



Virulence Gene profiling in *Salmonella Typhimurium* Isolated from Fecal Samples of Domestic and Wild Animals

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ABSTRACT

The virulence gene profile of twelve (12) *Salmonella* isolates obtained from faecal samples of animal origin, including livestock, primates and emu, was determined. All *Salmonella* isolates were identified by biochemical tests. Genus specific PCR further confirmed the *Salmonella* isolates revealing the expected bands of 496 bp. All isolates on serotyping were found to be *Salmonella Typhimurium* with antigenic structure 4,2:i:1,2. These *Salmonella* isolates were screened for different virulence-associated genes by PCR-based procedures. All the isolates were positive for *ssaQ*, *hilA*, *prgH* and *fljB* genes. The *mgtC* gene was present in varying frequencies in isolates from different animal species with an overall frequency of 75%. By conventional PCR, it was demonstrated that the four genes, as indicators for salmonella pathogenicity islands (SPIs), were present in all strains, independently of the host species and outcome of infection.

Keywords: Livestock, *Salmonella*, serotyping, virulence genes, wild animals

Salmonella is Gram-negative facultative intracellular pathogen of major zoonotic significance. It is widespread in the intestines of most wild and domestic birds, reptiles and mammals, including humans and domestic livestock. The pattern of distribution differs for individual *Salmonella* serotypes. *S. Typhimurium* infects all animal species and has a worldwide distribution (Ekperigin and Nagaraja, 1998). *Salmonella* infections are usually acquired through direct contact with faeces and indirectly from contaminated food or inanimate objects.

Salmonella can cause a variety of diseases ranging from acute gastroenteritis to systemic infections depending on the serotype, host, and sets of virulence determinants. The ability of *Salmonella* to cause a successful infection in the host can be attributed to an array of virulence genes located on plasmids or within the chromosome. The characterization of virulence-associated genes in *Salmonella* is important for its pathogenicity, understanding the potential transfer mechanisms, and developing an efficient detection method in epidemic disease control.

S. Typhimurium genome contains two pathogenicity islands (SPI) with genes encoding type III secretion systems; SPI1 is required for the penetration of the epithelial intestine layer, while SPI2 plays role in subsequent proliferation of bacteria in the spleen. Molecular analysis of SPI1 has revealed more than 28 genes encoding a complex type III secretion system (*spa*, *inv*, *prg*, and *org*), secreted effector proteins (*sip* or *ssp*; *spt*) and regulatory components (*invF*, *hilA*) involved in the invasion phenotype (Galan, 1996).

The aim of the present work therefore was the application and evaluation of selected PCR-protocols for the characterization of *Salmonella* isolates by means of virulence genes determination.

MATERIALS AND METHODS

Isolation, identification and biochemical characterization of *Salmonella*

Strains of *S. Typhimurium* harvested from animals (faeces



of domestic and wild animals, emu, and a single isolate from a clinical case of chronic diarrhoea in cattle) were included in the study.

The microbiological culture of the faecal samples for *Salmonella* isolation was done by the standard methods (Quinn *et al.*, 1994). Briefly, 1g of faeces was added to Tetrathionate broth (1:10) and incubated at 42°C for 24-48 hours in incubator. A loopful of the incubated broth culture was plated on Hektoen Enteric (HE) agar, Brilliant green agar (BGA), MacConkey Lactose agar (MLA), Xylose Lysine Deoxycholate agar (XLD agar) and Tryptone Soya Agar (TSA) plates and incubated at 37°C for 24-48 hours. A number of plating methods were used to recover *Salmonella* spp. due to the selectivity of some media for the growth of certain serotypes and also to avoid false positive reactions given by some of the *Enterobacteriaceae* such as *Citrobacter*, *Proteus*, *Pseudomonas* etc. The colonies showing typical characters were selected for biochemical characterization. The isolates were identified on the basis of morphological, cultural and biochemical characteristics. Morphology of the isolates was studied by examining the smear under oil immersion lens after Gram staining. Young cultures were looked for the typical shape, size, and arrangements.

Since, no single biochemical test can conclusively identify *Salmonellae*, a combination of biochemical tests were performed *viz.*, Methyl Red, Voges Proskauer, Indole, citrate utilization, carbohydrate fermentation, urea hydrolysis and production of H₂S on TSI. The carbohydrate fermentation tests used to identify the *Salmonella* were lactose, arabinose, maltose, sorbitol and dulcitol.

The biochemical test was also performed using KB011 Hi *Salmonella* identification kit (Himedia). For this, pure culture of *Salmonella* isolates were inoculated in brain heart infusion broth (BHI) and kept overnight at 37°C. Next day, loopful of broth culture was incubated into individual wells of the kit and kept for overnight at 37°C. After 24 hours, methyl red reagent was added in 1st well, Baritt reagent A and Baritt reagent B were added in 2nd well and change in colour was detected upto 12th well. The cultures were stained with Gram's staining technique. The isolates were maintained as BHI-glycerol suspension (50% v/v) at -80°C for long-term preservation.

Serotyping of *Salmonella*

The *Salmonella* isolates were sent to National Salmonella

and Escherichia Centre, Central Research Institute, Kasauli (Himachal Pradesh) for serotyping.

Molecular Characterization of *Salmonella* isolates

Bacterial DNA was extracted from the isolates using the Freeze and Thaw method described by Queipo-Ortun *et al.* (2008) with little modification. Briefly, the organisms were cultured onto Tryptone Soya Broth. After overnight incubation at 37°C, 1ml of broth culture was taken in eppendorf tube, centrifuged at 10,000 rpm for 2 minutes, the supernatant was discarded and the pellet cells were mixed with 200µl of nuclease free water. The mixture was then heated in boiling water for 10-15 minutes followed by snap cooling into ice for 10-15 minutes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and used as template DNA in PCR.

The PCR assays used approximately 10 pg of DNA template in a 25 µl reaction mixture with 13 µl of Taq DNA Master Mix (Qiagen) and 250 nM of each oligonucleotide primer (Genaxy) for amplification of various genes in a thermocycler (BioRad). After removing from thermocycler, PCR products were stored at -20°C. PCR products were analyzed using conventional agarose gel electrophoresis in 1% w/v agarose (Genaxy). The amplified products were run in agarose gel in 1x TBE buffer (Genaxy) containing ethidium bromide at 0.1 mg/ml. Quantitative DNA Markers (Genaxy) were used as molecular size markers. The DNA bands were visualized and imaged using the Molecular Imager[®] ChemiDoc[™] XRS+ imaging system (Bio-Rad). *S. Typhimurium* isolates were confirmed by PCR method by using *Salmonella* genus specific oligonucleotide primers (Cohen *et al.*, 1993). The reaction was run for 40 cycles and PCR was programmed to 30 sec for denaturation at 94°C, annealing at 60°C for 30 sec and extension at 72°C for 45 sec. The reaction concluded with a 5 min extension phase at 72°C.

Targeting of virulence genes in *S. Typhimurium*

The PCR for screening the isolates for presence of different virulence genes, *ssaQ*, *hilA*, *prgH*, *fljB* and *mgtC* was done as per the standard method followed by earlier workers with some modifications (Table 1).

RESULTS AND DISCUSSION

In the present study, 5 isolates of *Salmonella*- two from primates and three from cattle isolated previously

Table 1: Primers and thermocycler conditions employed to study virulence genes in *S. Typhimurium*

Sl. No.	Primer pair	Sequence (5'-3')	Gene target	Product size (bp)	Amplification conditions			Reference
					Temp	Time	No. of cycles	
1	<i>hilA</i> F <i>hilA</i> R	CGG AAC GTT ATT TGC GCC ATG CTG AGG TAG GCA TGG ATC CCC GCC GGC GAG ATT GTG	<i>hilA</i>	784	95°C	5min	30X	CIRG (Unpublished data)
					94.8°C	1min		
					60°C	1min		
					72.8°C	1min		
					72°C	10min		
2	<i>prgH</i> F <i>prgH</i> R	GCC CGA GCA GCC TGA GAA GTT AGA AA TGA AAT GAG CGC CCC TTG AGC CAG TC	<i>prgH</i>	756	95°C	5min	25X	Skyberg <i>et al.</i> (2006)
					94°C	1min		
					55°C	1min		
					72°C	2min		
					72°C	10min		
3	<i>ssaQ</i> F <i>ssaQ</i> R	GAA TAG CGA ATG AAG AGC GTC C CAT CGT GTT ATC CTC TGT CAG C	<i>ssaQ</i>	677	94°C	5min	30X	Soto <i>et al.</i> (2006)
					94°C	1min		
					60°C	1min		
					72°C	2min		
					72°C	5min		
4	<i>mgtC</i> F <i>mgtC</i> R	TGA CTA TCA ATG CTC CAG TGA AT ATT TAC TGG CCG CTA TGC TGT TG	<i>mgtC</i>	655	94°C	5min	30X	Soto <i>et al.</i> (2006)
					94°C	1min		
					60°C	1min		
					72°C	2min		
					72°C	5min		
5	<i>fliB</i> F <i>fliB</i> R	GAC TCC ATC CAG GCT GAA ATC AC CGG CTT TGC TGG CAT TGT AG	<i>fliB</i>	848	95°C	10min	39X	Lee <i>et al.</i> (2009)
					94°C	1min		
					60°C	1min		
					72°C	1min		
					72°C	15min		

(Hansda, 2016; Mir, 2016), were included. Besides fecal samples collected in earlier study were used for isolation of *Salmonella* and 7 isolates of *Salmonella* were further isolated in this study. *Salmonella* isolates were streaked on Hektoen Enteric (HE) Agar, Brilliant Green Agar (BGA), Brain Heart Infusion (BHI) Agar, Xylose Lysine Deoxycholate (XLD) Agar and Tryptone Soya Agar (TSA).

All the isolates were positive for methyl red (MR), triple sugar iron test (TSI), motility–indole-lysine test (MIL), ortho-nitro phenol glycoside (ONPG), and citrate utilization test. Glucose fermentation test included lactose, arabinose, maltose, sorbitol and dulcitol. These isolates yielded negative results for Voges-Proskauer (VP) and

urease. The result obtained from biochemical tests were similar to the results obtained by Iwade *et al.* (2006) who characterized an outbreak derived *Salmonella Enteritidis* strains with atypical triple sugar iron and Simmons citrate reactions. In the present study, the isolates were positive for methyl red, nitrate reduction and citrate utilization but negative for urease, lysine decarboxylation, gelatin liquefaction, indole and Voges-Proskauer. Acid and gas was produced from glucose, mannitol, maltose, arabinose, xylose, dulcitol, inositol and mannose. Either no or variable reaction in lactose was observed. All the tests were found similar as compared to the chart provided by the manufacturer (KB011 Hi *Salmonella* identification kit, Himedia).

Genus specific PCR, capable of discriminating genus *Salmonella* from other bacteria, further confirmed the *Salmonella* isolates revealing the expected bands of 496 bp (Fig. 1) as described previously by Cohen *et al.* (1993). All isolates (n=12) serotyped at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (Himachal Pradesh), from wild animals and livestock, were found to be *S. Typhimurium* with antigenic structure 4,2:i:1,2.

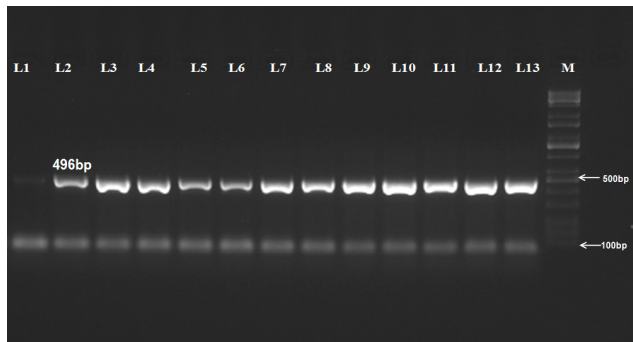


Fig. 1: PCR confirmation of *Salmonella* isolates using genus specific primer; Lane M-Ladder (100bp); Lane 1-*E. coli* Negative control and Lane 2-13-Positive isolates

All the *Salmonella* isolates were screened for virulence-associated genes by PCR based procedures. The 12 isolates were tested for the presence of five virulence genes, namely *ssaQ*, *hilA*, *prgH*, *fljB* and *mgtC* by conventional PCR using the respective primer pairs. Results showed that all *Salmonella* isolates tested carried *ssaQ*, *hilA*, *prgH* and *fljB* genes (Fig. 2). *mgtC* gene was present at different frequencies in strains isolated from different animal species with an overall frequency of 75.0%. The distribution of different virulence genes in various salmonella isolates is presented in Table 2.

The findings are broadly in agreement with Koochakzade *et al.* (2015), Oliveira *et al.* (2003) and Amini *et al.* (2010) in *Salmonella* isolates. The *Salmonella* isolates from primates showed the same virulence pattern. Krawiec *et al.* (2015) reported prevalence of various virulence genes *spvB*, *spiA*, *pagC*, *cdtB*, *msgA*, *invA*, *sipB*, *prgA*, *spaN*, *orgA*, *tolC*, *ironN*, *sitC*, *ipfC*, *sifA*, *sopB*, and *pefA* among the various *salmonella* isolates from free living birds in Poland.

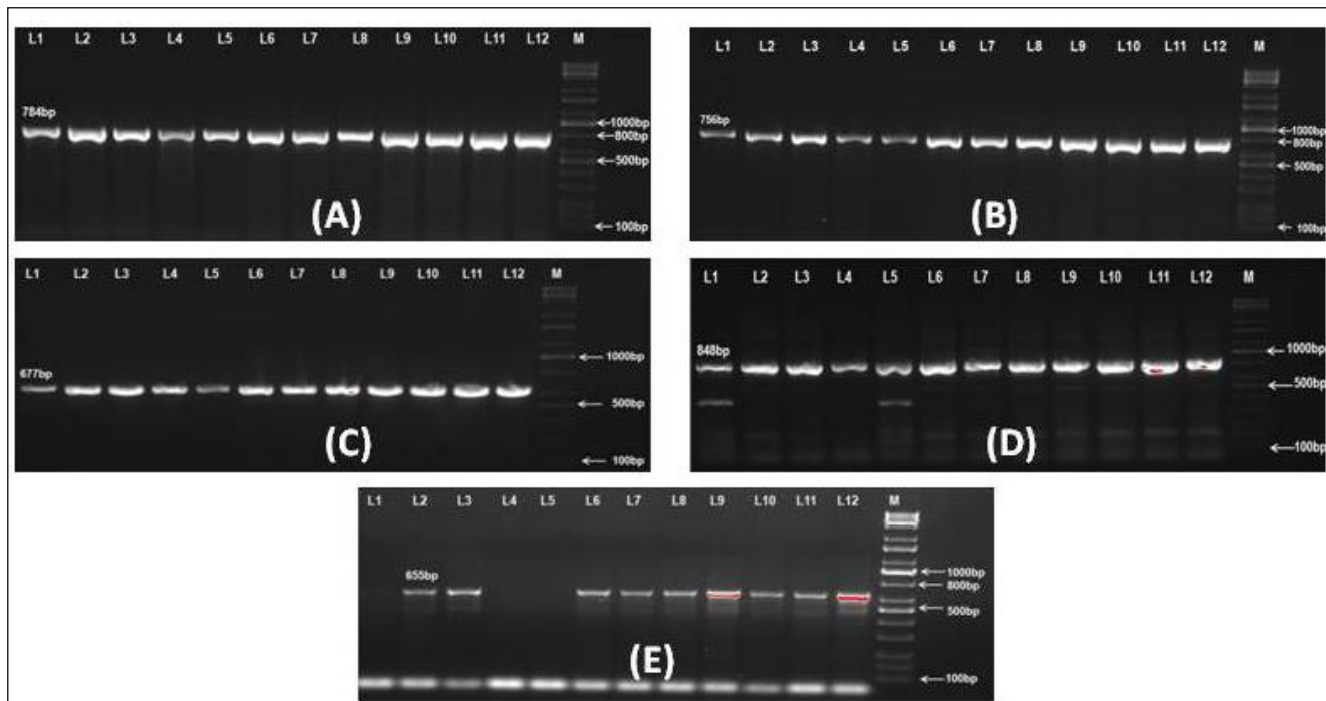


Fig. 2: Screening of *S. Typhimurium* for virulence genes including, *hilA* (A) *prgH* (B) *ssaQ* (C) *fljB* (D) *mgtC* (E); Lane M-Ladder (100bp) and Lane 1-12 depict positive isolates with respective amplification products

Table 2: Virulence gene profiling in *S. Typhimurium* of animal origin

Sl. No.	Isolate	Source	<i>ssaQ</i>	<i>hilA</i>	<i>prgH</i>	<i>fjB</i>	<i>mgtC</i>
1	Cattle	Case of chronic diarrhea, Hansda, (2016)	+	+	+	+	-
2	Primate		+	+	+	+	+
3	Primate	Mir, (2016)	+	+	+	+	+
4	Cattle		+	+	+	+	+
5	Cattle		+	+	+	+	+
6	Cattle		+	+	+	+	+
7	Buffalo		+	+	+	+	-
8	Buffalo		+	+	+	+	-
9	Buffalo	Isolated in present study	+	+	+	+	+
10	Buffalo		+	+	+	+	+
11	Buffalo		+	+	+	+	+
12	Emu		+	+	+	+	+
n=12 (% Positive)			12 (100%)	12 (100%)	12 (100%)	12 (100%)	9 (75%)

CONCLUSION

Salmonella Typhimurium with antigenic structure 4,2:i:1,2 on PCR-based screening for virulence-associated genes demonstrated all the isolates (n=12) to be positive for *ssaQ*, *hilA*, *prgH* and *fjB* genes, while 75% isolates were positive for *mgtC* gene.

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