Chapter-3

MATERIALS AND METHODS

3.1. Sources of Materials and Sampling

Faecal samples/rectal swabs and tissue samples were collected from various species of animals and birds reported to be clinically affected with diarrhoea or suspected to be cases of salmonellosis. Tissue samples were collected from dead animals and birds on postmortem examination. Human diarrhoeic stool samples were also collected for the present study. The materials were collected aseptically in sterile containers and swabs, immediately brought to the laboratory and kept at 4 ºC until further processing.

1. Samples were collected from different locations of Assam, Meghalaya and Mizoram as depicted in Fig (1 and 2) with respective latitude and longitude. Instructional Poultry Farm and AICRP on Poultry Breeding, College of Veterinary Science, Assam Agricultural University, Guwahati-22, Assam.
2. AICRP on Pig, College of Veterinary Science, Assam Agricultural University, Guwahati-22, Assam.
3. Private Farms in and around Assam, Mizoram and Meghalaya.
4. Cattle farm, Animal Husbandry and Veterinary Department, Govt. of Assam, Khanapara.
5. Guwahati Diagnostic Laboratory, Bhangagarh, Guwahati.
6. Guwahati Medical College Hospital, Bhangagarh, Guwahati.
7. Department of Veterinary Pathology, Khanapara, Assam.
8. Assam State Zoo cum Botanical Garden, Guwahati.
9. Deepor Beel, a Ramsar site near Guwahati.
10. Kaziranga Bird Rescue Centre of the Kaziranga National Park.
11. Regional Pig Farm and Regional Cattle Farm, Kyredemkulai, Shillong.

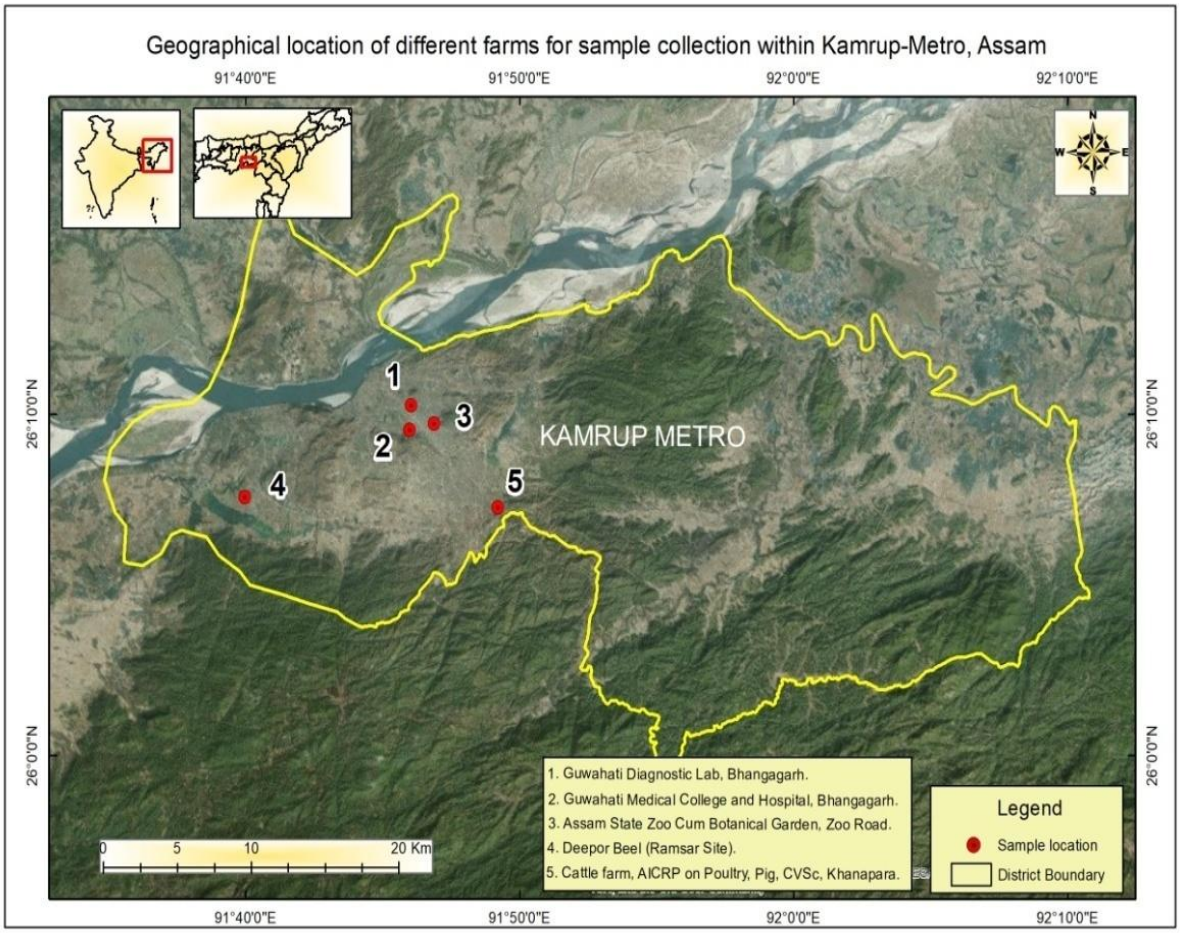

Fig 1: Geographical location of different farms for sample collection within Kamrup Metro, Assam (Courtesy: GIS laboratory of Aaranyak, Guwahati).

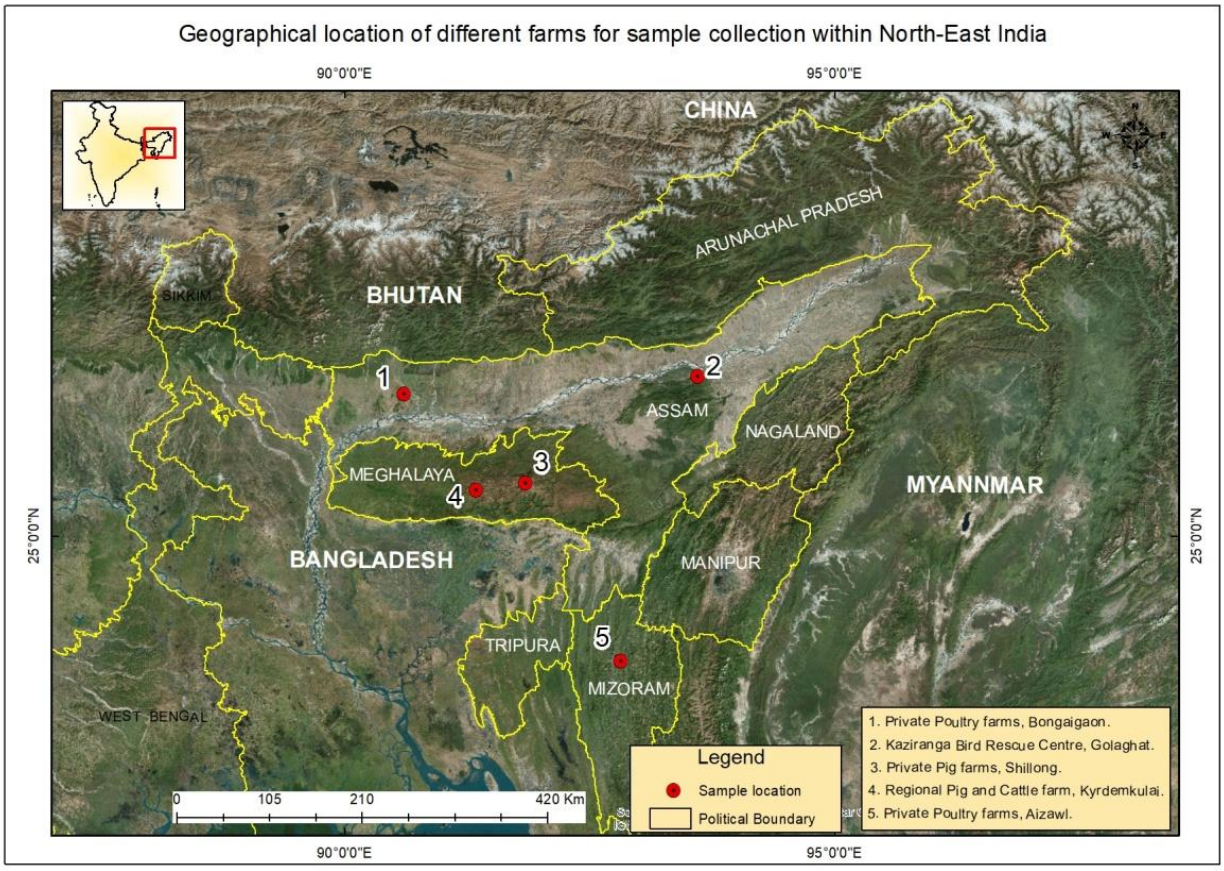

Fig 2: Geographical location of different farms for sample collection within North-east India (Courtesy: GIS laboratory of Aaranyak, Guwahati).

3.2. Isolation of Salmonella from Collected Samples

3.2.1. Isolation of *Salmonella*

The following media were used for isolation of *Salmonella*

(a) **Media used for primary isolation**:

- Rappaport Vassiliadis (RV) broth was used as an enrichment broth for primary isolation of *Salmonella* (June *et al*., 1995).
- Brilliant Green Agar (BGA) was used as a selective medium for confirming the isolates obtained in Rappaport-Vassiliadis (RV) media.

(b) **Media used for purification of colonies**: Mac Conkey's Lactose Agar (MLA) and Nutrient Agar (NA) were used for purification of suspected *Salmonella* colonies that appeared on BGA.

(c) **Media for maintenance of culture**: For maintaining the purified Salmonella isolates, Nutrient Agar (NA) medium containing 1.5 per cent agar was used. The isolates were also preserved in NA medium until further use.

3.2.2. Inoculation of samples

For isolation of *Salmonella*, faecal samples were inoculated in test tubes containing 5 ml of RV broth, which was used as an enrichment medium. The tubes were incubated aerobically at 37° C for 24 to 48 hours. A loopful of broth culture was then streaked onto BGA plates and incubated for a period of 24 hours at 37° C. Subsequently the plates were screened and suspected pink or red colonies were sub-cultured on MLA plates for purification. The plates were then incubated aerobically at 37°C for 24 hours. Single transparent colonies from MLA plates were stained by Gram's staining method for microscopic examination.

3.2.3. Maintenance of cultures

After purification, representative colonies of different isolates were streaked onto NA slants and incubated at 37° C for 24 hours. The slants were sealed with molten paraffin and maintained at 4° C in refrigerator and were used whenever required. Viability was maintained by sub-culturing the isolates at an interval of 4-6 weeks onto fresh NA slants.

Simultaneously, the cultures were also maintained in 50% glycerinated broth and preserved in -80°C.

3.2.4. Characterization and identification of the isolates

Characterization and preliminary identification of suspected *Salmonella* cultures were made on the basis of morphology, colony characteristics, and biochemical tests as per the method recommended by Cruickshank *et al*. (1975) and Edwards & Ewing (1986).

Morphological identification

Isolated cultures were characterized morphologically by Gram staining technique depending on its shape, size and arrangement along with staining reaction and were subsequently recorded.

Colony characteristics

The colonies were characterized according to their colour, size, shape and appearance on culture media.

Biochemical tests

Biochemical tests required for identification of *Salmonella* were performed as per the methods described by Cruickshank *et al.* (1975), and Edwards and Ewing (1986). The isolates were subjected to the following biochemical tests:

(a) **Carbohydrates fermentation:** Fermentation of glucose, lactose, sucrose, mannitol and dulcitol were recorded.

(b) **Indole test:** Production of indole was tested by a colorimetric reaction by using Kovac's reagent.

(c) **Methyl red (MR) test and Voges Proskauer's (VP) test:** These tests were performed in Glucose Phosphate Peptone water (GPPW) broth.

(d) **Citrate utilization test:** This test was performed by using Simmon's citrate agar slants.

(e) **Hydrogen sulphide (H_2S) production test:** Hydrogen sulphide production was studied using TSI (Triple Sugar Iron) agar slant. Interpretation was made on the basis of H_2S production and sugar fermentation pattern based on colour change.

3.3. Serotyping of *Salmonella*

The isolated *Salmonella* strains were sent for serotyping to the National Institute of Cholera and Enteric Diseases (NICED), Kolkata.

3.4. Molecular Detection of *Salmonella* by Simplex PCR

3.4.1. Reference strains

Standard reference strains of *Salmonella* used in this study were obtained from Microbial Type Culture Centre (MTCC), Chandigarh and National Institute of Cholera and Enteric Diseases (NICED), ICMR, Kolkata.

3.4.2. DNA extraction

The isolates were grown in Luria Bertani (LB) broth for overnight at 37°C and then centrifuged at 12,000 rpm for 10 minutes. The supernatants were discarded and were suspended in a total volume of 100µl of 1XTE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and boiled at 100°C for 5 minutes. After boiling, the cell suspensions were cooled on ice for 3-4 minutes and centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was then collected without disturbing the sediment and was stored in -20° C for future use as template DNA for PCR (Theron *et al*., 2001)

3.4.3. Confirmation of *Salmonella* isolates by simplex PCR

The isolates were confirmed as *Salmonella* by detecting *Salmonella* specific gene *invA* by simplex PCR.

Table 3.1.Primer sequences used for amplification of *Salmonella*-specific gene, *invA*

Primer	Primer sequence ($5^{/}$ - $3^{/}$)	Amplicon size
Forward	ACCACGCTCTTTCGTCTGG	942 bp
Reverse	GAACTGACTACGTAGACGCTC	

3.4.4. PCR reaction mixture

For detection of *invA*gene, simplex PCR was carried out in a reaction volume of 25 µl. The amount of each reagent in the reaction mixture is provided below:

Components	Volume
Master Mix (Fermentas) Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP).	12.5 µl
Template DNA	1.0 µl
Forward primer (10 pmol/µl)	0.5 µl
Reverse primer (10 pmol/µl)	0.5 µl
Nuclease free water (NFW)	10.5 µl
Total	**25.0 µl**

3.4.5 Cycling Conditions for Simplex PCR:

Steps	Temperature (ºC)	Time	No. of cycles
Initial denaturation	94	5 min	1
Denaturation	94	30 sec	30
Annealing	56	1 min	
Extension	72	30 sec	
Final extension	72	5 min	1
Hold	4	-	-

3.5. Molecular Detection of Virulence Genes of *Salmonella* by Multiplex PCR

All the standard strains and field isolates of *Salmonella* were screened for the presence of seven important virulence genes, viz. *invA, invH, stn, sopB, sopE, sefC* and *pefA* by multiplex PCR using the primers as shown in Table 3.2. For the present study, specific primers were designed using the corresponding gene sequences downloaded from NCBI database using Primer 3 software.

Table 3.2: Primer sequences designed for amplification of the virulence genes under study

Target Gene	Primer sequence (5' – 3')	Primer concentration (pMol/µl)	Product size (bp)	Accession No. (NCBI)
invA	Forward- CCACGCTCTTTCGTCTGG Reverse- AACTGACTACGTAGACGCTC	20	942	AE006468.1
invH	Forward- TATAGCTGTCTTCCTGTCTT Reverse - ATGTATTGTGGATGTTCCTG	10	305	AE006468.1
Stn	Forward- ATTGAGCGCTTTAATCTCCT Reverse- GCTGTTGAATCTGTACCTGA	10	543	AE006468.1
sopB	Forward- GCATCTCTAAACGCTACTG Reverse- GCTTCTATCACTCAGCTTCA	10	470	AE006468.1
sopE	Forward- GTAGGGCAGTATTAACCAG Reverse- TTTATCTCCCTAGGTAGCCC	10	254	AE014613.1
pefA	Forward- CCAAAGTACTGGTTGAAAG Reverse- TATTTGTAAGCCACTGCGAA	20	185	AE006471.1
sefC	Forward- GCAGGTCCAAAACTATACA Reverse- CGATAACGAAACACCATTT	10	609	AE014613.1

3.5.1. Cycling Conditions standardized for multiplex PCR:

The PCR condition suitable for the amplification of the genes of the multiplex was standardized as shown in Table 3.3 and 3.4. Negative control mixtures containing no template or genomic DNA from *Salmonella* were also included in each of the sample run.

Table 3.3: Cycling Conditions used for multiplexPCR:

Steps	Temperature (°C)	Time	No. of cycles
Initial denaturation	94	5 min	1
Denaturation	94	30 sec	35
Annealing	56	90 sec	
Extension	72	2 min	
Final extension	72	10 min	1
Hold	4	-	-

Table 3.4: Composition of PCR Reaction Mixture Used in Multiplex PCR

Components	Volume
2X PCR Master mix	12.5 µl
Primers (F & R) 20 pmol/µl (*invA,pefA*) 10 pmol/µl (*invH,sopB,sopC,stn and sefC*)	7.4 µl
25mM $MgCl_2$	1.5 µl
NFW	1.6 µl
Template (40-60 ng)	2.0 µl
Total	**25 µl**

3.5.2. Confirmation of Multiplex PCR Products by Agarose Gel Electrophoresis

Solutions and buffers

Working solution of TAE buffer (1X)

Tris-acetate	40.0 mM
EDTA	1mM

Agarose gel

Agarose	0.3 g
1X TAE	30.0 ml
Ethidium bromide (10mg/ml)	1.5µl

6X Loading Dye

Bromophenol blue	0.25%	25 mg
Xylene cyanol	0.25%	25 mg
Glycerol	30%	3.3 ml
Double distilled water (DDW)	6.7 ml (Total 10 ml)	

Agarose (1.5%) was prepared in 1X TAE (Tris Acetate EDTA) buffer in a conical flask and heated to dissolve the agarose completely using a microwave oven. The dissolved agarose was cooled down to a temperature of around 55-60^{o}C and ethidium bromide solution (stock conc. 10mg/ml) was added to make the final concentration 0.5 µg/ml. Dissolved agarose was poured into a gel casting tray assembly (Bio-Rad) having comb of appropriate number and sizes hung into that and then allowed to solidify at room temperature. Once solidified, the gel was placed in electrophoresis tank containing 1X TAE and then the combs were removed from the gel. PCR products and 6X loading dye were mixed and were loaded in the wells with the help of micropipette. Simultaneously, 6 µl of DNA ladder (100bp DNA ladder, NEB) was also loaded in one of the wells to access the size of PCR products. A negative control containing no template was also included in each of the sample runs.

Electrophoresis was carried out for 60 minutes at 80 volts, viewed under Gel Documentation System (Bio-Rad) and the result was recorded.

3.6. Bacterial expression and purification of outer membrane lipoprotein invH of *Salmonella*

3.6.1. Bacterial strains and plasmid

A *Salmonella* Typhimurium strain (MTCC 98) was used as the donor strain in the present study to clone *invH* gene. *Escherichia coli* DH5α was used as the host and plasmid pGEM-T (Promega) was used as the vector.

3.6.2. PCR amplification of *invH* gene

DNA sequence of the *invH* gene of *S.* Typhimurium strain MTCC 98 encoding the outer membrane lipoprotein invH was amplified by PCR using following set of primers:
invH F: 5′- GAGGTCTAGAATGAAAAAATTTTATAGCTGTCTTC -3′
invH R: 5′-TATACTCGAGTAAGGCTTGCAGTCTTTCAT -3′

Primers were designed using online primer BLAST software of NCBI using conserved regions of the gene sequences accessed from GenBank database. Restriction sites for XbaI were incorporated at the 5' end of one primer and sites for XhoI at the 5' end of the other primer to facilitate cloning. Amplification of the *invH* gene was carried out in a total volume of 50 µl as shown in Table 3.5.

Table 3.5: Composition of PCR Reaction Mixture

Components	Volume
NFW	38 µl
$MgCl_2$ (50mM)	1.5 µl
10X Buffer	5.0 µl
dNTPs (10 mM)	1.0 µl
*invH*Forward primer (20pmol/µl)	1.0 µl
invH Reverse primer (20pmol/µl)	1.0 µl
Template	2 µl
Taq DNA polymerase(5U/µl, invitrogen)	0.5 µl
Total volume	**50 µl**

The following conditions were used for the amplification of *invH* gene in a thermocycler (Eppendorf).

Steps	Temperature (^{o}C)	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	56	45 sec	
Extension	72	1 min	
Final extension	72	10 min	1

3.6.3. Agarose gel electrophoresis of the PCR amplified *invH* gene

After PCR, the amplified product was run in 1.5% TAE agarose gel as mentioned in the section 3.5.2. The *invH* fragments were gel purified by using commercial gel extraction kit (Wizard SV Gel and PCR Clean up system, Promega).

Procedure followed:

A. Dissolving the gel slice

The products were run on 1.5% agarose gel prepared in TAE buffer. After electrophoresis, the appropriate DNA bands were excised from the gel with the help of a Bard-Parker blade and taken in a micro centrifuge tubes. 10 µl of membrane binding solution was added per 10 mg of gel slice. The mixture was then vortexed and incubated at 60ºC until the gel slice completely dissolved.

B. Binding of DNA

The SV Minicolumn was inserted into the collection tube. The dissolved gel mixture was transferred to the minicolumn assembly and incubated at room temperature (RT) for 1 minute. The mixture was centrifuged at 16,000 x *g* for 1 minute. The flow through was discarded and the minicolumn was reinserted into the collection tube.

C. Washing

700 µl of Membrane wash solution (ethanol added) was added and centrifuged at 16,000 x *g* for 1 minute. The flow through was discarded and the minicolumn was

reinserted into collection tube. 500 µl of Membrane wash solution (ethanol added) was added and centrifuged at 16,000 x *g* for 5 minutes. The flow through was discarded and the minicolumn was reinserted into collection tube. The column assembly was subjected to empty spin for 1 minute at 16,000 x *g*.

D. Elution

The contents of minicolumn were transferred to a clean 1.5 ml micro centrifuge tube. 50 µl of nuclease free water was added to the minicolumn, incubated at room temperature for 1 minute and centrifuged at 16,000 x *g* for 1minute. Minicolumn was discarded and the DNA was stored at -20 ºC.

3.6.4. Cloning of outer membrane lipoprotein invH:

A. Ligation of the gel purified, *invH* gene fragment into pGEM-T Vector

The gel purified, *invH* gene fragment was ligated into pGEM-Tvector (Promega) in a vector insert molar ratio of 1:3 as follows:

$$\text{ng insert required} = \frac{\text{ng vector x kbp insert}}{\text{Vector size (kbp)}}$$

Components	Volume
pGEM-T vector (Promega)	2.0 µl
Insert invH fragment (262.8ng/µl)	1.75 µl (1:10 dilution)
2X ligation buffer	10.0 µl
T4 DNA Ligase (Fermentas, 3 weiss unit)	2.0 µl
NFW	4.25 µl
Total	**20.0µl**

The ligation mixture was incubated at 22ºC for 3-4 hours in an Eppendorf thermo cycler.

B. Preparation of *E. coli* (DH5α) competent cells

Solutions and buffers

1) RF I solution

Components (molecular weight)	Final concentration	Amount
Rubidium chloride (MW 120.9)	100 Mm	6.00 gm
Manganese chloride (MW 197.91)	50 Mm	4.95 gm
Potassium acetate (MW 98.15)	30 Mm	1.47 gm
Calcium chloride (MW 147.02)	10 mM	0.75 gm
Glycerol	15% (w/v)	75 ml
DDW upto		500 ml

The pH of the solution was set at 5.8 and filter sterilized before use.

2) RF II Solution

Components (molecular weight)	Final concentration	Amount
MOPS (4-Morpholinopropanesulfonic acid (MW 209.3)	10 mM	1.55 gm
Rubidium chloride (MW 120.9)	10 mM	0.60 gm
Calcium chloride (MW 110.98)	75 mM	5.63 gm
DDW upto		500 ml

The solution was sterilized by filtration.

Procedure:

DH5α strain of *E. coli* (obtained from frozen stock) was streaked on LB-agar plate and incubated for 16 hrs at 37°C. The bacterial colonies which appeared on LB- agar plate were then selected. Single colony was picked from the plate and transferred to 50 ml of LB medium in 500 ml flask and incubated at 37ºC overnight in a shaker incubator at 150 rpm. 2 ml of DH5α cells were inoculated for sub-culturing onto 100 ml of LB (1:100 ratios) and kept in shaker incubator for 3-4 hours at 37°C and 200 rpm to reach an OD of 0.6.The

culture was then transferred to sterile 50 ml centrifuge tubes and chilled on ice for 10 minutes. The tubes were then re-centrifuged at 6500 rpm at 4°C for 10 minutes, supernatant discarded and cells were resuspended in 30 ml of cold RF I solution and chilled on ice for 30 minutes. The tubes were centrifuged at 6500 rpm for 10 minutes at 4°C.The supernatant was discarded and the cells were resuspended in 1 ml of RF II solution and chilled on ice for 15 minutes.100 µl aliquots were prepared in pre-chilled sterile 1.7 ml MCTs and were stored at -80ºC until further use.

3.6.5. Transformation of DH5α competent cells

The newly constructed plasmid was transformed into *E.coli* DH5α competent cells as described by Sambrook and Russell (2001). All the steps were carried out under sterile conditions. For transformation, 100 µl aliquots of competent cells were mixed with 10 µl DNA samples (100-200 ng) and incubated on ice for 15-20 min. The cells were then subjected to heat shock at 42 °C for 90 sec and immediately placed on ice for 2-5 min. 1 ml of LB broth was added to the cells and incubated at 37°C with shaking for 1 hr. The cells were spread on LB agar plates supplemented with kanamycin (50 µg/ml) and incubated at 37°C for 16-18 hr.

3.6.6. Screening and selection of clones

The agar plates were observed for the development of colonies. The colonies were selected by blue-white screening. The white colonies were further screened by colony PCR for the presence of the desired insert.

A. Screening of recombinant clones by colony PCR

The recombinant clones were screened for the presence of desired insert by PCR using sequencing primers for the presence of insert. The following reaction mixture was prepared for amplification of *invH* gene by *Taq* DNA polymerase (Invitrogen) using universal pUC/M13 forward and reverse sequencing primers.

pUC/M13 F: 5′-GTTTTCCCAGTCACGAC-3′

pUC/M13 R: 5′-CAGGAAACAGCTATGAC-3′

Composition	Volume
NFW	40 µl
$MgCl_2$ (50mM)	1.5 µl
10X Buffer	5.0 µl
dNTPs (10 mM)	1.0 µl
pUC/M13 Forward primer (20pmol/µl)	1.0 µl
pUC/M13 Reverse primer (20pmol/µl)	1.0 µl
Template (bacterial colony)	Clone (picked by sterile pipette tips)
Taq DNA polymerase(5U/µl, invitrogen)	0.5 µl
Total volume	**50 µl**

The following conditions were used for the amplification of *invH* gene in a thermocycler (Eppendorf).

Steps	Temperature(^{o}C)	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	50	45 sec	
Extension	72	1 min	
Final extension	72	8 min	1

B. Selection of the clones

After PCR, the amplified product was run in 1.5% TAE agarose gel as mentioned in section 3.5.2. The recombinant clones which showed the presence of desired insert by PCR amplification were selected for further studies. Positive white Clones (5-6 nos.) showing desired gene inserts in PCR were picked by sterile pipette tips and inoculated separately onto 50 ml LB broth containing Ampicillin (100µg/ml) and incubated at 37^{o}C overnight in a shaker incubator at 150 rpm. After overnight incubation, the plasmids were isolated from the broth cultures by alkaline lysis method.

3.6.7. Plasmid Isolation by alkaline lysis method (Sambrook *et al.*, 2001)

Solutions for plasmid extraction

Alkaline lysis solution I

Components	Final concentration	Amount
Glucose	50 mM	0.9008 gm
Tris-Cl (pH-8.0)	25 mM	0.37224 gm
EDTA (pH-8.0)	10 mM	0.394gm
Double Distilled Water (DDW)		100ml

Solution I was sterilized by autoclaving for 15 minutes at 15lbs/sq inch pressure and stored at 4°C.

Alkaline lysis solution II

Components	Final concentration	Amount
NaOH (0.2 N stock)	0.2 N	0.78 g
SDS	1.0 %	1.0 g
DDW		100 ml

Solution II was prepared freshly.

Alkaline lysis solution III

Components	Final concentration	Volume
5M potassium acetate	5 M	60 ml
Glacial acetic acid	11.5 %	11.5 ml
DDW		100 ml

Solution III was stored at 4°C.

Procedure:

The Bacterial plasmids were extracted from the broth cultures as per the method of Sambrook *et al.* (2001) with slight modifications. Briefly, broth cultures (8 ml) were centrifuged at 8,000 rpm for 10 minutes. Supernatant was discarded and the pellet was

resuspended in 200 µl ice cold alkaline lysis solution I, mixed by pipetting and kept at room temperature for about 10 minutes. 200 µl of freshly prepared alkaline lysis solution II was added and mixed gently by inverting the tubes to obtain clear lysate. The tubes were kept on ice for 5 minutes. 300 µl of alkaline lysis solution III was added, mixed by inverting the tubes several times and then kept on ice for 10 minutes. The tubes were centrifuged at 12,000 rpm for 10 minutes at 4°C.The supernatant was transferred to a new tube, equal volume of phenol: choloform: isoamyl alcohol (25:24:1) was added and mixed by inverting the tube several times. The suspension was centrifuged at 12,000 rpm for 10 minutes at 4°C.The aqueous phase was collected in a new tube and RNaseA treatment (1U/µl) was given for about 1hour at 37ºC. After that 1/10th volume of 3M sodium acetate (pH-5.2) and equal volume of isopropanol was added to it. These were gently mixed and kept at -80°C for 1-2 hrs.The tubes were then centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatant was discarded and the pellet was washed with 70% ethanol by centrifuging at 12,000 rpm for 10 minutes at 4°C. Washing step was repeated once more. The pellet was air dried and then 50 µl of TE buffer was added to dissolve the pellet. The plasmids were stored at -20°C for further use.

3.7. Cloning of *Salmonella invH* gene into pET 303 CT His expression vector

The pET 303CT His expression vector, procured from SABT, GADVASU, Ludhiana, was used for expression of *Salmonella* outer membrane invH.

3.7.1. RE digestion to release the invH coding sequences from the pGEM-T plasmids and to linearise pET 303 CT/ His vector for cloning

The recombinant pGEM-T vector having *invH* insert and the pET303 CT His expression vector were subjected to*XhoI* and *XbaI* RE digestion separately. The double restriction enzyme digestion (*XhoI* and *XbaI*) reactions were set up in 30 µl and 75 µl volumes, respectively for the release of *invH* insert from pGEM-T plasmid as well as to linearise the pET303 CT His vector for cloning purpose. The reaction mixture was incubated at 37ºC in a water bath overnight.

Components	Volume
Plasmid(2051 ng/µl)	8.00 µl
Reaction buffer(Fast Digest Buffer)	3.00µl
Enzyme(XhoI)(10U/µl)	8.00µl
Enzyme(XbaI)(10U/µl)	8.00µl
NFW	3.00µl
Total	**30.0 µl**

Digestion invH insert

Components	Volume
Plasmid (315 ng/µl)	50.0 µl
Reaction buffer(2X Tango Buffer)	15.0 µl
Enzyme(XhoI)(10U/µl)	1.0 µl
Enzyme(XbaI)(10U/µl)	1.0 µl
NFW	8.0µl
Total	**75.0 µl**

Digestion pET303CT/His vector

3.7.2. Purification of RE digested products by agarose gel electrophoresis

Agarose gel electrophoresis and purification of RE digested products were done according to the procedure described in section 3.5.2 and 3.6.3.

3.7.3 Ligation of the gel purified, *invH* gene fragment into pET303 CT His Vector

The gel purified, *invH* gene fragment was ligated into pET 303 CT His vector in a vector insert molar ratio of 1:3 as follows:

$$\text{ng insert required} = \frac{\text{ng vector x kbp insert}}{\text{Vector size (kbp)}}$$

Components	Volume
pET 303 CT His vector(13.5ng/µl)	7.5 µl
Insert *invH* fragment (13ng/µl)	2.0 µl
10X ligation buffer	2.0 µl
T4 DNA Ligase (Fermentas, 3 weiss unit)	1.5 µl
NFW	7.0 µl
Total	**20.0µl**

The ligation mixture was incubated at 22ºC for 3-4 hours in an Eppendorf thermo cycler.

3.7.4 Transformation of DH5α competent cells

After ligation, 10 µl of the ligation mixture was added into DH5α competent cells for transformation as per the protocol described in section 3.6.5.

The clones (10-15 Nos.) obtained after transformation were picked and streaked on the LB agar plates containing Ampicillin (100µg/ml) and incubated at 37ºC overnight.

3.7.5 Screening of recombinant clones by colony PCR

The recombinant clones were screened for the presence of desired insert by PCR using sequencing primers for the presence of insert. The following reaction mixture was prepared for amplification of *invH* gene by *Taq* DNA polymerase (Invitrogen) using universal T7 forward and T7 reverse sequencing primers.

T-7F: 5′-TAATACGACTCACTATAGGG-3′

T-7R: 5′-TAGTTATTGCTCAGCGGTGG-3′

Components	Amount
NFW	40 µl
$MgCl_2$ (50mM)	1.5 µl
10X Buffer	5.0 µl
dNTPs (10 mM)	1.0 µl
T7 Forward primer (20pmol/µl)	1.0 µl
T7 Reverse primer (20pmol/µl)	1.0 µl
Template (bacterial colony)	Clone (picked up by sterile pipette tips)
Taq DNA polymerase(5U/µl, invitrogen)	0.5 µl
Total volume	**50 µl**

The following conditions were used for the amplification of *invH* gene in a thermocycler (Eppendorf).

Steps	Temperature(^{o}C)	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	50	45 sec	
Extension	72	1 min	
Final extension	72	8 min	1

A. Selection of the clones

After PCR, the amplified product was run in 1.5% TAE agarose gel as mentioned in section 3.5.2. Plasmids were isolated from the broth cultures of the positive white clones (5-6 nos) by alkaline lysis method as per the protocol described earlier (section 3.6.7).

B. Sequencing of the positive clones

The positive clones were sent for sequencing to 1st base sequencing system; Malaysia. Sequencing was done using T7 forward and reverse sequencing primers to confirm the presence and orientation of the insert.

3.8. Expression of *invH* gene in bacterial expression BL21 (DE3 star Chem. Competent Cells, Life technologies) system

3.8.1. Transformation of BL21 competent cells

The recombinant pET 303 CT His plasmid DNA extracted from the positive DH5α clone was transformed into BL21 (DE3) *E. coli* competent cells for protein expression.

3.8.2. Optimization of protein expression by IPTG induction

pET 303 CT His plasmid carrying BL21 clones were subjected to induction by IPTG for optimization of recombinant *invH* protein expression.A single recombinant colony was picked from the LB Ampicillin plate and inoculated into 5 ml of LB carbenicillin (100µg/ml) broth. The broth was incubated overnight at 37^{o}C with the rotary shaking speed of 200 rpm. Overnight culture 0.2 ml was inoculated into 20 ml of LB carbenicillin broth (5 tubes) and grown at 37°C with agitation. An OD_{600} of 0.5-1.0 was achieved within 3hrs. Two ml of uninduced culture was removed and centrifuged for 1min in a microcentrifuge tube. The supernatant was discarded and the pelleted cells were stored in -20°C. IPTG at 1.0 mM concentration was added to the culture tubes and continued to incubate the culture as described above.2 ml aliquots of cells were removed at 2, 4, 6 and 8 hours post-induction from each tube for protein expression study. The cells were centrifuged as mentioned above and the pellets were stored in -20°C till further use.

3.8.3. Solutions/buffers for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Solution A (30% acrylamide stock solution):

Acrylamide	29.2 g
Bis (N, N'-Methylenebisacrylamide)	0.80
DDW upto	100 ml
Stored in dark at 4°C	

Solution B (1.5% M Tris-HCl buffer, pH 8.8):

Tris base (Tris hydroxymethyl aminoethane)	18.20 g
SDS (Sodium dodecyl sulfate)	0.40 g
DDW up to	100 ml
pH adjusted to 8.8 with 1N HCl. Stored at 4°C	

Solution C (0.5 M Tris-HCl buffer, pH 6.8):

Tris	6.10 g
SDS	0.40 g
DDW upto	100 ml
pH adjusted to 6.8 with 1N HCl. Stored at 4°C	

Solution D (10% ammonium persulfate):

Ammonium persulfate	100 mg
Dissolved in 1 ml DDW (Prepared fresh before use)	

15 % SDS-PAGE resolving gel cocktail (100 ml):

Water	25.5 ml
30% Acrylamide (Solution A)	49.5 ml
1.5 M Tris-HCl, pH 8.8 with 0.4% SDS (Solution B)	25 ml

5% SDS-PAGE stacking gel cocktail (100 ml):

Water	58 ml
30% Acrylamide (Solution A)	17 ml
0.5 M Tris-HCl, pH 6.8 with 0.4% SDS (Solution C)	25 ml

15% Resolving gel (5ml)

15% resolving gel cocktail	4.95 ml
Ammonium persulphate (APS)	0.05 ml
TEMED	0.002 ml

5% Stacking gel (1ml)

5% resolving gel cocktail	0.99 ml
Ammonium persulphate (APS)	0.01 ml
TEMED	0.001 ml

Tris- Glycine buffer (Running buffer 5X)

Glycine	144 g
Tris	30 g
10% SDS	50 ml
Volume made up to 1000 ml with DDW	

1X Running Buffer:

Glycine	9.40 g
Tris base	1.50 g
10% SDS	5 ml
DDW up to	500 ml

2X loading buffer

10% SDS	4.0 ml
β- Merceptoethanol	0.8 ml
Sucrose	2.0 g
1 M Tris (pH 6.8)	1.25 ml
Bromophenol blue	1.00 ml
DDW upto	10.0 ml

Staining solution

Acetic acid	10%
Methanol	45%
DDW	45%
Coomasie brilliant blue	0.15%

Destaining solution

Acetic acid	10%
Methanol	45%
DDW	45%

3.8.4 Expression study of recombinant outer membrane lipoprotein invH by SDS-PAGE analysis

SDS-PAGE analysis was carried out in a vertical minigel electrophoresis apparatus (Bio-Rad) as follows:

Glass plates were cleaned and set in a gel casting assembly of the Bio-Rad's electrophoresis system and 15% resolving gel solution was prepared and poured between the two SDS-PAGE glass plates. Immediately, 1 ml of distilled water was poured over the gel for making an even surface for the stacking gel. After polymerization, water was removed by tilting the plates. Then 5% stacking gel was prepared, poured over the resolving gel and a suitable comb was inserted. The polymerized gel was mounted into the electrophoresis chamber after removing the combs and buffer reservoir was filled with 1 X Tris glycine running buffer. Before loading, sample buffer (2X) was added to equal volume of each sample (bacterial cell pellet) and then boiled for 10 minutes in a water bath at 95ºC. The samples were then centrifuged at 10,000 rpm for 15 mins and the supernatants (25µl from each sample) were loaded into the wells of SDS-PAGE gels. A prestained protein molecular weight marker (Thermo scientific) was included in a well along with the samples. Electrophoresis was carried out at a constant current of 80V, till the tracking dye reached the bottom of the gel. The gel was taken out from the plates and stained with coomassie brilliant blue for 2 hours and then destained with several changes of the destaining solution. The desired size of the expressed recombinant protein was compared with the protein ladder.

3.9 Solutions for protein purification at native conditions

A. Binding/Lysis buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
15 mM Imidazole	0.10 g
DDW upto	100 ml

B. Washing buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
Imidazole	40-100 mM
DDW	100 ml

C. Elution buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
250 mM Imidazole	2.38 g
DDW	100 ml

3.9.1. Purification of recombinant outer membrane lipoprotein invH by Ni-NTA column chromatography under native conditions

3.9.1.1 Preparation of lysate

10ml culture was inoculated in 1000 ml LB broth and incubated for 3 hours at 37°C with shaking at 250 rpm until OD_{600} reached 0.6.After 3 hrs of incubation, uninduced culture (10 ml) was collected into a 15 ml centrifuge tube and IPTG was added into the rest of the culture (990 ml) at a final concentration of 1mM. The cells were harvested at 6hrs post induction. The harvested culture was pelleted by centrifugation at 8000 rpm for 5 min, washed with PBS and the pellet was resuspended in 2ml per gram wet weight of lysis/binding buffer (pH 7.4). 8 mg lysozyme was added and incubated on ice for 30 minutes. Using a

sonicator equipped with a microtip, the contents were sonicated the solution on ice using six 10-second bursts at high intensity with a 10-second cooling period between each burst. RNase A (10 μg/ml) and DNase I (5 μg/ml) were added and incubated on ice for 10–15 minutes. The lysate was then centrifuged at 3,000 × *g* for 15 minutes to pellet the cellular debris and the supernatant was transferred to a fresh tube for purification.

3.9.1.2 Purification of recombinant invH protein

Preparation of the column:

The Ni-NTA Agarose (Invitrogen) was resuspended in its bottle by inverting and gently tapping the bottle repeatedly. Two ml of 50% Ni-NTA slurry (invitrogen, Cat.No.R901-15) was poured into a purification column. The slurry was allowed to settle completely by gravity until the ethanol was removed. Then 6 ml of distilled water was poured over it and the resin was resuspended by alternately inverting and tapping the column. The resin was again allowed to settle under gravity. The column was equilibrated using the denaturing lysis buffer as described in the step 2-3.

Purification under native conditions:

After preparing the column, 10ml of lysate was mixed with Ni-NTA slurry by shaking gently for 1-2 hrs at room temperature or overnight at 4°C on a rotary mixer (Tarsons), for binding of the His-tagged recombinant protein. Then the mixture was loaded into an empty column and the resin was allowed to settle by gravity and the flow through was collected. After removal of the flow through, the column was washed with 10 ml of washing buffer (pH 7.4) three times containing different concentrations of imidazole, i.e. 50mM, 100mM and 150mM, respectively. The 1 ml fractions of flow through were collected at each step of washing for SDS-PAGE analysis. The recombinant protein was eluted with 8 ml of elution buffer (pH 7.4) and stored at -20ºC after adding protease inhibitor, PMSF (phenylmethylsulfonyl fluoride) at a final concentration of 1mM.After purification, the resin was washed using 0.5 M NaOH for 30 min and 30 ml of distilled water was poured into the column. For equilibration, binding buffer was poured into the column and kept at 4°C till next use (within a week).

3.9.2 SDS-PAGE analysis of purified recombinant protein invH

SDS-PAGE analysis was carried out in a vertical minigel electrophoresis apparatus (Bio-Rad) as mentioned earlier in the section 3.8.4. Here the samples were bacterial cleared lysates, purified invH protein samples and wash flow throughs at different stages of washing. The yield of the protein was very less under native conditions indicating that the protein was present mostly in insoluble form in the cells, not in the soluble form. To check whether the protein was present in the insoluble form, the bacterial cell pellet left after the native cell lysate preparation was resuspended in the denaturing lysis buffer and checked by SDS-PAGE analysis. A high level protein expression was seen in denaturing conditions, which enabled us for further purification under denaturing conditions using 6M Urea in large scale.

3.10 Solutions for protein purification at denaturing conditions

A. Binding/Lysis buffer (100ml, pH 7.7)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
6M Urea	57.19g
15 mM Imidazole	0.10 g
DDW upto	100 ml

B. Washing buffer (100ml, pH 7.5)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
6M Urea	57.19g
Imidazole	40-100 mM
DDW up to	100 ml

C. Elution buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
6 M Urea	57.19 g
250 mM Imidazole	2.38 g
DDW upto	100 ml

3.10.1. Large scale Purification of recombinant invH protein by Ni-NTA column chromatography under denaturing conditions

3.10.2. Preparation of lysate

Fifty ml culture was inoculated in 5000ml LB broth and incubated for 3 hours at 37°C with shaking at 250 rpm until OD_{600} reached 0.6.After 3 hrs of incubation, uninduced culture (10 ml) was collected into a 15 ml centrifuge tube and IPTG was added into the rest of the culture (4990 ml) at a final concentration of 1mM. The cells were harvested at 6hr post induction. The harvested culture was pelleted by centrifugation at 8000 rpm for 5 min, washed with PBS and the pellet was resuspended in 5ml per gram wet weight of lysis/binding buffer (pH 7.7). The tube containing bacterial cell suspension was stirred for 15-60 minutes in a rotary mixer (Tarsons) at room temperature with by gentle vortexing intermittently, taking care to avoid foaming. Cell lysis was considered as complete when the solution became translucent. The lysate was centrifuged at 10,000 rpm for 30 min at 22°C to pellet the cellular debris. After centrifugation, the supernatant was transferred to a new tube for purification.

3.10.3. Purification of recombinant invH protein

The recombinant protein was purified under denaturing conditions using the buffers mentioned in the section 3.10. The protocol for purification of the protein was similar to the protocol mentioned in the section 3.9.1.2.

3.10.4. SDS-PAGE analysis of purified recombinant proteininvH

SDS-PAGE analysis was carried out in a vertical minigel electrophoresis apparatus (Bio-Rad) as mentioned earlier in the section 3.8.4. Here the samples were bacterial cleared lysates, purified invH protein samplesand wash flow throughs at different stages of washing.

3.11. Refolding of recombinant outer membrane lipoprotein invH

Solutions for protein refolding

1. 1X Phosphate Buffer Saline (PBS 500 ml, pH 7.4)

NaCl	4 g
Potassium Chloride (KCl)	0.1 g
Disodium Hydrogen Phosphate (N_2HPo_4)	0.72 g
Potassium Dihydrogen Phosphate (KH_2Po_4)	0.12 g
Water upto	500 ml
pH adjusted to 7.4 and autoclaved	

2. Refolding BufferA (400 ml, pH 8.0)

4M Urea	96.08g
0.1M glutathione reduced	0.0122g
0.01mM glutathione oxidized	0.0024g
1mM EDTA	0.15g
5% Glycerol	20 ml
1X PBS upto	400ml

3. Refolding Buffer B (400 ml, pH 8.0)

2M Urea	48.05g
0.1M glutathione reduced	0.0122g
0.01mM glutathione oxidized	0.0024g
1mM EDTA	0.15g
5% Glycerol	20 ml
1X PBS up to	400ml

4. Refolding Buffer C (400 ml, pH 8.0)

1M Urea	24.02g
0.1M glutathione reduced	0.0122g
0.01mM glutathione oxidized	0.0024g
1mM EDTA	0.15g
5% Glycerol	20 ml
1X PBS up to	400ml

The purified recombinant protein was stepwise dialyzed by using Snake Skin Pleated Dialysis tubing (3.5 kDa, Fisher Scientific) at 4°C with refolding buffer A, B and C for 3-4 hours, respectively with continuous stirring using teflon coated magnetic bar over the magnetic stirrer. Then the refolded protein was dialyzed against 2-3 changes of PBS (pH 7.4).The protein was concentrated by keeping the dialysis bags in PEG-8000 (polyethylene glycol 8000) which absorbed the PBS and reduced the volume. Then the concentrated protein was recovered from the dialysis bag and protease inhibitor cocktail was added and stored at -80°C deep freezer until further use.

3.12. Estimation of Protein Concentration

Reagents for protein estimation (Lowry *et al*., 1951)

1. Reagent A

Sodium carbonate (Na_2Co_3)	2% (w/w)
Sodium hydroxide (NaOH)	0.1N

2. Reagent B

Copper sulphate	0.5% (w/w)
Potassium Sodium tartrate	1% (w/w)

3. Reagent C (for 51ml)

Reagent A	50ml
Reagent B	1 ml

Quantification of protein was done using method described by Lowry *et al*. (1951).The Lowry reagent C was prepared freshly by mixing reagent B in the ratio of 20:1(v/v).The solutions of different concentration of BSA were prepared in PBS (pH 7.4).A volume of 100µl from each concentration of BSA solution, PBS and test solution were taken in separate tubes and 500µl of reagent C was added to all tubes. The suspension was incubated at RT for 10 mins. Reagent D (SRL) was added to all tubes.The suspension was mixed by vortexing and incubated at RT in dark for 30 mins. The OD was measured at 660nm.The concentration of test protein was estimated using regression analysis in MS excel spreadsheet (MS office 2007).The variable of OD values was taken as dependant variable(variable- Y).The results were considered significant only when the coefficient of determination (R^2) was more than 90%.

3.13. Solutions for Western Blotting

Transfer buffer

Tris Base	25 mM
Glycine	192 mM
Methanol	20%
SDS	0.1%

Blocking buffer

Bovine Serum Albumin (BSA)	0.5%
Skimmed milk powder	5%
Lactalbumin Hydrolysate (LAH)	1%
Dissolved in PBS	

Antibody dilution buffer

50% of Blocking buffer in PBS.

Primary antibody

Anti-His Tag Protein mouse antibody (Calbiochem, Cat.No. D00159204)

BCIP-NBT substrate:

Readymade solution of chromogenic substrate BCIP-NBT alkaline phosphatase conjugated (femtoChromo-AP) was used.

3.13.1 Western Blot analysis of the recombinant outer membrane lipoprotein

Expressed recombinant invH protein was characterized by western blot analysis in order to confirm the specificity/reactivity with Anti-His antibody. The following protocol was used to perform the western blotting:

Transfer of proteins into PVDF membrane

After SDS-PAGE, the gel was taken out from the plates, washed twice with DDW and then kept on transfer buffer. On the other side, cassette was placed with the gray side down on a clean surface and pre-wetted fibre pad was then kept on the gray side of the

cassette of Mini Trans blot cell module, Biorad (Cat.No. 1703810).A sheet of filter paper was placed on the fibre pad and the equilibrated gel was placed on the filter paper. After that, the pre-wetted PVDF membrane (Novex) was placed on the gel and the sandwich was completed by placing a piece of filter paper on the PVDF membrane. The PVDF membrane was dipped in 20% methanol for 1 min before use, dried and then equilibrated in transfer buffer for 20 minutes. At last, the last fiber pad was placed. Air bubble, if any was removed using roller. The cassette was closed firmly to ensure careful gel and filter paper sandwich. The cassette was locked using white latch. The cassette module was placed in an assembly in the proper direction and frozen blue cooling unit was placed in the buffer tank, to keep the buffer cool during electrophoresis. Then the buffer tank was filled with transfer buffer upto the blotting mark. Teflon coated magnetic bar was placed at the bottom of the assembly. The assembly was placed onto the magnetic stirrer to maintain even buffer temperature and ion distribution in the tank. The speed was set to keep ion distribution even. Electrophoresis was done at 80 V, constant 350 mA for 2 hrs. After transfer, the gel was stained with staining solution to check the efficiency of transfer and the membrane was subjected to immunological detection.

Development of the Western Blot

The membrane after transfer was blocked by incubating over night at 4°C in presence of blocking buffer prepared in PBS. After blocking, the membrane was washed thrice with PBS for 5 minute each, and incubated with 1:650 diluted anti –His Tag protein mouse antibody(1µg/ml) for 1hr at 37°C on a dancing shaker. The membrane was washed thrice with PBS (pH 7.4) as mentioned above. After three washings with PBS, the blot was developed by incubating the membrane with 5-7 ml of BCIP-NBT substrate (femto Chromo-AP).The colour reaction was terminated by washing the membrane with distilled water to prevent background colouration.

3.13.2. Analysis of recombinant invH protein by Dot Blot Procedure

A strip of Polyvinylidene fluoride (PVDF) membrane was used. The membrane was activated with 20 % methanol for 2 sec and washed with distilled water.The recombinant protein invH was blotted into the PVDF membrane along with the uninduced cell lysate and air-dried for 15-20 mins.The membrane was then blocked in antibody

dilution buffer (2 ml blocking buffer + 2ml of PBS +16 µl anti-his antibody alkaline phosphatase) for 30-45 mins with antibody dilution of 1:250 on a dancing shaker. The membrane was then washed 3 times (5 minutes each) with PBS (pH7.4) on a dancing shaker. The blot was developed by using BCIP-NBT substrate (femto Chromo-AP). After development of the blot, the PVDF membrane was washed with distilled water and photograph was taken.

3.14. Raising of hyperimmune sera

For determination of the immunogenicity of the purified invH protein, antisera were raised in rabbits against the purified invH protein. Two female New Zealand White rabbits of 7-8 months of age were used for raising hyper immune serum. The animals were housed separately and provided with food and water with regular supply of vitamins and minerals. The inocula were prepared by mixing the purified invH protein (200µg protein/0.5ml) with equal volume of Freund's Complete Adjuvant (FCA) and injected intramuscularly into thigh muscles of rabbit.A booster dose was given two weeks later by mixing the above antigen with equal volume of Freund's incomplete adjuvant (FICA). Blood was collected for serum collection a week after the last dose and serum was analysed for presence of antigen specific antibodies by Western blot and indirect ELISA.

3.15. Titration of polyclonal antibodies for ELISA

3.15.1 Reagents for Indirect ELISA

100 mM Carbonate-bicarbonate buffer (pH 9.6) for antigen coating

Na_2CO_3	3.03 g
$NaHCO_3$	6 g
DDW	1000 ml

PBS (pH 7.4)

Phosphate Buffer Saline Tween-20 (PBST)

PBS	100 ml
Tween-20	0.5 ml

Blocking buffer

BSA	2 % (w/v)
Dissolved in PBS	100 ml

Antibody-dilution buffer (pH 7.0)

BSA	250 mg
Dissolved in PBS	100 ml

Substrate solution

One tablet of Sigmafast buffer with urea H_2O_2 (golden foil) and one tablet of Sigmafast OPD (silver foil) of the Sigmafast OPD tablet set (P9187-50SET) were properly dissolved in 20 ml of distilled water in a amber coloured 50 ml centrifuge tube and used immediately.

3 M H_2SO_4 stop solution

Volume of H_2SO_4 (mol wt 98)	29.4 ml
DDW upto	100 ml

3.15.2 Procedure:

The levels of IgG antibody were measured by ELISA (Engvall and Perlmann, 1971). Briefly, 100 µl of antigen mixed with coating buffer (0.1 M carbonate buffer, pH 9.6) was added to each well of 96-well micro titer plates and the plates were incubated at 4°C overnight. The plates were washed once with PBS-T (PBS with 0.05% Tween 20) and blocked with 2% BSA in PBS-T for 1 hour at 37°C. After washing the plates thrice with PBS-T, different dilutions of rabbit raised invH antisera were added to the wells as primary antibody keeping appropriate controls (PBS and serum from unimmunized rabbit). Plates were incubated at 37°C for 1 hour. After washing the plates thrice with PBS-T, the plates were incubated (37ºC, 1 hr) with 100 µl of 1:3000 diluted HRP-conjugated goat anti-rabbit IgG (Sigma, USA). Plates were washed thrice with PBS-T and 100 µl of OPD/H_2O_2 substrate (Sigma, USA) was added and incubated for 10 min at RT in dark. The reaction was terminated by addition of 100 µl of 3M H_2SO_4 and the absorbance was read at 492 nm on an ELISA reader (Synergy H1 Hybrid Reader, BioTek).

3.15.3 Optimization of antigen and antibody concentration

a) Coating Antigen

The optimum antigen concentration was selected by checkerboard titration using serial dilution of antigen (recombinant invH protein). Ni-NTA column purified recombinant invH protein (conc. 7µg/µl) was serially diluted (1:6 to 1:0.5) in bicarbonate-carbonate coating buffer (pH 9.6) and coated the ELISA plate by putting 100 µl diluted antigen per well.

b) Antibody Optimisation

Primary antibody (rabbit raised invH anti sera) at 2 fold dilutions (1:100, 1:12,800) were tested for reactivity. Anti- rabbit IgG HRP-conjugate at 1:3,000 dilutions was used every time as the secondary antibody after testing its reactivity at two other dilutions (1: 1,000 and 1: 5,000)

c) Standard Curve of Indirect ELISA

The OD values obtained against different dilutions of primary antibody and different antigen concentrations were plotted to draw a standard curve and the coefficient of determination (R^2) values were derived.

The cut-off value was determined by mean OD of negative control plus 2 X Standard deviations. The titer was determined using regression analysis taking OD value as independent variable (x-variable) and reciprocal of log of serum dilution as dependant variable (y-variable).

3.15.4 Determination of Antigen Specific Antibody by Western Blot.

The production of antigen specific antibodies in hyperimmune serum of the rabbits was determined by Western Blot analysis following the process described in section 3.13.1. Rabbit raised invH anti sera (1:400) was used as primary antibody and anti- rabbit IgG HRP-conjugate at 1:3,000 dilutions was used as the secondary antibody for the process.

3.16. Immunization of mice

Six to eight weeks old male Swiss albino mice were used for immunizations with recombinant invH protein and bacterin vaccine. For separation of serum, whole blood was collected from the tail vein of the animals of all the experimental groups on 0, 7th, 14th, 21st and 28th day of immunization, taking the day of primary immunization as '0' day. Serum samples were aseptically stored in sterile vials and kept at -20°C. The details of the adjuvant, dose and route of administration of the vaccines are given in Table 3.6.

3.17. Preparation of bacterin from Salmonella by phenol-water extraction.

Salmonella Typhimurium (MTCC 98) culture was grown unshaken in trypticase soy broth (SRL, India) at 37° C for 18 hrs (log phase growth). The bacteria were then killed by addition of formalin (1% final solution) and allowed to stand at room temperature for 12 hr. The culture was then centrifuged at 4,000 x g for 20 min and the sediment washed and centrifuged 3 times in PBS (pH 7.2). The final sediment was then lyophilized. A solution of 1 gm of formalin-killed bacteria and 17.5 ml water was heated to 65-68°C while an equivalent amount of 90% phenol was preheated to 65-68° C. The phenol was then added and the mixture kept at 65-68° C with vigorous stirring for 20 min. The mixture was then placed into an ice bath and centrifuged for 30 mins at 3,000 g at 4° C. Three distinct layers appeared after centrifugation (lower phenol, middle white precipitate, and upper aqueous), and the upper aqueous layer was collected. The same amount of water taken is then replaced and the mixture was again heated, stirred, chilled, and the aqueous layer again collected. The combined opalescent aqueous layers were dialyzed against water (molecular weight cut off = 12,000-14,000) for 2 days to remove the phenol. The dialyzed material was centrifuged at 3,000 x g for 20 min at 4º C to remove anyinsoluble material. The resulting solution containing LPS wasthen lyophilized, reconstituted in PBS, and used as ELISA antigen. Thimersol was added as a preservative.

Table 3.6 Details of Vaccine Formulation, Dose, Concentration and Route of administration in different groups of mice.

Group of mice (n=10)	Formulations	Conc. of recombinant InvH protein/mice	Volume of inoculum	Route
Group A1	r(invH) of *S*. Typhimurium withFCA	50µg/mouse	200µl/mouse	s/c
Group A2	r(invH) of *S*. Gallinarum with FCA	50 µg/mouse	200 µl /mouse	s/c
Group A3	r(invH) of *S*. Enteritidis with FCA	50 µg/mouse	200 µl /mouse	s/c
Group B1	Bacterin of *S*. Typhimurium with FCA	50 µg/mouse	200 µl /mouse	s/c
Group B2	Bacterin of *S*. Enteritidis with FCA	50 µg/mouse	200 µl /mouse	s/c
Group B3	Bacterin of *S*. Gallinarum with FCA	50 µg/mouse	200 µl /mouse	s/c
Group C	PBS control	-	200 µl /mouse	s/c

The mice belonging to Group A (sub-groups A1, A2 and A3) were injected subcutaneously with 50 µg recombinant InvH protein of *S*. Typhimurium, *S*. Enteritidis and *S*. Gallinarum, respectively adjuvanted with FCA. Mice in Group B (sub-groups B1, B2 and B3) were injected with 50µg of bacterin of *Salmonella* Typhimurium adjuvanted with FCA. Mice in Group C were injected with 200 µl of PBS and maintained as the control group.

All protocols were approved by the Institutional Ethical Committee of Gauhati University for the use of animals for experimentation. The mice were bred at the Dept.of Pharmacology& Toxicology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati where housing and care met as per research requirements.

3.18. ELISA Assay for Determination of Serum Antibody Titers

The production of antigen specific antibodies in mice raised serum of the mice was estimated by plate-ELISA following the process described in section 3.15.2.

3.18.1 Optimization of antigen and antibody concentration

a) Coating Antigen

The optimum antigen concentration was selected by checkerboard titration using serial dilution of antigen (recombinant invH protein). Ni-NTA column purified recombinant invH protein (conc. 7 µg/µl) was serially diluted (1: 6 to 1: 0.5) in bicarbonate-carbonate coating buffer (pH 9.6) and coated the ELISA plate by putting 100 µl diluted antigen per well.

b) Antibody Optimisation

Primary antibody (mice raised InvH anti sera) at 2 fold dilutions (1: 100, 1: 51,200) were tested for reactivity. Anti- mice IgG HRP-conjugate at 1: 3,000 dilutions was used every time as the secondary antibody after testing its reactivity at two other dilutions (1: 1,000 and 1: 5,000)

c) Standard Curve of Indirect ELISA

The OD values obtained against different dilutions of primary antibody and different antigen concentrations were plotted to draw a standard curve and the coefficient of determination (R^2) values were derived.

The cut-off value was determined by mean OD of negative control plus 2X Standard deviations. The titer was determined using regression analysis taking OD value as independent variable (variable-X) and reciprocal of log of serum dilution as dependant variable (variable-Y).

Two-way ANOVA was performed to analyse the difference in antibody titre between two groups and within group treated for different days. After ANOVA multiple comparison of means was performed using Tukey's HSD test.

3.19. Analysis of LD_{50} (50% lethal dose)

The LD_{50} was estimated by the method prescribed by Reed and Muench (1938).The log phase grown culture of *S.*Typhimurium (MTCC 98) was diluted to the OD 1.0.A 10-fold dilution of the culture was made. Each dilution was plated on the blood agar

plate and incubated at 37°C overnight. The colony was counted to estimate the cfu per ml of culture at OD 1.0.Each dilution was injected to different groups of mice. The doses at which more than 50% and less than 50% mortality was induced were taken for estimation for LD_{50} using statistical method of Reed and Muench (1938).

$$DL = \frac{50\% - (MNB\ 50\%)}{(MNA\ 50\%) - (MNB\ 50\%)} \text{ X Log of dilution factor}$$

Log of 50% end point dilution = Log (reciprocal of starting point dilution) – DL

DL = difference of logarithms

MNA 50% = mortality next above 50%

MNB 50% = mortality next below 50%

3.20. Challenge Studies in Mice

Homologous and heterologous challenges of the mice were done on 28th day post-booster vaccination. Ten birds from each group were challenged with *S.*Typhimurium for homologous challenge and 10 each for *S.* Enteritidis and *S.* Gallinarum, respectively for heterologous challenge.The details of the challenge dose and route are given in Table 3.7. One week after challenge,the mice were euthanized and bacteriological analysis was carried out to determine persistance and clearance of the challenge strain from liver and spleen to evaluate the protective efficacy of various vaccine formulations against homologous and heterologous challenges. Aseptically collected organs were homogenized in PBS and 500µl of homogenates was plated on BGA plates for detection of bacterial count on direct culture.The number of direct-culture bacterial colonies was determined and expressed as mean $\log_{10}$ CFU/g of tissue. After reisolation of suspected Salmonella from the tissue samples on BGA plates, the cultures were confirmed by *Salmonella*-specific PCR.

Analysis of CFU (colony forming unit)

The CFU/ml was analysed by the pour plate method.The log phase grown culture of *Salmonella* Typhimurium, *S.* Enteritidis and *S.* Gallinarum were diluted to the optical density 0.1. Serial ten-fold dilutions of the culture were made. Each dilution was plated on

blood agar plate and incubated at 37°C overnight. The colony was counted to estimate the CFU/ml of culture at optical density 0.1.

Table 3.7: Details of Challenge Organism, Dose and Route of Challenge in Different Groups of Mice.

Group	Sub-group	No. of Mice	Challenge	Dose and Route
A	A1	10	*Salmonella* Typhimurium	3 X $10^{9.5}$ CFU, oral
	A2	10	*S.*Enteritidis	3 X 10^{8} CFU, oral
	A3	10	*S.*Gallinarum	3 X 10^{9} CFU, oral
B	B1	10	*S.*Typhimurium	3 X $10^{9.5}$ CFU, oral
	B2	10	*S.*Enteritidis	3 X 10^{8} CFU, oral
	B3	10	*S.*Gallinarum	3 X 10^{9} CFU, oral
C	C1	10	*S.*Typhimurium	3 X $10^{9.5}$ CFU, oral
	C2	10	*S.*Enteritidis	3 X 10^{8} CFU, oral
	C3	10	*S.*Gallinarum	3 X 10^{9} CFU, oral

3.21. Statistical Analysis

Statistical analysis of the association of vaccine groups and protection was done by Chi-square test of independence using Yates's correction. The data were expressed as mean ±standard deviation (SD) of triplicate data. The P-value of less than 0.05 was considered significant. All the statistical analyses were done using the online software, R (version 3.3.2).