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

M. Das[1], E. Motina[1], D. Deka[1], T.K. Dutta[2], P. Roychowdhury[2], A. De[3] and S. Chakraborty[2*]

[1]Department of Veterinary Public Health & Epidemiology, College of Veterinary Sciences and animal Husbandry, Central agricultural University, Selesih, Aizawl, Mizoram - 796014, India
[2]Department of Veterinary Microbiology, College of Veterinary Sciences and animal Husbandry, Central agricultural University, Selesih, Aizawl, Mizoram - 796014, India
[3]Department of Veterinary Physiology and Biochemistry, CVSc & AH, R.K. Nagar, West Tripura – 799008, India

**Corresponding author*

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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 *inv*A, *stn*, *pef*A, *sef*C, *spv*C by Polymerase Chain Reaction (PCR) technique. All *Salmonella* serovars detected positive for *inv*A and *stn* genes, *spv*C and *pef*A gene was found positive in *Salmonella typhimurium* and one *Salmonella* Vircho of Aizawl origin (overall 40%). *sef*A was absent in all isolates. All the *Salmonella* serovars possessed invasive gene (*inv*A) 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.

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 *inv*A 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-*mgt*C (Blanc-potard and Groisman, 1997) or in the actual manifestation of pathogenic processes, viz., *sop* (Wallis and Galyor, 2000), *stn* (Chopra *et al.,* 1994). As pork is the major preferred meat and consumption of it is very high in North-eastern states of India, there are potential chances of transmission of *Salmonella* infection through meat to the people of this part of the country. Thus, the present study has been carried out to detect the presence of virulence associated genes in *Salmonella* serovars isolated from raw pork collected from Aizawl and Imphal cities.

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 *inv*A (responsible for invasiveness), *pef*A (plasmid encoded fimbrial), *sef*C (*Salmonella enteritidis* fimbrial), *spv*C (*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 *sef*C and *pef*A 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 *inv*A and *stn* genes (100% positive). None of the isolates were found positive for *sef*C (0%). However, two isolates (One *S. typhimurium* and one *S. vircho*) from Aizawl, were also found to be positive for *pef*A and *spv*C 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 *inv*A 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 *inv*A gene. In this present study, we also found, 60% (3/5) isolates positive for *pep*A and 20% (1/5) positive for *spv*C and *sef*C genes. Similar finding was obtained by Das *et. al.,* (2012) who reported presence of *inv*A, *stn*, *pef*A, *sef*C and *spv*C 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 *inv*A 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

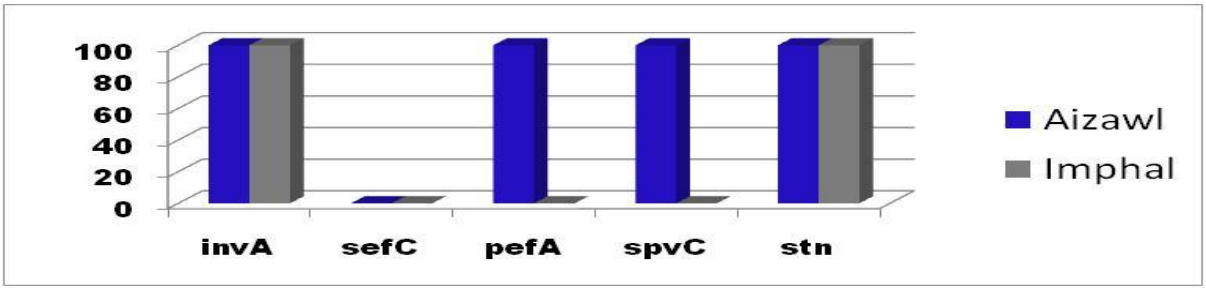


Fig.2 Distribution of virulence associated genes according to *Salmonella* serovars

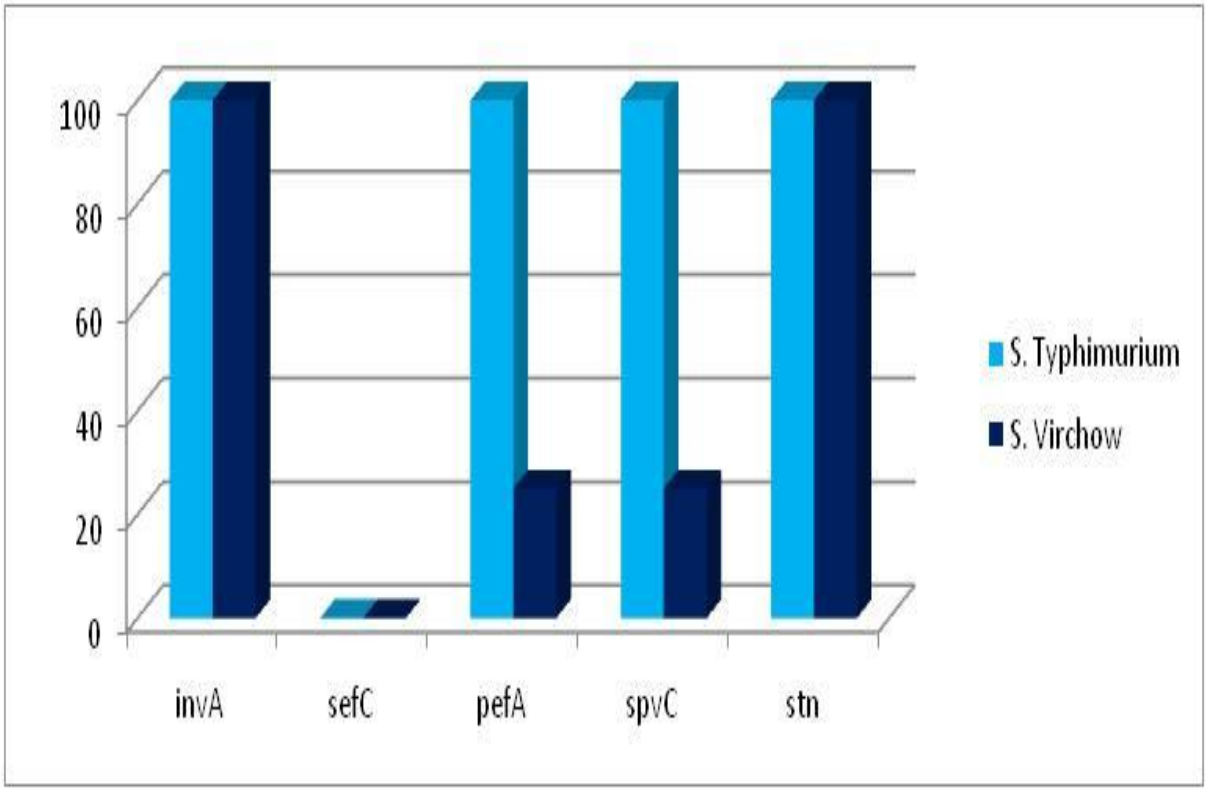
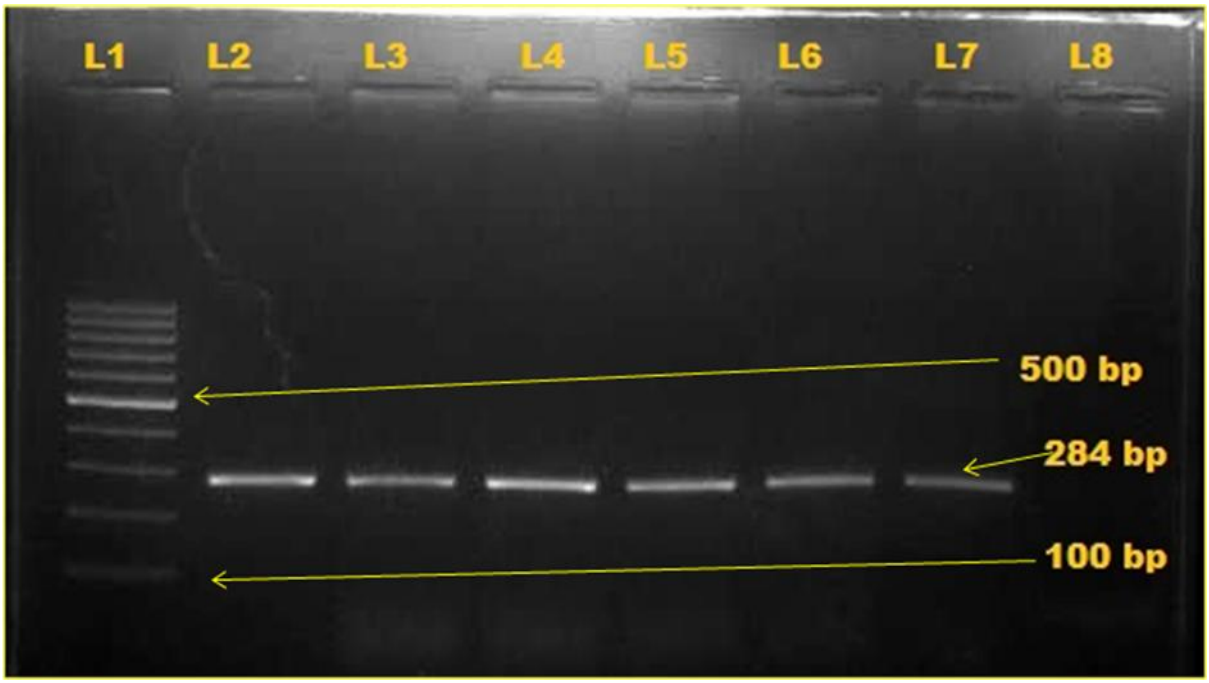
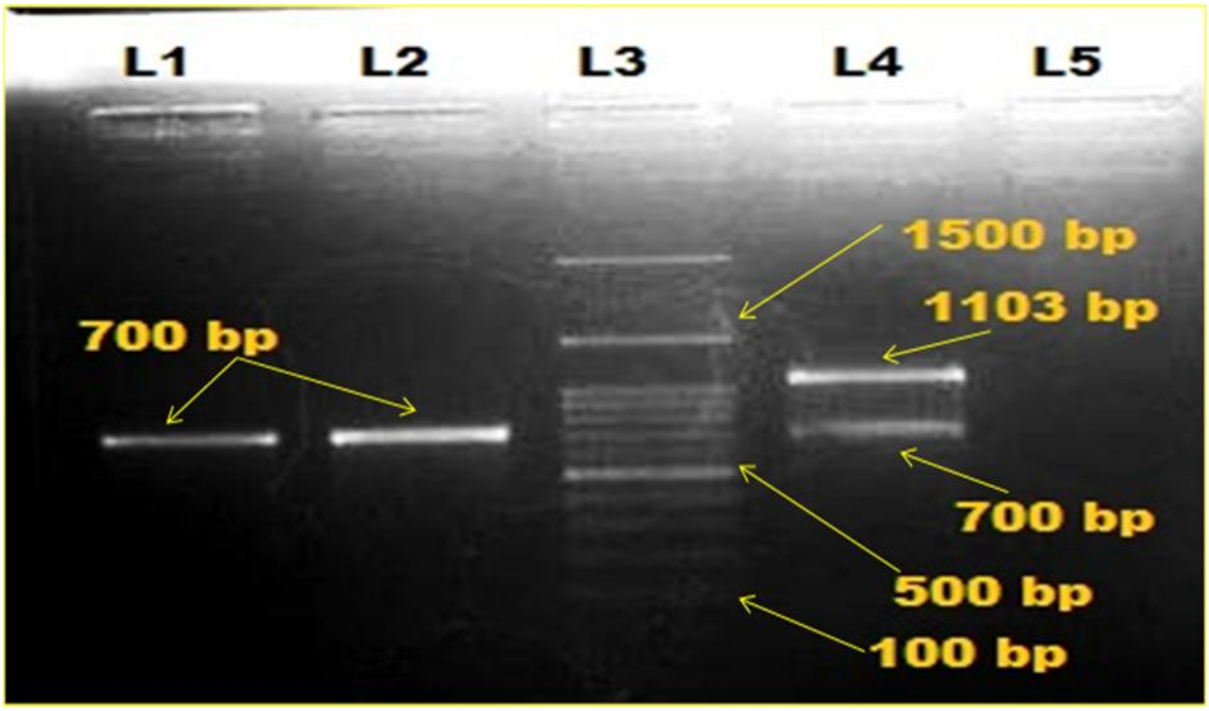


Fig.3 PCR amplification of *inv*A gene (284 bp) in *Salmonella* isolates



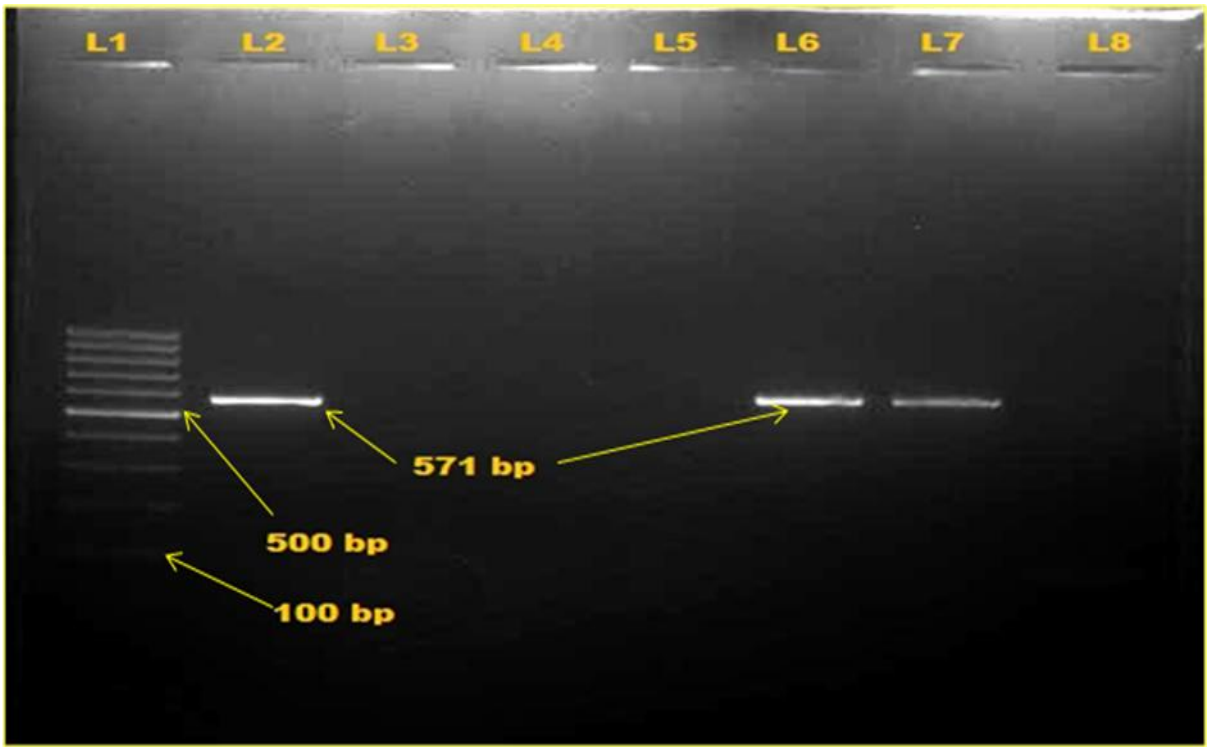
L1 100 bp DNA ladder; L2 Positive control; L3,L4, L5,L6, L7 Samples; L8 Negative control.

Fig.4 PCR amplification of *pef*A (700bp) and *sef*C (1103 bp) gene in *Salmonella* isolates



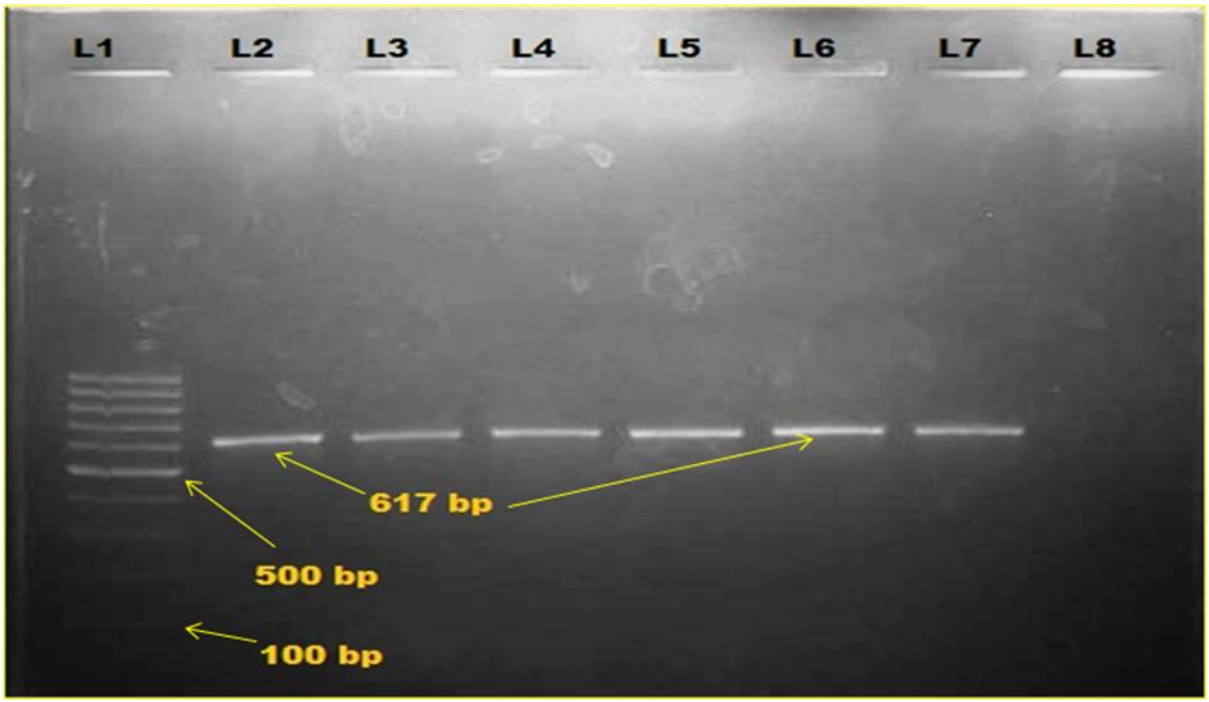
L1, L2 Positive samples; L3 3000 bp DNA ladder; L4 Positive control; L5 Negative control.

Fig.5 PCR amplification of *spv*C (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



L1 100 bp DNA ladder; L2 Positive control; L3,L4, L5,L6, L7 Samples; L8 Negative control

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
***inv*A**	*inv*A F	GTG AAA TTA TCG CCA CGT TCG GGC AA	284 bp	Rahn *et al.*, (1992), Kumar *et al.*, (2008)
	*inv*A R	TCA TCG CAC CGT CAA AGG AAC C		
***sef*C**	*sef*C F	GCG AAA ACC AAT GCG ACT GTA	1103 bp	Rahman *et al.*,(2000), Murugkar *et al.*, (2003)
	*sef*C R	CCC ACC AGA AAC ATT CAT CCC		
***pef*A**	*pef*A F	TGT TTC CGG GCT TGT GCT	700 bp	Clouthier *et al.*,(1994), Murugkar *et al.*,(2003)
	*pef*A R	CAG GGC ATT TGC TGA TTC TTC C		
***spv*C**	*spv*C F	ACT CCT TGC ACAACCAAATGCGGA	571 bp	Chiu *et al.*, (2006)
	*spv*C R	TGT CTCTGCATTTCGCCACCATCA		
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	95^0C (5 min)	94^0C (4 min)	94^0C (4 min)	95^0C (5 min)
Subsequent 30 cycles(step 2,3 and 4)				
Denaturation	95^0C (30 sec)	94^0C (55 sec)	94^0C (45sec)	94^0C (1 min)
Annealing	64^0C (30 sec)	55^0C (55 sec)	56^0C (45sec)	59^0C (1 min)
Extension	72^0C (45 sec)	72^0C (55 sec)	72^0C (1 min)	72^0C (1 min)
Final extension for 1 cycle	72^0C (10 min)	72^0C (7 min)	72^0C (7 min)	72^0C(10min)

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

Genes	Amplicon size	Aizawl (n=2)	Imphal (n=3)	Total
***inv*A**	284 bp	2 (100%)	3 (100%)	5 (100%)
***sef*C**	1103 bp	0 (0%)	0 (0%)	0 (0%)
***pef*A**	700 bp	2 (100%)	0 (0%)	2 (40%)
***spv*C**	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	*inv*A	*sef*C	*pef*A	*spv*C	*stn*
Salmonella typhimurium	1	1 (100%)	0	1 (100%)	1 (100%)	1 (100%)
***Salmonella* Virchow**	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 *inv*A 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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