Chapter-4

RESULTS

4.1. Isolation and Identification of *Salmonella*

During the present investigation, a total of 495 samples were collected from diarrhoeic (85) and healthy (34) human, diseased (56) and healthy (14) cattle, diarrhoeic (60) and healthy (28) pig, healthy (101) wild birds, diarrhoeic (72) and healthy (31) poultry, healthy (8) kako (*Gecko gecko*) and healthy (6) tiger. Among the 280 clinical samples collected from man and animals with a history of diarrhoea, 53 (18.93%) were positive for *Salmonella* (Table 4.1), while out of 215 samples collected from healthy animals and birds, 23 (10.7%) were positive for *Salmonella.* Of 85 diarrhoeic stool samples collected from human, 14 (16.47%) were positive for *Salmonella* while out of the 56 faecal samples collected from diarrhoeic cattle, 7 (12.5%) yielded *Salmonella*. Out of 60 diarrhoeic faecal samples collected from pig, 14 (23.33%) were positive for *Salmonella.* Out of the 72 faecal/intestinal samples/cloacal swabs collected from diarrhoeic poultry, 18 (25%) yielded *Salmonella*, while out of 31 healthy samples, 4 (12.9%) yielded *Salmonella*. The overall rate of recovery 76 samples (15.35%) out of 495 samples.

Table.4.1: Isolation of *Salmonella* from Human and Different Species of Animals and Birds

Host	No. of samples examined			No. of isolates recovered	
	Total	Diarrhoeic	Healthy	Diarrhoeic	Healthy
Poultry	103	72	31	18 (25%)	4 (12.9%)
Cattle	70	56	14	7 (12.5%)	-
Wild Birds	101	-	101	-	15 (14.85%)
Human	119	85	34	14 (16.47%)	-
Pig	88	60	28	14 (23.33%)	-
Gecko	8	-	8	-	2 (25%)
Tiger	6	-	6	-	2 (33.33%)
Total	**495**	**273**	**222**	**53 (18.93%)**	**23 (10.7%)**

4.1.1. Biochemical Characterization of *Salmonella* Isolates

All the 76 *Salmonella* isolates recovered from different sources fermented glucose, mannitol and dulcitol, but did not ferment lactose and sucrose. All the isolates were

positive for methyl red test, citrate utilization and hydrogen sulphide production but were negative for indole production, Voges-Proskauer (VP) test and urease production. The isolates showed yellow butt black middle and pink top on inoculation into Triple Sugar Iron (TSI) Agar slants.

4.1.2. Serotyping of *Salmonella*

The results of serotyping of the *Salmonella* isolates are presented in Table 4.2. Out of the 76 isolates, 23 (30.3%) belonged to the serovar *Salmonella enterica* subsp. *enterica* serovar Weltevreden with the antigenic structure 3{10}, r, z6; whereas 15 isolates (19.74%) belonged to serovar Enteritidis with the antigenic structure 9,12:g,m:-; 2 isolates (2.63%) belonged to serovar Typhi with antigenic structure 9,12,Vi,d:- ; 7 isolates (9.21%) belonged to serovar Newport with the antigenic structure 6, 8, e, h, 1, 2,:-; 10 isolates (13.15%) belonged to serovar Typhimurium with antigenic structure 1,4,5,12,i,1,2:-; 4 isolates (5.3%) belonged to serovar Gallinarum with antigenic structure 9(D1),1,9,12:-; 6 isolates (7.9%) belonged to serovar Choleraesuis with antigenic structure 7(C1),6,7,c,1,5:- and 5 isolates (6.6%) belonged to serovar Dublin with the antigenic structure 9(D1),1,9,12,(Vi),g,p.

Table 4.2: Distribution of Various Serovars among *Salmonella* Isolates Recovered from Different Sources

Sl. No.	Serotype	Antigenic Structure	No. of isolates	Source/ Host
1	*Salmonella* Enteritidis	9,12:g,m	15	Human (3), Poultry (6),Wild Bird (6)
2	*S.* Weltevreden	3{10},r,z_6	23	Poultry (9), Pig (5),Wild Bird (7), Gecko (2)
3	*S.* Typhi	9,12,Vi,d	4	Human (4)
4	*S.* Newport	6, 8,e, h, 1, 2	7	Cattle (2), Human (2), Poultry (3)
5	*S.* Typhimurium	1,4,5,12,i,1,2	10	Human (5), Pig (5)
6	*S.* Litchfield	8,6,8,l,v,1,2	2	Poultry
7	*S.* Kentucky	8,8,20,I,z6	2	Tiger
8	*S.* Gallinarum	9(D1),1,9,12	4	Wild bird (2),Poultry (2)
9	*S.* Choleraesius	7(C1),6,7,c,1,5	4	Pig
10	*S.* Dublin	9(D1),1,9,12,(Vi),g, p	5	Cattle

4.2. Standardization of Multiplex PCR for Detection of Virulence Genes

For standardization of the multiplex PCR (m-PCR) protocol, each of the seven genes was amplified individually by simplex PCR using standard strains of *Salmonella* (Fig 4.1 and 4.2). A total of 17 standard strains belonging to different serovars of *Salmonella* were used for this purpose (Table 4.3). The seven virulent genes targeted to be amplified for m-PCR were *invA, invH, stn, sefC, pefA, sopB* and *sopE*. The annealing temperature and cycling conditions for the m-PCR were standardized to achieve the optimum amplification which required a number of trials to be carried out using different annealing/extension time/temperature in a gradient thermal cycler. Different primer concentrations were also used for standardization of the m-PCR. Of the seven pairs of primers used, those for *invH, stn, sefC, sopB,* and *sopE* genes were used at a final concentration of 10 pMol/µl, while 20 pMol/µl concentration was used for the primers of *invA* and *pefA* genes. The PCR condition standardized for the m-PCR was 94^{o}C for 5 min, 94^{o} C for 30 sec, 56^{o} C for 90 sec, 72°C for 2 min and 72^{o} C for 10 min.

Out of the 17 standard strains of *Salmonella* screened, the one belonging to serovar Idikan (IDH 5073) showed presence of all the seven virulence genes and hence it was used as a reference strain for all the multiplex PCR assays. The serovars Typhi, Enteritidis, Choleraesuis and Typhimurium showed variable presence of the seven virulence genes while the serovar *S.* Schwarzengrund showed the presence of only four virulence genes, details of which are shown in Table 4.3.

Table 4.3: Virulence Gene Profile of Standard Strains of *Salmonella*

Sl. No.	Isolates	Strain No	Source	Virulence genes						
				invA	***invH***	***stn***	***sopB***	***sopE***	***sefC***	***pefA***
1	*S.* Newport	MTCC 3229	MTCC	+	+	+	+	+	-	-
2	*S.* Schwarzengrund	MTCC 3230	-do-	+	+	+	+	-	-	-
3	*S.* Typhimurium	MTCC 98	-do-	+	+	+	+	+	-	+
4	*S.* Typhimurium	MTCC 3917	-do-	+	+	+	+	-	-	+
5	*S.* Virchow	MTCC 1166	-do-	+	+	+	+	+	-	-
6	*S.* Abony	NCTC 6017	NICED	+	+	+	+	+	-	-
7	*S.* Choleraesius	ATCC 10708	-do-	+	+	+	+	+	+	-
8	*S.* Enteritidis	NICED 503984	-do-	+	+	+	+	+	+	-
9	*S.* Idikan	IDH 5073	-do-	+	+	+	+	+	+	+
10	*S.* Infantis	NICED 505330	-do-	+	+	+	+	+	-	-
11	*S.* Paratyphi A	NICED C6915	-do-	+	+	+	+	+	-	-
12	*S.* Paratyphi B	NICED NK3727	-do-	+	+	+	+	+	-	-
13	*S.* Poona	NCTC 4840	-do-	+	+	+	+	+	-	-
14	*S.* Typhi	NICED C6953	-do-	+	+	+	+	+	+	-
15	*S.* Vellore	ATCC 15611	-do-	+	+	+	+	+	-	-
16	*S.* Virdi	IDH 377	-do-	+	+	+	+	+	-	-
17	*S.* Worthington	IDH 3642	-do-	+	+	+	+	+	-	-

4.2.1. Screening of *Salmonella* Isolates for Virulence Genes

All the 76 *Salmonella* isolates obtained in the present study were subjected to m-PCR for detection of different virulence genes (Table 4.4). Variable virulence gene profiles were detected among the isolates. The virulence genes *invA, invH, stn* and *sopB* were detected in all (100%) the *Salmonella* isolates under the present study, while the rest three virulence genes *sefC, sopE* and *pefA* genes were present in 23 (30.26%), 39 (51.32%) and

20 (26.32 %) isolates respectively and they were found in varying percentages among the *Salmonella* serovars Enteritidis, Weltevreden, Typhi, Newport, Litchfield, Kentucky, Typhimurium, Choleraesuis, Dublin and Gallinarum.

The distribution of virulence genes according to the source of recovery of the isolates revealed that *invA, invH, stn and sopB* genes were present in all the isolates irrespective of their source of origin. The *sefC* gene was present in 23 (30.26%) while pefA and *sopE* gene were present in 20 (26.32 %) and 39 (51.32%) respectively in the isolates recovered from human, cattle, wild bird, pig, tiger, gecko and poultry (Table 4.4).

Table 4.4. Virulent Gene Profile of Field Isolates of *Salmonella*

Sl. No.	Isolates	Source	No. of isolates	Virulent genes						
				invA	*invH*	*stn*	*sopB*	*sopE*	*sefC*	*pefA*
1.	*S.* Weltevreden	Poultry	9	9	9	9	9	4	3	3
		Wild bird	7	7	7	7	7	3	3	2
		Pig	5	5	5	5	5	3	2	3
		Gecko	2	2	2	2	2	1	1	0
	Total		**23**	**23**	**23**	**23**	**23**	**11**	**9**	**8**
2.	*S.* Enteritidis	Poultry	6	6	6	6	6	3	2	0
		Wild bird	6	6	6	6	6	4	1	0
		Human	3	3	3	3	3	0	1	1
	Total		**15**	**15**	**15**	**15**	**15**	**7**	**4**	**1**
3.	*S.* Typhimurium	Pig	5	5	5	5	5	4	2	3
		Human	5	5	5	5	5	1	0	1
	Total		**10**	**10**	**10**	**10**	**10**	**5**	**2**	**4**
4.	*S.* Newport	Poultry	3	3	3	3	3	2	0	1
		Cattle	2	2	2	2	2	1	0	0
		Human	2	2	2	2	2	1	1	0
	Total		**7**	**7**	**7**	**7**	**7**	**4**	**1**	**1**
5.	*S.* Gallinarum	Wild Bird	2	2	2	2	2	1	1	0
		Poultry	2	2	2	2	2	1	0	0
	Total		**4**	**4**	**4**	**4**	**4**	**2**	**1**	**0**
6.	*S.* Choleraesius	Pig	4	4	4	4	4	3	1	2
7.	*S.* Dublin	Cattle	5	5	5	5	5	3	2	3
8.	*S.* Typhi	Human	4	4	4	4	4	1	1	0
9.	*S.* Kentucky	Tiger	2	2	2	2	2	2	1	0
10.	*S.* Litchfield	Poultry	2	2	2	2	2	1	1	1

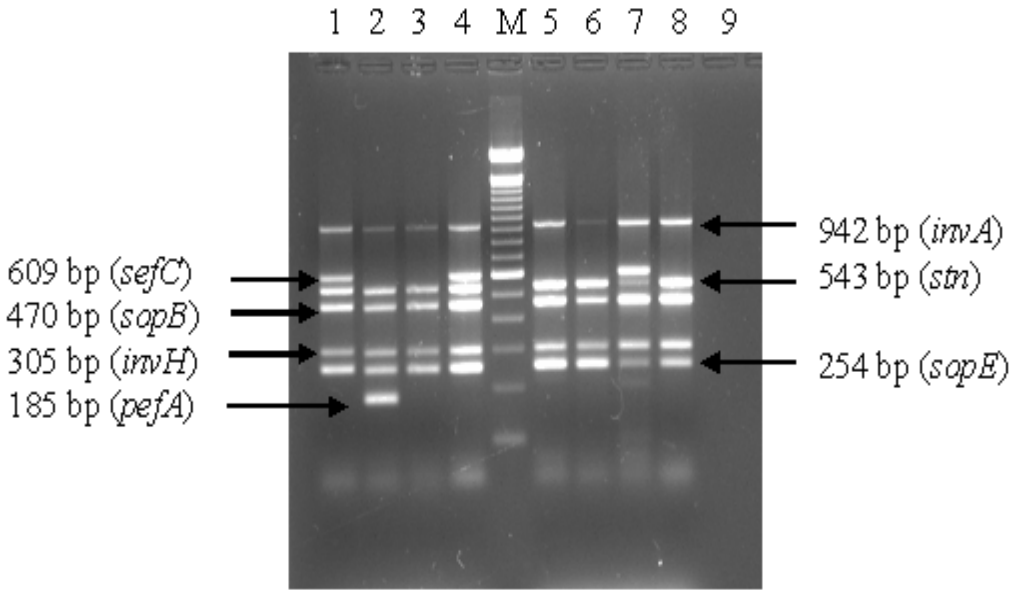

Figure 4.1: Detection of Virulence Genes of *Salmonella* by Multiplex-PCR (m-PCR).

Lane M: 100 bp ladder

Lane 1: *S.* Typhi (NICED C6953)

Lane 2: *S.* Typhimurium (MTCC 98)

Lane 3: *S.* Virchow (MTCC 1166)

Lane 4: *S.* Choleraesius (ATCC 10708)

Lane 5: *S.* Paratyphi A (NICED C6915)

Lane 6: *S.* Paratyphi B (NICED NK3727)

Lane 7: *S.* Enteritidis (NICED 503984)

Lane 8: *S.* Newport (MTCC 3229)

Lane 9: Negative control

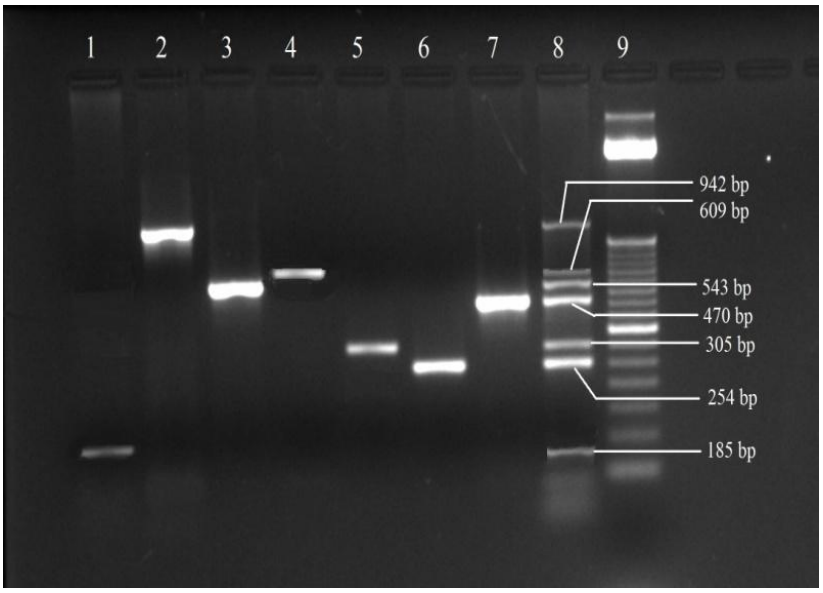

Figure 4.2: Detection of 7 genes in simplex and multiplex PCR

Lane 1: *pefA* (185 bp)

Lane 2: *invA* (942 bp)

Lane 3: *stn* (543 bp)

Lane 4: *sefC* (609 bp)

Lane 5: *invH* (305 bp)

Lane 6: *sopE* (254 bp)

Lane 7: *sopB* (470 bp)

Lane 8: m-PCR of *S.* Idikan isolate

Lane 9: 50 bp ladder (Invitrogen)

4.3 Bacterial expression of outer membrane protein InvH

4.3.1. Genomic DNA isolation of *Salmonella* Typhimurium (MTCC 98) and PCR amplification of complete *invH* gene

The genomic DNA was isolated from a strain of *S.* Typhimurium (MTCC 98) using hot-cold lysis extraction method (Theron *et al*., 2001). The complete *invH* gene was amplified using specific forward and reverse primers. Figure 4.3 shows amplification of the PCR product of expected size (461 bp) on 1.5% agarose gel.

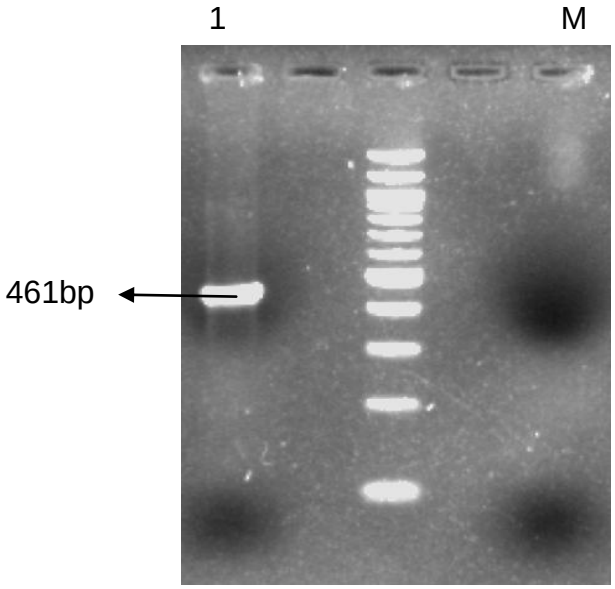

Fig 4.3: PCR amplification of *invH* gene (461bp) of *Salmonella* Typhimurium.

Lane 1: amplified PCR product

Lane M: 100 bp ladder

Lane 2: Negative Control

4.3.2. Gel purification of PCR amplified product of *invH* gene

The amplified PCR product was purified by using Gel Extraction Kit (Promega) for removal of free dNTP's and other reactants on non-specific amplified products, before proceeding for cloning. The purified PCR product devoid of unutilized primers and dNTP's was visualized in agarose gel.

4.3.3. TA Cloning of outer membrane lipoprotein encoding *invH* gene

4.3.3.1. Ligation and transformation

The PCR purified product of *invH* gene was ligated with the vector insert {pGEM-T plasmid vector (Promega)} at a molar ratio of 1: 3 and the ligated products were transformed into *E. coli* (DH5α competent) cells. Numerous white colonies developed on the selective medium (LB agar plates supplemented with ampicillin, 100 µg/ml). The recombinant white clones thus obtained following overnight incubation were screened for the presence of desired insert (Fig 4.4).

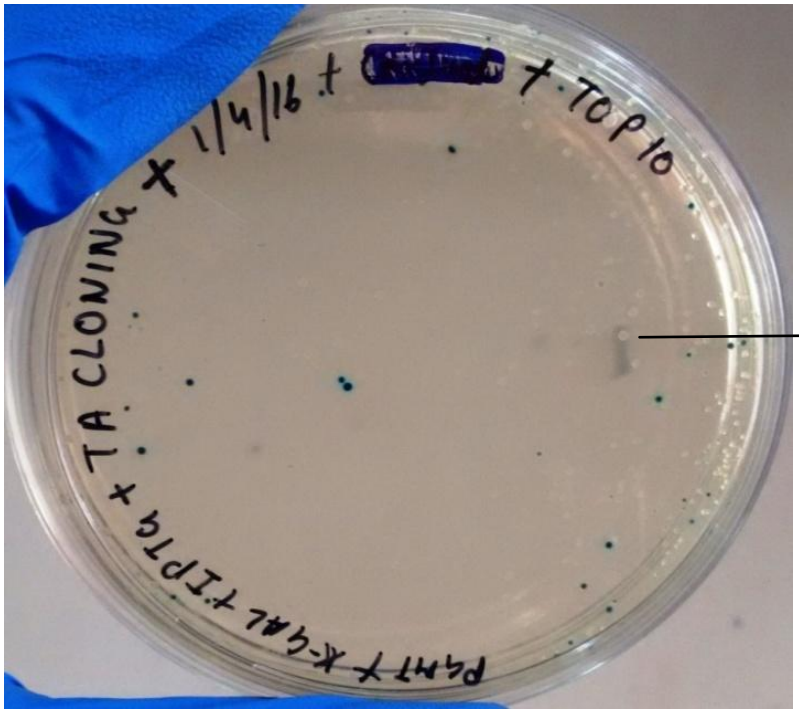

Fig 4.4: Blue-white screening of clones in pGEM-T vector

4.3.3.2. Screening of recombinant pGEM-T clones by colony PCR

Screening of the recombinant white clones was done by PCR using M13 forward and M13 reverse sequencing primers. PCR amplification resulted in a band of 493 bp size on 1.5% agarose gel (Fig 4.5). Plasmid was extracted from the PCR positive clones (Fig 4.6)

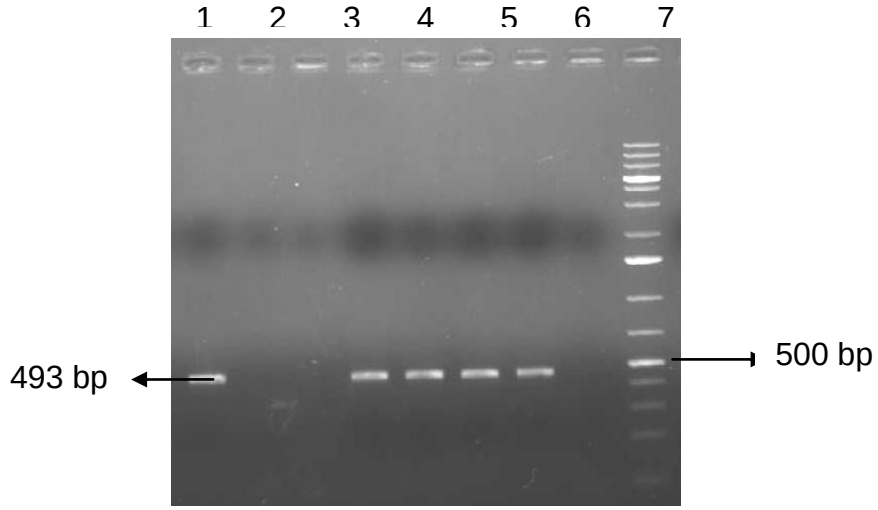

Fig 4.5: Screening of pGEMT clones for the presence of insert by colony PCR with pUC/M13 Forward & Reverse primers

Lane 1,4,5,6 and 7: Positive clones
Lane 2, 3 and 7: Negative clones (no insert)
Lane M: 100 bp ladder

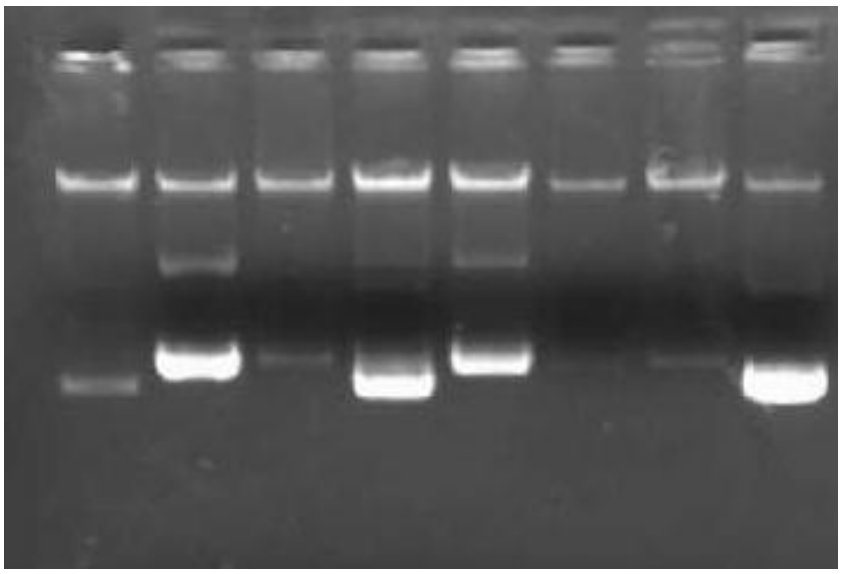

Fig 4.6: Plasmid isolation of the pGEM-T positive clones

4.3.4. Release of the invH sequence from the pGEM-T plasmids by RE digestion and linearization of pET 303 CT/His expression vector

The *XbaI* and *XhoI* double RE digestion could successfully release the *invH* gene insert from the recombinant pGEM-T plasmid with a product size of ~461 bp (Fig. 4.7a) and the pET 303 CT/His expression vector was linearized with a product size of ~5369 bp (Fig.4.7b). Both products were then purified from gels using the commercial gel extraction kit (Promega), as evident by the specific band of the eluted product during agarose gel electrophoresis. Ligation of the eluted products with a vector (pET 303CT/His) insert molar ratio of 1:3 followed by transformation into DH5α competent *E. coli* cells resulted in development of several white colonies on LB agar plates with ampicillin (100 µg/ml) indicating optimum transformation efficiency (Fig.4.8). The recombinant white clones thus obtained following overnight incubation were screened for the presence of the desired insert.

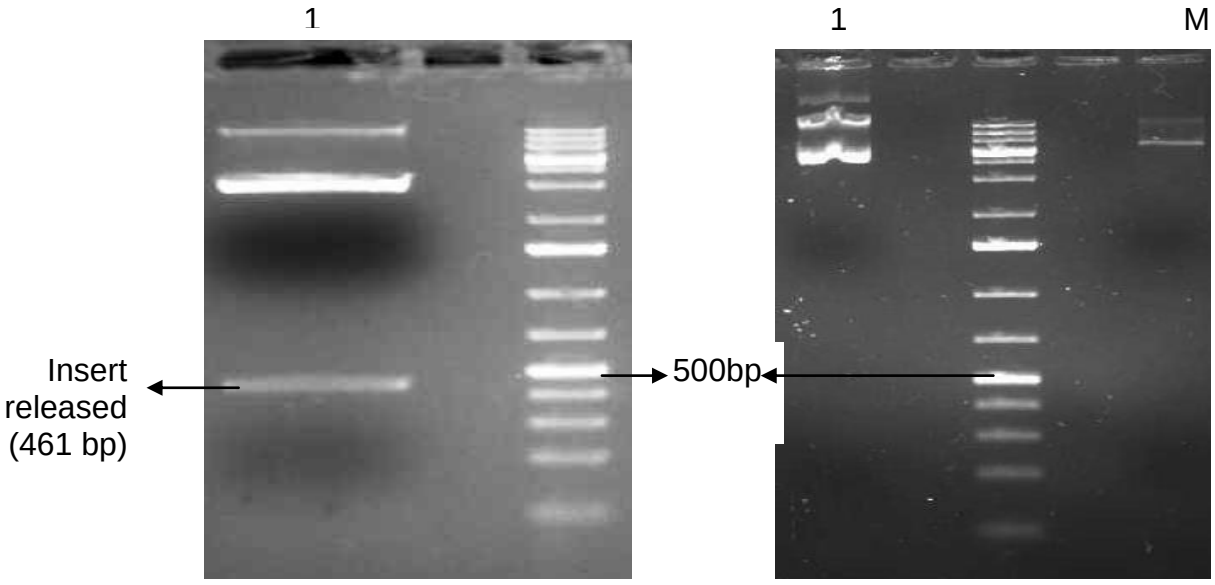

Fig 4.7(a): Release of invH gene insert from pGEM-T vector.

Lane 1: invH insert released from pGME-T
Lane M : 1kb ladder vector

Fig 4.7(b): Linearised pET303 CT/His vector using *XbaI* and *XhoI* RE's

Lane 1: Uncut pET303 CT/His vector
Lane 2: Linearised pET303 CT/His vector
Lane M: 1kb ladder

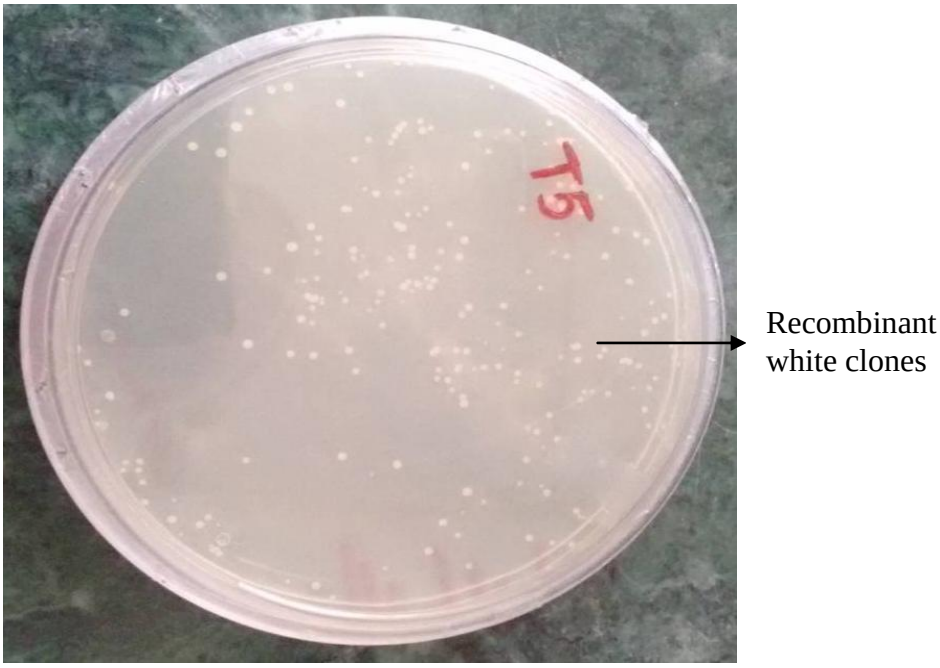

Fig 4.8: Screening of pET303 clones in LB Agar + Ampicillin plates(100µg/ml)

4.3.5. Screening of recombinant pET 303CT His clones by colony PCR

The white colonies were further screened by PCR using both T7 forward and reverse sequencing primers. PCR amplification resulted in a band of 645 bp size on 1.5% agarose gel (Fig.4.9). The correct orientation of the gene insert in frame with the C-terminal 6X His tag of the pET 303 CT/His vector was further confirmed by commercial sequencing of the plasmids extracted from the PCR positive clones. The gene sequence was submitted to GenBank with an assigned Accession No. KX275044.1 (www.ncbi.nlm.nih.gov). Phylogenetic comparison of the nucleotide sequence of the gene revealed 99% similarity with *S.* Typhimurium LT2 (Accession no. NC_003197).

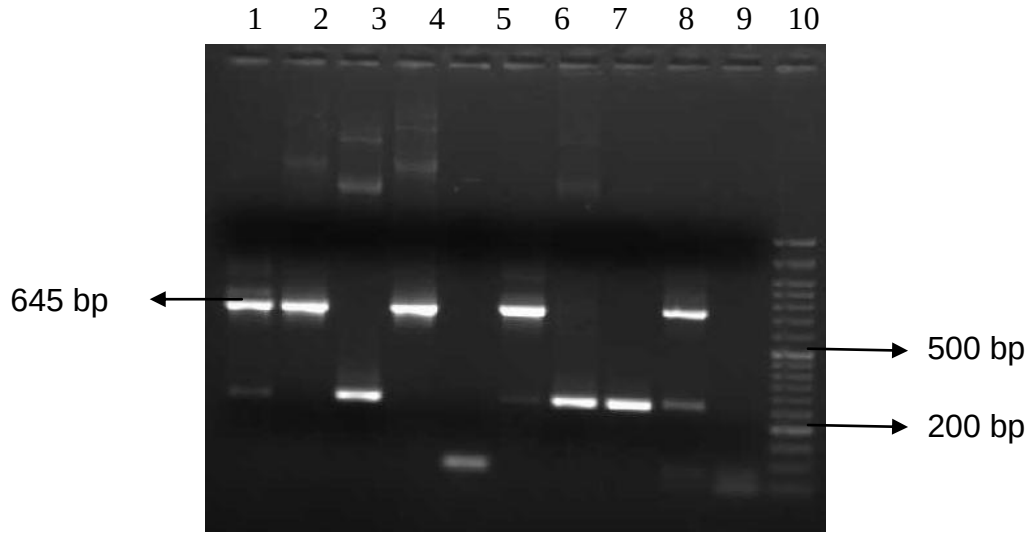

Fig 4.9 : Screening of recombinant pET 303 CT/His clones for the presence of insert by colony PCR with T7 Forward & Reverse Primers (645 bp)

Lane 1,2,4,6 & 9: positive clones of pET303 with insert

Lane 3, 5, 7, 8 & 10: Clones without insert

Lane M: 50 bp ladder.

4.3.6. Expression of *invH* gene in BL21 DE3 Star *E. coli* cells

The positive clones screened were picked up with the help of sterile pipette tips and were grown in LB broth containing ampicillin while plasmids were isolated from the cultured clones. The purified plasmids carrying the *invH* gene (lacking the nuclear localization signal) in pET303 CT/His expression vector were then used to transform the competent BL21DE3 star competent *E. coli* cells for expression of InvH protein. The transformed mixture was then plated over the LB ampicillin (100 µg/ml) plates. Then 5-6 white colonies were randomly selected and were grown overnight in LB broth containing carbenicillin (100 µg/ml). Next day, the fresh cultures were subjected to IPTG induction (1 mM) for *invH* gene expression at 37 ºC. The protein expression was observed after 2 hours of IPTG induction with the expression level found to be the highest at 6 hrs post-induction as was evident from SDS-PAGE analysis with the recovery of a ~15 kDa sized protein band that was detected after staining with Coomassie brilliant blue R-250 (Fig. 4.10).

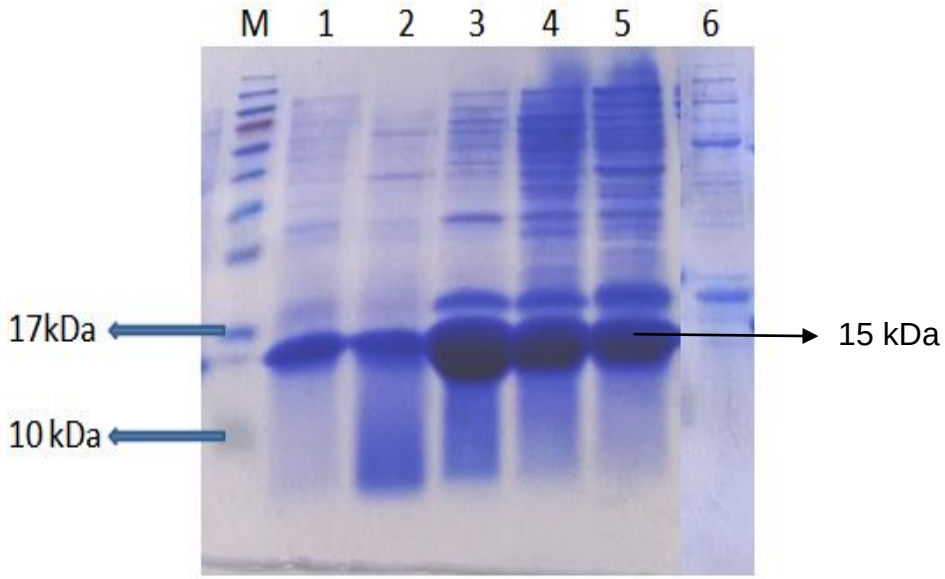

Fig. 4.10: SDS-PAGE analysis of the recombinant InvH protein expressed at different time intervals following induction

M : Prestained protein ladder (Thermo scientific)

Lane 1 : Cell lysate of 2 hr P.I

Lane 2 : Cell lysate of 4 hrs P.I

Lane 3 : Cell lysate of 6 hrs P.I

Lane 4 : Cell lysate of 8 hrs P.I

Lane 5 : Cell lysate of 10 hrs P.I

Lane 6 : Uninduced cell lysate

4.4. Purification of the bacterial expressed recombinant InvH protein by Ni-NTA chromatography and confirmation by SDS-PAGE

The initial attempts to purify the recombinant protein under native conditions using the Ni-NTA affinity chromatography was not satisfactory as the expressed protein was mostly present in insoluble form rather than the soluble form. Protein expression was found to be drastically reduced when attempts were carried out to express the protein by growing the culture at lower temperature (25-30ºC) for harvesting the protein under native conditions. Therefore to enable the purification of the recombinant protein, denaturing

conditions in the presence of 6M urea were utilized. The recombinant InvH protein was expressed in large scale by inducing 5 litres of the bacterial culture grown at 37ºC for 6 hrs with 1mM IPTG. The bacterial cell pellet was lysed in the denaturing binding buffer and the cleared lysate was then subjected to Ni –NTA column chromatography. The purified protein was then analysed in 15% SDS-PAGE and a protein band of ~15 kDa could be detected after staining with Coomassie brilliant blue R-250, which was absent in the lysate prepared from the un-induced cultures (Fig. 4.11). The purified denatured protein was refolded by stepwise dialysis with decreasing amount of urea in refolding buffer to bring the protein to its natural native conformation. While performing this method, no visible precipitation was noticed and the protein was collected and stored for further use. The concentration of protein was determined by Folin Lowry's method and was found to be 7 mg/ml.

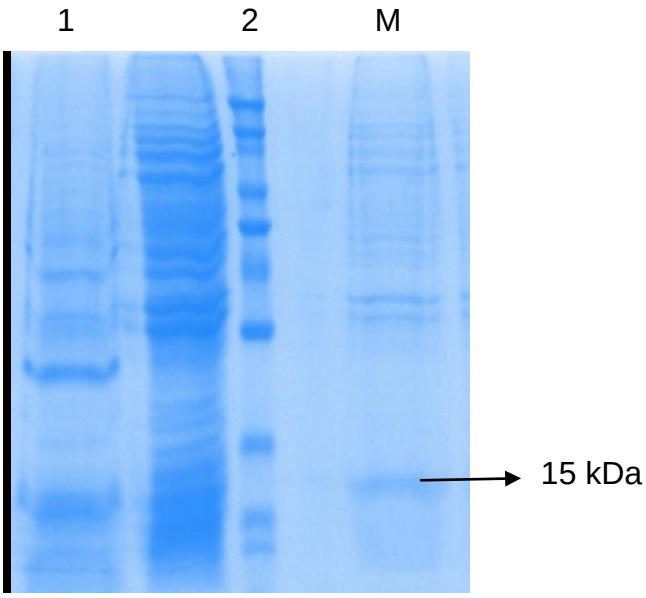

Fig. 4.11: SDS-PAGE analysis of the Ni-NTA purified recombinant proteins

Lane 1: Ni-NTA Purified proteins (Native condition)

Lane 2 : Cell lysate of uninduced culture

Lane M : Prestained protein ladder (NEB)

Lane 3 : Ni-NTA Purified proteins (Denaturing condition)

4.5. Characterization of the bacterial expressed recombinant InvH protein by western blot and dot blot analysis

After refolding, the small sized contaminant proteins and salt (urea) was removed from the refolded protein by dialysis using Snake Skin Pleated dialysis bags (Spectrum, USA) against 1X PBS (pH 7.4) and was concentrated using PEG 8000. Dot blot and western blot were performed for the characterization of the refolded, dialyzed recombinant protein using anti-His Tag protein mouse antibody (1 µg/ml) at 1: 650 dilutions. During dot blot, brown coloured spots appeared on the site where proteins were spotted on the PVDF membrane while control sites on the PVDF membrane did not show any colour formation (Fig. 4.12a). Western blot was performed to confirm the size of expressed recombinant protein using the same antibody used in the dot blot that resulted in intense colour reaction of the ~15 kDa protein indicating the correct size of the expressed protein (Fig. 4.13a, 4.13b).

Fig 4.12: Dot blot analysis of the purified recombinant invH protein with anti-His antibody.

Lane 1: PBS control

Lane 2: Uninduced lysate

Lane 3: Purified protein (native) showing reactivity with anti-His antibody

Lane 4: Purified protein (denatured) showing reactivity with anti-His antibody

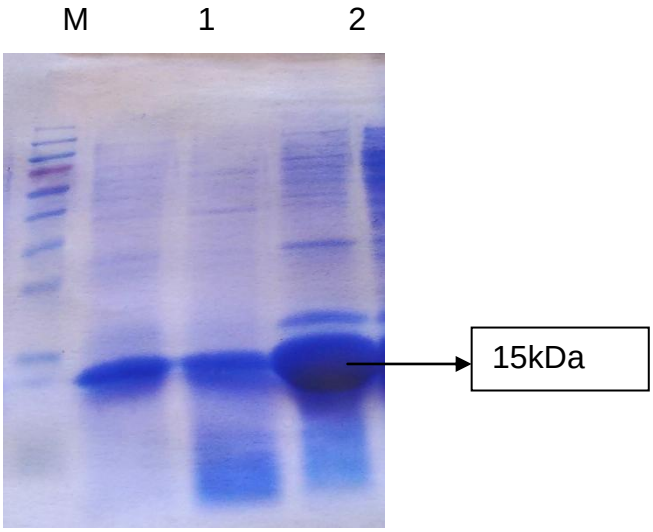

Fig. 4.13(a): SDS PAGE analysis of purified InvH protein

Lane M : Prestained Protein ladder (Thermo)

Lane 1&2: Purified protein (Denaturing condition)

Lane 3: Unpurified Protein

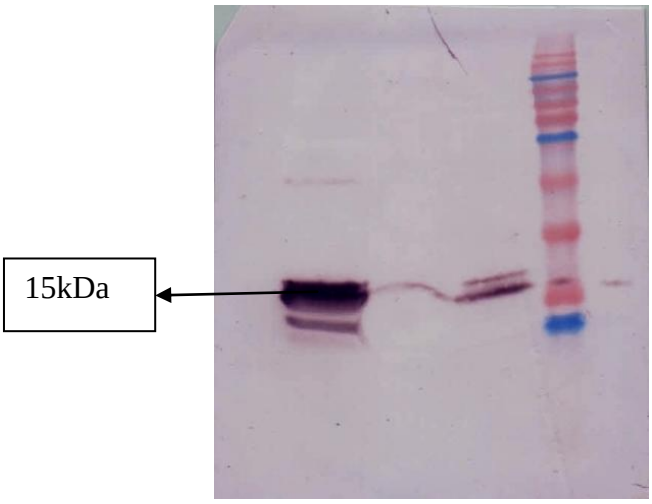

Fig 4.13(b): Reactivity of purified InvH protein with anti- His antibody

Lane 1: Unpurified Protein

Lane 2: Purified protein (native) showing reactivity with Anti-His antibody

Lane 3: Purified protein (denatured) showing reactivity with Anti-His antibody

Lane M: Prestained Ladder (Puregene)

4.6. Raising of anti-r (invH) Serum in Rabbit

The purified r (invH) was used for production of polyclonal immune serum in rabbit. After each immunization, rabbits were bled and the sera were separated. The immunoreactivity of the antibodies in the immune sera was assessed by western blotting while the antibody titer was determined by indirect-ELISA. After final immunization, the collected sera were pooled and the antibody titre of the serum samples of r (invH) immunized rabbit was determined by indirect-ELISA using the rabbit preimmune sera (Figure 4.14a). The polyclonal immune serum and the preimmune serum were serially diluted from 1:100 to 1:1, 28,000. The r (invH) protein was found to be highly immunogenic inducing a titer upto 6,400. The immune reactivity of the recombinant protein was evaluated by Western blot analysis (Fig. 4.14b) whereby a ~15 kDa protein identified through SDS-PAGE was to react specifically with the corresponding anti-recombinant invH hyper immune serum raised in rabbits.

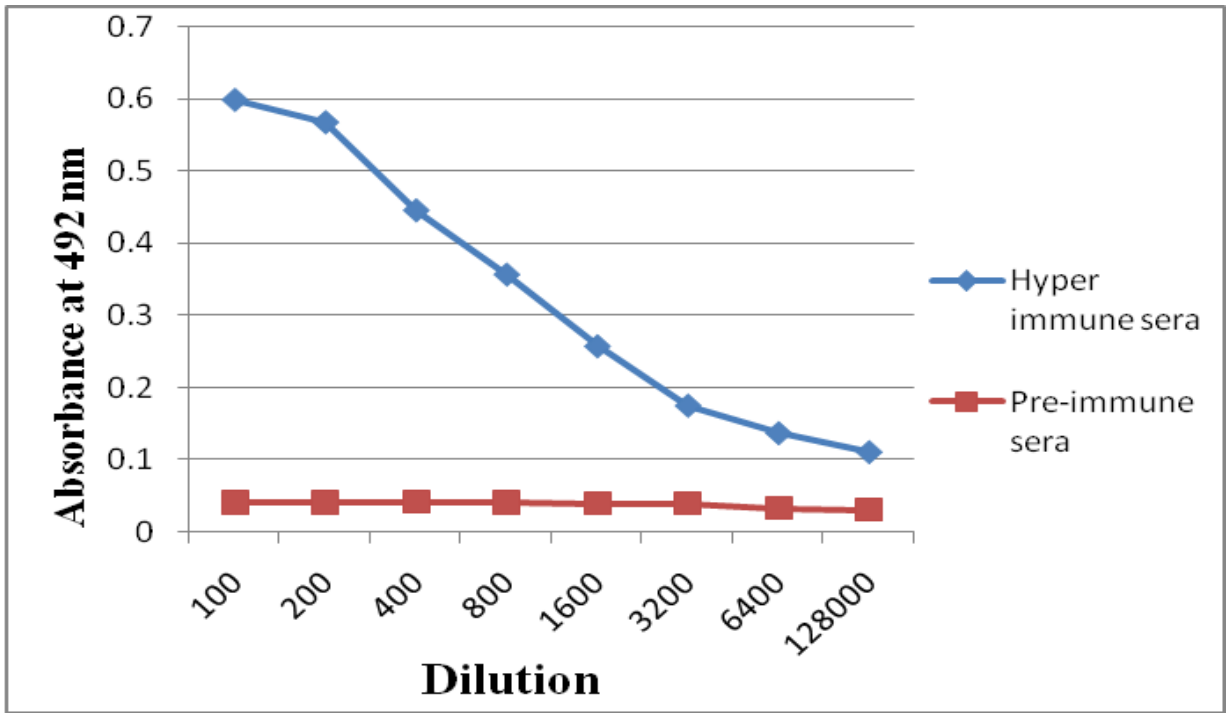

Fig 4.14(a): Determination of antibody titre by ELISA

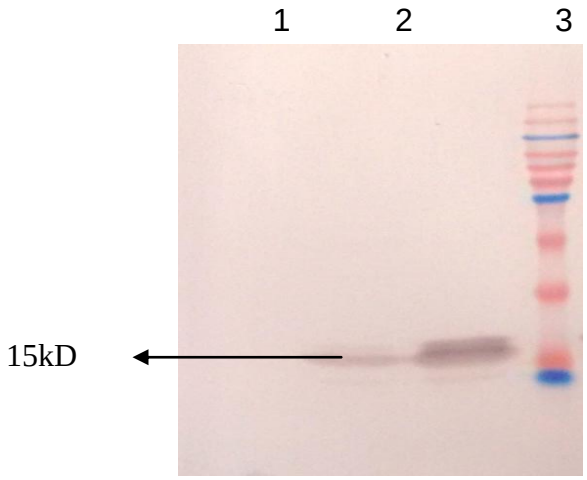

Fig 4.14(b): Western blot analysis of the purified r (InvH) protein of *S.* Typhimurium detected with anti-rabbit polyclonal anti-His antibody.

Lane 1 : Pre immune sera

Lane 2&3: r (invH) showing reactivity with anti-recombinant invH hyper immune serum raised in rabbit.

Lane 4: Prestained Ladder (Puregene)

4.7. Immune Response in Mice

In the present study, humoral immune response was assessed by indirect ELISA from serum samples collected at 7 days interval after primary immunization and after booster dose up to 42nd day post-primary immunization (PPI). The standard concentrations of antigen, antibody and conjugate used in ELISA were determined by checkerboard titration. On the basis of the checkerboard titration, 6 µg/µl of antigen and 1:200 dilution of serum concentration were used for subsequent studies.

Both the groups of mice that were injected with recombinant InvH protein adjuvanted with FCA (Group A) and bacterin vaccine adjuvanted with FCA (Group B) showed no significant difference in IgG response on 7th day PPI (P-value > 0.05). The mean antibody titre against r (invH) in the groups of mice vaccinated with r (invH) +FCA increased upto 14 days. Following booster vaccination on 14th day, the titre increased up to 21 days which started declining on 28th day and there was a significant difference between the antibody titres recorded on 21st and 28th days PPI ($p < 0.01$). The mean anti-r (invH) IgG response in serum of mice is depicted in the Table 4.5 and represented graphically in Figure

4.15. Group A showed a steady rise in IgG response from 7th to 21st day, followed by a sharp decline after 21st to 28th day and the IgG response remained low till 42nd day PPI.

The group of mice injected with *Salmonella* bacterin vaccine (Group B) showed a similar trend of antibody response as that of r (invH). There was a significant decline in IgG response after 21st day till 42nd day ($p < 0.05$) even after administration of booster dose on 14th day.

The mean antibody titre for different day's sera was found to be significantly different ($p < 0.05$) between groups of mice vaccinated with recombinant InvH protein and bacterin vaccine. The mean antibody titre of the group of mice injected with bacterin vaccine was found to be lesser compared to that of the group injected with r (InvH). There was highly significant difference ($p < 0.01$) in mean antibody titre between the groups (Table 4.5).

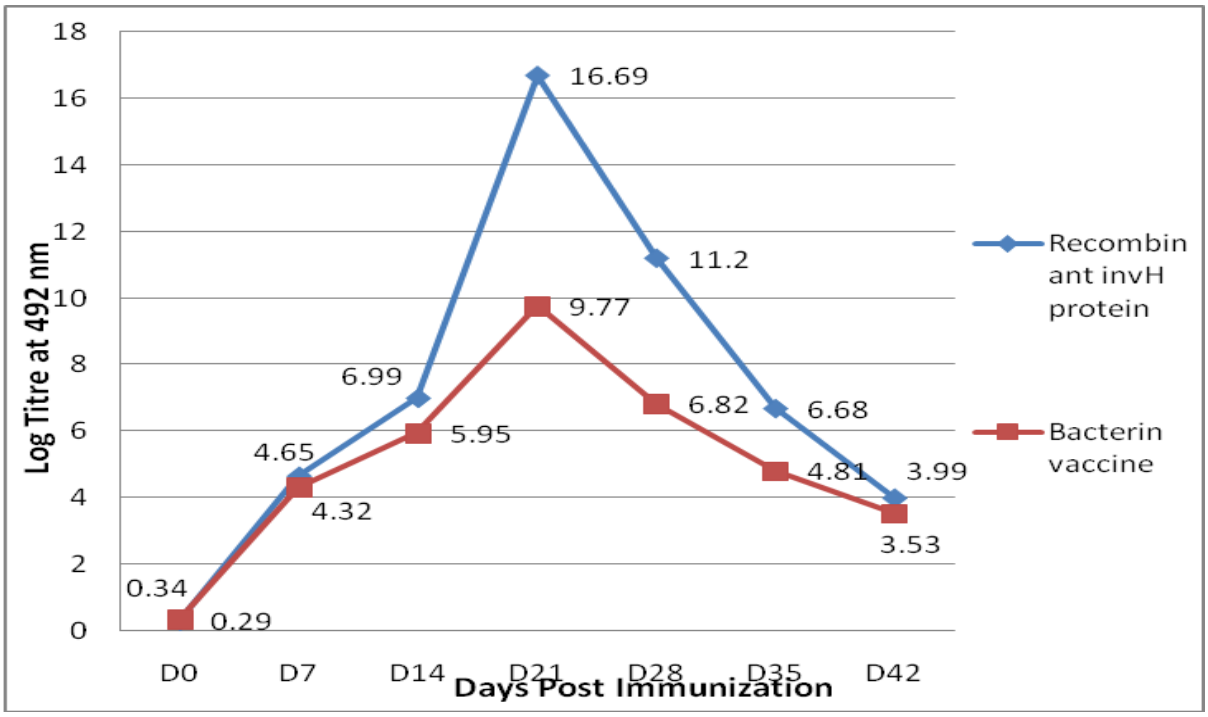

Fig 4.15: Graphical representation of mean antibody titre of different experimental groups of mice at different time intervals.

Table 4.5: Mean antibody titre (log value) of different groups of mice injected with recombinant INVH protein and bacterin vaccine of *Salmonella*

VACCINE GROUP		DAYS POST IMMUNIZATION					
	D0	D7	D14	D21	D28	D35	D42
A	$_{I}0.00^{A}$ ± 0.00	$_{I}4.65^{B}$ ± 0.04	$_{I}6.99^{C}$ ± 0.09	$_{I}16.69^{D}$ ± 0.16	$_{I}11.20^{E}$ ± 0.13	$_{I}6.68^{C}$ ± 0.12	$_{I}3.99^{G}$ ± 0.01
B	$_{I}0.00^{A}$ ± 0.00	$_{I}4.32^{B}$ ± 0.03	$_{II}5.95^{C}$ ± 0.06	$_{II}9.77^{D}$ ± 0.14	$_{II}6.82^{E}$ ± 0.15	$_{II}4.81^{F}$ ± 0.03	$_{I}3.53^{G}$ ± 0.01

N.B. Means bearing a common superscript in a column and subscript in a row do not differ significantly.

4.8. Challenge Studies

The LD_{50} doses of *Salmonella* Typhimurium, *S.* Enteritidis and *S.* Gallinarum strains were found to be 3 x $10^{7.5}$, 3 x 10^{6} and 3 x 10^{7} CFU per mouse, respectively. To determine the efficacy of recombinant invH protein in conferring protection to mice against infection by homologous (*S.* Typhimurium) and heterologous (*S.* Enteritidis and *S.* Gallinarum) virulent strains of *Salmonella* two weeks after the last booster dose, the mice of the immunized (r- invH with FCA and Bacterin with FCA vaccinated) groups and the non-immunized control (PBS inoculated) group were challenged with respective lethal dose (100 x LD_{50}) of *Salmonella* through the oral route. All the animals in the control group started showing the symptoms of salmonellosis like ruffled hair, lethargic movements, slow responsiveness to external stimuli and anorexia at the end of the day 1 post-infection. All the mice in the control group died within 3 days of infection while the group of mice immunised with r-invH with FCA showed complete protection (100%) against *Salmonella* Typhimurium and *Salmonella* Enteritidis infections, while protection was 90 percent against *Salmonella* Gallinarum. However, the death of one mouse recorded in this group was not presumably due to *Salmonella,* as the organism could not be recovered from the blood and tissues of the dead mouse. The bacterin vaccinated group showed a protection of 60 percent against all the three serovars under study *viz. S.*Typhimurium, *S.* Enteritidis and *S.*

Gallinarum. Ninety percent of the immunized mice survived till the end of study period of 90 days in the recombinant protein vaccinated group (Table 4.6).

Statistical analysis employing Chi-square test (with Yate's correction) showed that the immunised mice were conferred significantly better protection compared to the control group of mice. There were significant association between vaccination and survivability of mice in the group vaccinated with recombinant invH protein ($p < 0.01$). There was lack of significant association between vaccination and survivability in case of the bacterin vaccinated group ($p > 0.05$).

Table 4.6: Protection of Mice Immunised with Recombinant InvH Protein of *Salmonella* Typhimurium and Bacterin Vaccine of *Salmonella*

Group	Dose of immunization/mice	Challenge Dose (cfu)	Post challenge survival	Post challenge death		
				24 hrs	48 hrs	72 hrs
r(InvH) of *S.* Typhimurium with FCA	50µg	$3 \times 10^{9.5}$	100% (10/10)*	0	0	0
r(InvH) of *S.* Enteritidis with FCA	50µg	-do-	100% (10/10)*	0	0	0
r(InvH) of *S.* Gallinarum with FCA	50µg	-do-	90% (9/10)*	0	0	1
Bacterin of *S.* Typhimurium with FCA	50µg	-do-	60% (6/10)^{NS}	0	1	2
Bacterin of *S.* Enteritidis with FCA	50µg	-do-	60% (6/10)^{NS}	0	2	4
Bacterin of *S.* Gallinarum with FCA	50µg	-do-	60% (6/10)^{NS}	0	1	2
PBS control	-	-do-	0% (0/10)	0	4	6

* Significant association between vaccination and survivability.
^{NS}No significant association between vaccination and survivability

4.8.1. Results of Re-isolation from the Challenged Mice

Salmonella could be re-isolated from faecal samples of all (100%) the mice that died during challenge infection. Re-isolation of *Salmonella* from liver, spleen and intestinal lymph nodes was possible from 90%, 78% and 85% of dead mice, respectively. The identity of the challenged strain was confirmed by genus-specific PCR. The PCR was performed targeting amplification of *inv*A gene specific for the genus *Salmonella* using specific primers. The reaction showed an amplified product of 942 bp (Figure 4.16).

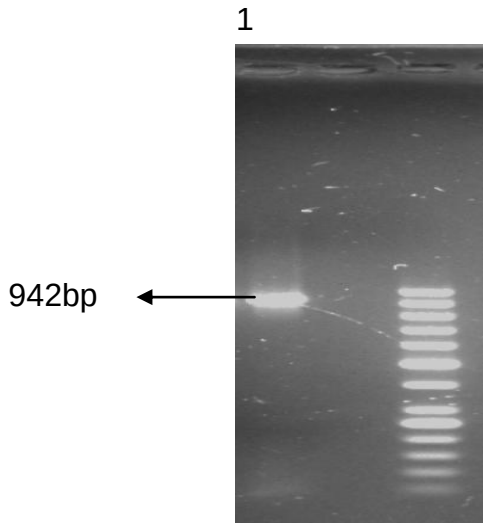

Fig 4.16: Confirmation of the *Salmonella* Typhimurium isolates from faecal samples of dead mice by PCR targeting amplification of *invA* gene

Lane 1: Amplified PCR product

Lane M: 100 bp ladder