Molecular Detection of Virulence Associated Genes in *Salmonella* Serovars Isolated from Raw Pork of Aizawl and Imphal

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**Abstract**

The study was aimed to detect virulence associated genes of *Salmonella* serovars, which were isolated from raw pork samples of unorganised butcher shops of Aizawl and Imphal. A total of 5 *Salmonella* isolates (Aizawl=2, Imphal=3), belonging to *Salmonella vircho* (n=4), and *Salmonella typhimurium* (n=1), were obtained from 200 samples (100= Aizawl and 100= Imphal) and screened for five virulence associated genes, namely invA, stn, pefA, sefC, spvC by Polymerase Chain Reaction (PCR) technique. All *Salmonella* serovars detected positive for invA and stn genes, spvC and pefA gene was found positive in *Salmonella typhimurium* and one *Salmonella* Vircho of Aizawl origin (overall 40%). sefA was absent in all isolates. All the *Salmonella* serovars possessed invasive gene (invA) and enterotoxin gene (stn), which make them capable for producing gastroenteritis in human. Presence of *Salmonella typhimurium* positive with 4 virulence associated genes in Aizawl is a matter of worry from hygienic point of view.

**Keywords**

*Salmonella*, Virulence, PCR, Pork, Imphal, Aizawl

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**Introduction**

Food is one of the most essential needs for the survival of the living organisms and often acts as a major route for contaminants for the entry to the body (Das *et al.*, 2018). Meat is considered as one of the most important food item for human consumption from the ancient time. A major proportion of the worldwide population chiefly relies on meat as a potent source of good quality protein (Bradeeba and Siyakumaar, 2013). If raw meat is not cooked properly with an adequate time and temperature conditions, it may cause serious harm to consumer’s health. Visual detection of microbial contamination in raw meat and meat
products not possible (Movassagh et al., 2010). The microbial population, that comes in contact with meat during the production, processing, transportation and distribution, acts as potential challenge to meat industry, and potentially creates hazards in form of infection, spoilage and intoxications (Dhanze et al., 2012). Out of different deadly food-borne infections, Salmonellosis is associated with hyperendemic diarrhoeal disease around the world affecting both human and animal alike (Prakash et al., 2005). Among six subspecies, Salmonella enterica causes 99.5% of food-borne illness in humans and animals (Pignato et al., 1998). The incidence of zoonotic transmission of nontyphoidal Salmonella serovars are largely associated with food of animal origin such as eggs, milk, poultry, beef and pork meat (Alcaine et al., 2007; Fernandez et al., 2012). The virulence of Salmonella is linked to a combination of chromosomal and plasmid factors. Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. The chromosomally located invasion gene invA being thought to trigger the invasion of Salmonellae into cultured epithelial cells (Galan and Curtiss, 1989). Some genes are also known to be involved in adhesion and invasion, viz., sef (Clothier et al., 1993), pef (Baumler et al., 1996), inv (Galan et al., 1992); whereas some others are associated with survival in the host system-

**Materials and Methods**

A total of 5 Salmonella isolates were isolated from 200 raw pork samples, collected from different local unorganized butcher shops of Aizawl (n=100) and Imphal (n=100) cities. For serotyping, isolates were sent to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh, India, and identified as Salmonella enteric serovar Vircho (n=4), and Salmonella enterica serovar Typhimurium (n=1). Those isolates were subjected for detection of virulence associated genes under this study. The selected virulence associated genes were invA (responsible for invasiveness), pefA (plasmid encoded fimbrial), sefC (Salmonella enteritidis fimbrial), spvC (Salmonella plasmid virulence) and stn (Salmonella enterotoxin). Oligonucleotide primers (Eurofins Genomics India Pvt. Ltd., Bangalore, India), used for detection of the targeted genes are given in Table 1 (references). For positive control of all the mentioned genes in this study, standard culture of Salmonella enteritidis (ATCC 13076) was used.

The template DNA for PCR detection of virulence associated genes was prepared as per standard methods of Das et al., (2018). Salmonella isolates was grown in 5 ml Luria Bertani (LB) broth and incubated at 37°C overnight under constant shaking. After incubation, 1 ml of the bacterial broth culture was taken in a sterile microcentrifuge tube and centrifuged at 8000 rpm at 4°C for 10 mins. The bacterial pellet thus obtained was washed thrice with sterile Normal Saline Solution (NSS, 0.85% w/v) by centrifuging at 8000 rpm at 4°C for 5 mins and finally pellet was re-suspended in 100μl of nuclease free sterile distilled water. The bacterial suspension was boiled for 15 mins in a boiling water bath followed by immediate chilling for 15 mins at -20°C (Snap chilling). The lysate was
centrifuged again at 5000 rpm for 5 mins to sediment the cell debris and the supernatant was used as template DNA for PCR assay.

The PCR reactions for identification of virulent genes were carried out following the protocol mentioned by Rahn et al., (1992), Murugkar et al., (2003) and Chiu et al., (2006), with slight modifications. Briefly, 25 µl of PCR mixture contained 12.5 µL of 2X Dream taq PCR Master Mix (Thermo Scientific), 1 µl (10 pmol) each of forward and reverse primer and 5 µl of template DNA (culture lysate). For sefC and pefA genes, duplex PCR was used. PCR amplification was performed in a Master Cycler Gradient (Bio Rad, USA). The thermal conditions maintained for the amplification of different virulence associated genes are given in Table 2. To monitor the quality control of PCR assays, a reagent blank, which contained all the components except template DNA for which sterile deionised water was substituted and a negative control containing non Salmonella DNA were included in every PCR procedures. The amplified products were analysed by horizontal submarine agarose gel electrophoresis (Sambrook et al., 2001).

Results and Discussion

All the isolates screened for the detection of virulence-associated genes, were found to be positive for invA and stn genes (100% positive). None of the isolates were found positive for sefC (0%). However, two isolates (One S. typhimurium and one S. vircho) from Aizawl, were also found to be positive for pefA and spvC genes (Table 3, 4; Fig. 1 to 6).

In the present study, we tried to identify the virulence associated genes in the Salmonella isolates from raw pork. S. virchow and S. typhimurium were the two different Salmonella species identified among the raw pork samples. The presence of invA and stn genes in all the isolates in our study is in agreement with the result obtained by other researchers (Chaudhary et al., (2015); Borges et al., (2013) and Karmi, (2013). However, Ateba and Mochaiwa (2014) investigated for presence of Salmonella isolates from 32 raw beef samples collected from North West Province, South Africa and found 10 out of 96 presumptive isolates were positive for invA gene. In this present study, we also found, 60% (3/5) isolates positive for pepA and 20% (1/5) positive for spvC and sefC genes. Similar finding was obtained by Das et. al., (2012) who reported presence of invA, stn, pefA, sefC and spvC genes in 100%, 100%, 51.42%, 25.71% and 42.85% isolates, respectively. Write here the importance of the detected genes in virulence of Salmonella and if possible the probable reason of absence of some genes in few isolates. Now a day, detection of invA gene by PCR is taken as internationally accepted standard identification tool for Salmonella (Darwin and Miller, 1999) as it is rapid, sensitive and specific for detection of Salmonella from any clinical samples (Lampel et al., 2000).

Fig.1 Distribution of virulence associated genes of Salmonella serovars from Aizawl and Imphal
Fig. 2 Distribution of virulence associated genes according to *Salmonella* serovars

![Bar graph showing distribution of virulence associated genes](image)

**Fig. 3** PCR amplification of *invA* gene (284 bp) in *Salmonella* isolates

![Agarose gel showing PCR amplification](image)

L1 100 bp DNA ladder; L2 Positive control; L3,L4, L5,L6, L7 Samples; L8 Negative control.
**Fig.4** PCR amplification of *pefA* (700bp) and *sefC* (1103 bp) gene in *Salmonella* isolates

L1, L2 Positive samples; L3 3000 bp DNA ladder; L4 Positive control; L5 Negative control.

**Fig.5** PCR amplification of *spvC* (571 bp) gene in *Salmonella* isolates

L1 100 bp DNA ladder; L2 Positive control; L3, L4, L5 (negative), L6, L7 (positive) Samples; L8 Negative control.
**Fig.6** PCR amplification of *stn* (617 bp) gene in *Salmonella* isolates

![PCR amplification of stn (617 bp) gene in Salmonella isolates](image)

**Table.1** List of oligonucleotide primers used in PCR for detection of virulence-associated genes in *Salmonella* serovars

| Gene | Primer name | Primer sequence (5’→3’) | Amplicon size | Reference |
|------|-------------|-------------------------|---------------|-----------|
| *invA* | *invA* F | GTG AAA TTA TCG CCA CGT TCG GGC AA | 284 bp | Rahn *et al.*, (1992), Kumar *et al.*, (2008) |
|       | *invA* R | TCA TCG CAC CGT CAA AGG AAC C |           |           |
| *sefC* | *sefC* F | GCG AAA ACC AAT GCG ACT GTA | 1103 bp | Rahman *et al.*, (2000), Murugkar *et al.*, (2003) |
|       | *sefC* R | CCC ACC AGA AAC ATT CAT CCC |           |           |
| *pefA* | *pefA* F | TGT TTC CGG GCT TGT GCT | 700 bp | Clouthier *et al.*, (1994), Murugkar *et al.*, (2003) |
|       | *pefA* R | CAG GGC ATT TGC TGA TTC TCC C |           |           |
| *spvC* | *spvC* F | ACT CCT TGC ACAACCAAATGCGGA | 571 bp | Chiu *et al.*, (2006) |
|       | *spvC* R | TGT CTCTGCAATTCGCCACCATA |           |           |
| *stn* | *stn* F | TTG TGT CGC TAT CAC TGG CAA CC | 617 bp | Prager *et al.*, (1995), Murugkar *et al.*, (2003) |
|       | *stn* R | ATT CGT AAC CCG CTC TCG TCC |           |           |
Table 2: PCR thermal cycling conditions for Salmonella virulence genes (invA, sefC, pefA, spvC and stn)

| Stages                        | invA          | sefC and pefA | spvC          | stn          |
|-------------------------------|---------------|---------------|---------------|--------------|
| **First cycle**               |               |               |               |              |
| Initial denaturation          | 95°C (5 min)  | 94°C (4 min)  | 94°C (4 min)  | 95°C (5 min) |
| Subsequent 30 cycles (step 2, 3 and 4) |
| Denaturation                  | 95°C (30 sec) | 94°C (55 sec) | 94°C (45 sec) | 94°C (1 min) |
| Annealing                     | 64°C (30 sec) | 55°C (55 sec) | 56°C (45 sec) | 59°C (1 min) |
| Extension                     | 72°C (45 sec) | 72°C (55 sec) | 72°C (1 min)  | 72°C (1 min) |
| **Final extension for 1 cycle** | 72°C (10 min) | 72°C (7 min)  | 72°C (7 min)  | 72°C (10 min) |

Table 3: Distribution of virulence-associated genes in Salmonella isolates in Aizawl and Imphal

| Genes | Amplicon size | Aizawl (n=2) | Imphal (n=3) | Total |
|-------|---------------|--------------|--------------|-------|
| invA  | 284 bp        | 2 (100%)     | 3 (100%)     | 5 (100%) |
| sefC  | 1103 bp       | 0 (0%)       | 0 (0%)       | 0 (0%)  |
| pefA  | 700 bp        | 2 (100%)     | 0 (0%)       | 2 (40%) |
| spvC  | 571 bp        | 2 (100%)     | 0 (0%)       | 2 (40%) |
| stn   | 617 bp        | 2 (100%)     | 3 (100%)     | 5 (100%) |

Percentage is given in the parentheses

Table 4: Distribution of virulence-associated genes in Salmonella isolates according to serovars

| Salmonella serovars | Total number of isolates | invA | sefC | pefA | spvC | stn |
|---------------------|--------------------------|------|------|------|------|-----|
| *Salmonella typhimurium* | 1                        | 1 (100%) | 0 | 1 (100%) | 1 (100%) | 1 (100%) |
| *Salmonella Vircho* | 4                        | 4 (100%) | 1 (25%) | 1 (25%) | 4 (100%) |

Percentage is given in the parentheses

It is concluded that, detection of virulence associated genes from Salmonella isolates concludes that Salmonella is highly pathogenic organism and 100 % positivity of invA and stn gene supports the fact that, these two genes are present irrespective of serovars and can act as the rapid identification tool for Salmonella in future. Presence of *Salmonella typhimurium* with maximum virulence associated gene is an alarming situation for Aizawl in public health and hygienic point of view. Albeit, more number of samples need to be studied to confirm the pattern of presence of virulence related genes of Salmonella from the isolates from raw pork available at Aizawl and Imphal cities as well as other major cities in North-eastern India, it is quite pertinent to take appropriate measures to improve the hygiene of local pork markets to minimize the health hazards to the consumers.
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