obtained sequences showing SipC.

The protein expression was induced by IPTG. The recombinant histidine-tagged protein was purified from E. coli inclusion bodies by breaking aggregates in mild concentration (3 M Urea) of chaotropic agents and refolding into active renatured protein followed by Ni-NTA affinity chromatography. The 1254 bp open reading frame insert encoded the 409amino acid polypeptides with a molecular mass of ~44 kDa including His-tagged protein at the C-terminal of the rSipC protein. Furthermore, all the process and steps involving SipC IB solubilization, renaturation, and purification steps were expedited, turn over time being within a day, represents a significant improvement over the conventional three-day purification procedure of the other *E. coli* recombinant protein expressed in the form of IB aggregates[23]. Subsequently, the SipC was eluted from the Ni-NTA resin between an imidazole concentration of 100mM-250mM. The collected fractions containing SipC was analyzed using 15% SDS-PAGE(Figure 3i). As revealed in the Figure 3, SipC migrates to a position in the gel close to the ~44 kDa band in the molecular mass markers in the first lane one and is close to the calculated molecular weight of 44 kDa (Figure 3 ii).

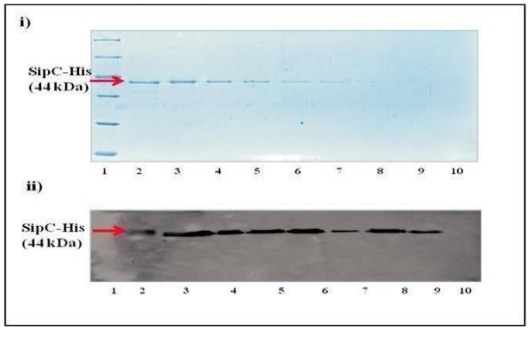


Figure 3. SDS-PAGE analysis and confirmation with immunoblotting for the purified SipC

Purification of 1 L of E. coli culture yielded approximately ~27 mg of protein. Further, the immunoblot with anti-His antibody confirmed the presence of ~44 kDa His-SipC protein.

Early and precise diagnosis is essential for the timely and efficient treatment of typhoid. Thus, there is a continuing quest for superior diagnostic tools. Lack of blood culture facilities in the developing countries compels the researchers to develop novel antigen for the typhoid diagnostics. The Widal test is the primary diagnostic tool of typhoid fever in most laboratories but it has drawbacks as it might give false-positive results in patients who have had previous vaccination or infection with S. Typhi[24-26].Following the purification of the protein, ELISA was developed using the recombinant SipC protein as an antigen for the detection of IgG and IgM antibodies against SipC. As depicted by a strong color change in the test wells and minimal change in the controlled wells, the Anti-SipC antibody was binding specifically with greater sensitivity to the developed His-rSipC protein (Figure 4).

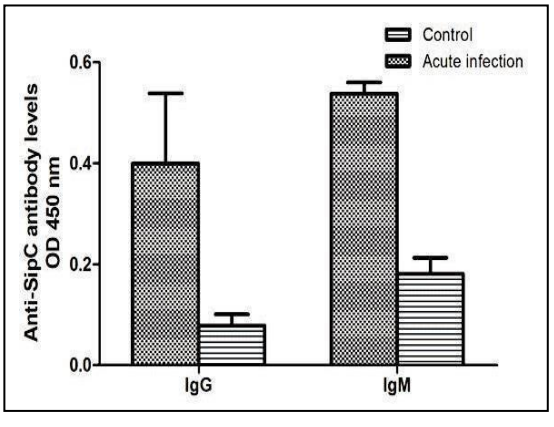


Figure 4. Indirect ELISA for seroreactivity of SipC for specific antibody detection

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An advantage of the chosen approach is the high concentration of obtained protein and application of His-tag instead of highly immunogenic (especially in cows) GST [27]. The His-tag has relatively lower immunogenicity and it also avoids additional steps in the ELISA procedure, such as preincubation of GST-tag with tested [27]. Additionally, the use of His-rSipC and its affinity towards nickel-chelate plates, allowed us to reduce the blocking time, a part of the general standard protocol of ELISA. Considering all advantages of established ELISA with SipC recombinant protein as antigen, we advocate the utility of His-rSipC as a high-quality simple and quick bio-reagent for the detection of specific anti-SipC antibodies in the biological specimens associated to Salmonella infections.

Conclusion:

For optimal diagnostic output, it is vital to test for a rise of IgM and IgG antibodies to diagnose typhoid fever because IgM and IgG antibodies against *S. typhi* alters depending on the stage of infection [28].Moreover, a 4-fold rise in antibody titers between acute and convalescent sera after 1014 days offers meaningful diagnostics interpretation of typhoid [29]. Our results have demonstrated that SipC can be used for the evaluation of both IgM and IgG (Figure 4) and shown the clinical usability of SipC. However, this is a preliminary study and needs to be confirmed by a prospective study on a larger number of samples. After confirmation with more clinical samples, a lateral-flow or smart-phone-based device could be developed for the point-of-care application.

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