Prevalence and virulence gene profiles of *Salmonella* serovars isolated from animal origin

Y Suresh, Ch. Bindukiranmayi, T Srinivasa Rao, M Srivani and A Uday Siva Naga Sankar

**Abstract**

*Salmonella* is a foodborne pathogen having a worldwide public health concern. The present study was undertaken to characterize *Salmonella* species of animal origin based on cultural isolation, molecular confirmation of serovars and their virulence profiles. A total of 516 samples comprising poultry cloacal swabs (249), raw foods of animal origin (118 chicken samples, 65 mutton and 30 pork), poultry liver swabs (17) and poultry farm water samples (37) were examined for presence of *Salmonella* serovars. Overall prevalence of *Salmonella* isolates was found to be 4.06% (21/516) with highest prevalence in chicken samples (6/118, 5.08%) followed by cloacal swabs of poultry (12/249, 4.81%), mutton (2/65, 3.07%) and pork (1/30, 3.33%). All the isolates carried all the 7 virulence genes *i.e.* invA, invH, sopB, sopE & stn (100%), while pefA gene was found only in *S. typhimurium* isolates and sefC gene was found only in *S. enteritidis* isolates.

**Keywords:** *Salmonella* typhimurium, enteritidis, virulence genes profile

**Introduction**

*Salmonella* species is responsible for a wide range of acute and chronic diseases in both poultry and humans. Contaminated poultry products are among the most important sources for foodborne outbreaks in humans. *Salmonella* is reported more frequently from poultry and poultry products than from any other animal species. The genus *Salmonella* belongs to the family Enterobacteriaceae and consists of more than 2500 serovars. According to the CDC (Centre for Disease Control and Prevention, Atlanta), *Salmonella* alone affects about 1.4 million people each year in the United States with about 16,000 hospitalizations and more than 500 deaths annually. In 1996, the USDA, Economic Research Service estimated that the total costs for medical care and lost productivity, resulting from foodborne *Salmonella* infections of humans was between 0.6 – 3.5 billion dollars annually (USDA, 1996) [58]. Salmonellosis is a major health problem, worldwide and is responsible for high rates of morbidity. Infection with *Salmonella enterica* occurs mainly through the consumption of contaminated food and the estimated number of human infections per year is greater than 93.8 million cases, with 155,000 deaths per year worldwide (Boyle et al., 2007; Majowicz et al., 2010 and Hendriksen et al., 2011) [14, 15, 26]. Genetically, *Salmonella enterica* is subdivided into 7 subspecies based on biochemical criteria and DNA homology although, as many as 2500 serovars of *S. enterica* have been identified, the most common serotypes attributed to foodborne outbreaks are *Salmonella* Typhimurium (*S. Typhimurium*) and *Salmonella* Enteritidis (*S. Enteritidis*), which account for more than 75% of reported cases (Popoff, 2001) [123].

*Salmonella* pathogenicity depends on a variety of virulence factors that help the pathogen in adhesion, invasion, intracellular survival, fimbrial expansion, systemic infection, antibiotic resistance, toxin production and magnesium and iron uptake (Fardsanei et al., 2017) [23]. Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. Some genes are involved in the adhesion and invasion *viz.* sef, pef, spv or inv (Galen et al., 1992; Krause et al., 1992; Clouthier et al., 1993 and Baumler et al., 1996) [24, 34, 19, 11] and others are connected with the survival in the host system like mglC (Blanc-Potard and Grosman, 1997) [121] or in the actual manifestation of pathogenic processes *viz.* sop, stn, pip A, B, D (Wallis and Galyov, 2000; Chopra et al., 1994 and Wood et al., 1998) [61, 17, 63]. Some of the virulence genes *viz.* *Salmonella* enterotoxin (stn) (Praeger et al., 1995 and Rahman, 1999) [44, 45] *Salmonella* Enteritidis fimbriae (sef) and plasmid-encoded fimbriae (pef) genes (Rahman et al., 2000) [46] are being diagnosed by employing nucleic acid based diagnostic techniques.
Therefore the present study has been carried with the aim to study the prevalence and virulence gene profile of different Salmonella serovars isolated from different samples of animal origin.

Materials and Methods

Standard control and primers
ATCC (American Type Culture Collection) cultures of Salmonella Typhimurium (ATCC 14028) and Salmonella Enteritidis (ATCC 13076) were used as standard controls. Oligonucleotide primers were custom synthesized from M/s. Bioserve Biotechnologies Pvt. Ltd. (Hyderabad).

Sample collection
A total of 516 samples were collected from different sources including chicken meat samples (118), mutton (65) and pork (30) from retail shops, poultry cloacal swabs (249) from poultry diagnostic laboratories, water samples (37) from different poultry farms in and around Krishna district of Andhra Pradesh and they were collected in sterile polythene bags/ sterile swabs/ sterile bottle. Each bag/swab/bottle was labeled with sample number and particulars about samples. The samples were transferred in an ice box to the laboratory for further processing.

Ten grams each of raw meat sample collected from various sources were mixed with 90 ml of BPW (Merck, Germany) chilled for 10min and centrifuged at 10,000 rpm for 5min. Supernatant was discarded, 50 μl of nuclease free water was added and placed in boiling water bath at 100 °C for 10 min. Immediately snap chilled for 10min and centrifuged at 10,000 rpm for 5min. The supernatant was taken as template and subjected to different mPCRs and the PCR products were subjected to gel electrophoresis using 1.5% agarose with ethidium bromide as fluorescent dye and visualized using Gel Documentation unit (BIORAD, USA).

Molecular confirmation of Salmonella Genus and serovars by MPCR
Salmonellae isolated from different samples by cultural methods were confirmed using Salmonella specific mPCR targeting random sequence gene, fliC & sfa genes for confirmation of Salmonella genus and S. Typhimurium and S. Enteritidis, respectively (Table-1). PCR assay was optimized in 25 µl reaction mixture containing 2 µl of DNA template, 2.5 µl of 10x master mix (Go Taq Green Master Mix, Promega), 1µl each of forward and reverse primers (10 pmol/μl) and the rest of the volume is made by adding nuclease free water, under standardized cycling conditions: initial denaturation at 95°C for 5 min; 35 cycles of 94°C for 30 s, 56 °C for 90 s and 72 °C for 30 sec and a final elongation step at 72 °C for 10 min (Soumet et al., 1999) [52].

Detection of virulence genes in Salmonella isolates
All the confirmed Salmonella isolates from different sources were screened for the presence of virulence genes such as invA, invH, stn, sopB, sopE, sfaC and pefA genes (Table-2). PCR assay was optimized in 25 µl reaction mixture containing 2 µl of DNA template, 2.5 µl of 10x master mix (Go Taq Green Master Mix, Promega), 0.5µl each of forward and reverse primers (10 pmol/μl) and the rest of the volume is made by adding nuclease free water, under standardized cycling conditions: initial denaturation at 94°C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 90 s and 72 °C for 120 sec and a final elongation step at 72 °C for 10 min (Choudhury et al., 2016) [18].

### Table 1: Salmonella genus and serovar specific m-PCR primers and sequences (Soumet et al. 1999) [52]

<table>
<thead>
<tr>
<th>S. No</th>
<th>Primer</th>
<th>Primer sets</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Salmonella Genus specific</td>
<td>ST 11</td>
<td>GCAACCATTGCTAAATTTGGCGCA</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST15</td>
<td>GGTAGAAATTCACCAGCGGTACTGG</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>S. Typhimurium fliC</td>
<td>Fli 15</td>
<td>CGGGTGTGCGCCGTTGGAATAT</td>
<td>559</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tym</td>
<td>ACTCTGCTGCGGTGGCGCATTT</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>S. Enteritidis sfaA</td>
<td>SeI67</td>
<td>AGGGTACCGCACGCGTACT</td>
<td>312</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SeI478</td>
<td>GGGACATTTAGCGTTTTCG</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2: Primers used for detection of virulence genes in Salmonella (Choudhury et al., 2016) [18]

<table>
<thead>
<tr>
<th>Target gene</th>
<th>primer</th>
<th>Primer sequence</th>
<th>amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>invA</td>
<td>InvA-F</td>
<td>ACCACGCTTTTCCTGCCTGG</td>
<td>942</td>
</tr>
<tr>
<td></td>
<td>InvA-R</td>
<td>GAACGCTACGTCGAGTAC</td>
<td></td>
</tr>
<tr>
<td>invH</td>
<td>InvH-F</td>
<td>TATAGCCTGTCTTCTTCTT</td>
<td>305</td>
</tr>
<tr>
<td></td>
<td>InvH-R</td>
<td>ATGTATGGTGTGTCTTCTT</td>
<td></td>
</tr>
<tr>
<td>stn</td>
<td>Stn-F</td>
<td>ATGTAGGTCTTAAATCTTCTT</td>
<td>543</td>
</tr>
<tr>
<td></td>
<td>Stn-R</td>
<td>CTGTCGCAATCTACCTGGA</td>
<td></td>
</tr>
<tr>
<td>sopB</td>
<td>SopB-F</td>
<td>AGCATCTCTAAAGCGTACT</td>
<td>470</td>
</tr>
<tr>
<td></td>
<td>SopB-R</td>
<td>GCTCTGTAACGCTGCTTCA</td>
<td></td>
</tr>
</tbody>
</table>
Results and Discussion
Out of 516 samples comprising foods of animal origin and other sources (249 Poultry cloacal swabs, 118 Chicken, 65 Mutton, 30 Pork, 17 Liver swabs of poultry and 37 poultry farm water) analyzed, 21 samples were found positive for *Salmonella* spp. with overall prevalence of 4.06%, the prevalence of the *Salmonella* in various samples was ranging from 5.08% in chicken followed by 4.81% in poultry cloacal swabs, 3.33% in pork and 3.07% in mutton. All the water samples and poultry liver swab samples were found negative for *Salmonella*.

All 21 *Salmonella* isolates were subjected to LA test (Hi Salmonella Latex kit, (Hi media). A clearly visible agglutination is seen for all 21 *Salmonella* isolates. S. Typhimurium ATCC 14028 and E. coli ATCC 25922 were used as positive and negative controls, respectively. The overall prevalence of *Salmonella* in the present result was in accordance with earlier research works done in different states of India, on animals and foods of animal origin where 4.58% (Mekwana et al., 2015) [36], 6.1% (Samanta et al., 2014) [50] and 2.5% (Nair et al., 2015) [39] prevalence of *Salmonella* spp. were reported. However Murugkar et al. (2005) [37] and Kaushik et al. (2014) [41] reported a higher prevalence of *Salmonella* spp. in foods of animal origin and animal samples i.e. 14.5% and 23.7%, respectively. Among the 21 positive *Salmonella* isolates, S. Typhimurium (7) was the most dominant serovar, followed by *Salmonella* group II (7), *Salmonella* group G (3), *Salmonella* Enteritidis (2), *Salmonella* Daytona and *Salmonella* Linderburg (one each).

*Salmonella* group II, considered as *Salmonella enterica* subsp. *salamae*, comprising of *Salmonella* Sofia, *Salmonella* Tranoroa, *Salmonella* Hagenbeck, *Salmonella* Nairobi serovars will be commonly associated with cold blooded animals but can be an occasional pathogen in man and other animals (Wuthe, 1969) [64]. It may also be responsible for severe infection in the young, very old or immunocompromised patients who then require hospitalization. Most of the infections with this subspecies were accompanied by gastroenteritis, with systemic infection apparent only in a very few cases. Although it is generally believed that cold-blooded animals are the major reservoirs of *S. enterica* subsp. *salamae*, it is also important to consider for food-borne infections (Angulo and Swerdlow, 1995 and CDC, 1999) [5, 16].

*Salmonella* groupG includes serovars like S. Poona, S. Worthington, S. Mississippi, S. Grumpensis, S. Atlanta, S. Cubana, S. Wichita etc. (Bridges and Scott, 1935) [15]. S. Poona infections associated with pet turtle exposure have been reported. Nine outbreaks of S. Poona linked to the consumption of contaminated (water melon & cantaloupe) food between 1998 and 2006 in different places of USA (Patil Waller, 2015) [41] have been reported. S. Cubana epizootic was reported in animals due to feeding of synthetic starter food and was also isolated from diarrhoeic infants in Cuba (Seligmann et al., 1945) [51].

Serovars like S. Typhimurium, S. Enteridis and S. Daytona belong to family *Salmonella enterica* sub. spp. *enterica*. Among *Salmonella enterica* subsp *enterica*, serotypes S. Typhimurium and S. Enteritidis are the most important agents of foodborne salmonellosis in humans (Baay and Huisin’tveld, 1993 and Tan and Shelef, 1999) [8, 55]. It was estimated that approximately 75% of human salmonellosis cases were due to consumption of contaminated food products such as beef, pork, poultry, eggs (Kent et al., 1981) [32] and meat products like sausages (Wall et al., 1994) [60].

Present work revealed 4.81% (12/249) prevalence of *Salmonella* in poultry cloacal swabs, which was in agreement with the 4% prevalence as reported by Gracia et al. (2011) [25], 6.9% by Amini et al. (2010) and 5.8% by Jafari et al. (2007) and findings of present study were higher than 0.6% as reported by Rodriguez et al. (2017). In contrast, higher prevalence of 12.5%, 15%, and 84% were reported by the Eyigor et al. (2002) [22] Samanta et al. (2014) [50] and Ramya et al. (2012) [47].

Out of 12 *Salmonella* isolates recovered from the poultry cloacal swabs, 6 were belonging to *Salmonella* group II, 3 were *Salmonella* group G, 2 were S. Enteritidis and one S. Daytonia. The prevalence of S. Enteritidis in cloacal swabs of poultry (0.8%, 2/249) in present study was very low when compared to the prevalence of 2.8%, 51.4%, & 62% as reported by Gracia et al. (2011) [25], Amini et al. (2010) [41] and Eyigor et al. (2002) [22], respectively. Present study revealed (6/249) 2.40% of *Salmonella* group II in poultry cloacal swabs and almost similar prevalence rate of 4% (4/100) was reported by Ahmed et al. (2011) [1].

Six samples (5.08%) out of 118 chicken samples examined were positive for *Salmonella* spp., which correlates well with the results of Saha et al. (2016) [49] who reported a prevalence rate of 4%, whereas different studies conducted in India and China reported higher prevalence of 12.5%, 23.7%, 33.33%, 45.2% as per Dogru et al. (2009) [21], Kaushik et al. (2014) [51], Balakrishnan et al. (2018) [19] and Bai et al. (2015) [7], respectively and lower prevalence of 1%, 3.2% and 3.7% were reported by many authors like Wang et al. (2009) [62], Issa et al. (2017) [28] and Yoon et al. (2014) [66], respectively.

Out of 6 *Salmonella* isolates recovered from the chicken samples, 5 (4.23%, 5/118) were S. Typhimurium and 1 (0.84%, 1/118) S. Linderburg. The results were in agreement with Kaushik et al. (2014) [51] and Anumotu et al. (2012) [6] who also reported 6% and 2% prevalence of S. Typhimurium in chicken meat samples.

Out of 65 mutton samples evaluated, 2 (3.07%, 2/65) samples revealed the presence of *Salmonella* spp. S. Typhimurium and *Salmonella* group II. This was almost in agreement with the findings of Dabassa (2013) [20] and Yadav et al. (2006) [65] who reported 2.7 % and 3.0% in mutton samples respectively while, higher prevalence rates of 11% and 43% were reported by Zweifel et al. (2004) [46] and Bonke et al. (2012) [13] and low prevalence of 1.1% was reported by Nousihi and Hamdi (2009) [50] in mutton.

Out of 17 poultry liver swabs processed, no *Salmonella* isolate was detected while 6.3%, 35.1% and 23.3% of prevalence were reported by Al- Nakhi et al. (1999) [11], Islam et al. (2006) [27] and Karim et al. (2017) [30], respectively. Good managerial practices of poultry flock at the farm level may be the reason for absence of *Salmonella* in liver samples.
swabs collected during P.M examination. Out of 30 pork samples processed, only one sample was positive for *S. Typhimurium* (3.33%, 1/30) which was in correlation with the findings of work done by TEAGASC (The Agriculture and Food Development Authority) in Ireland and Kiran *et al.* (2016) \[33\] where *Salmonella* spp. prevalence was reported to be 3.3% and 1.57%. In contrast to the present study, higher prevalence of 47.75% and 58.1% were reported by Villal Pando- Guzman *et al.* (2016) \[59\] and Zaidi *et al.* (2006) \[67\].

Out of 37 poultry farm water samples processed, no single *Salmonella* was detected. The finding of the present study was in correlation with the findings of Alali *et al.* (2010) \[2\], who also reported similar results in poultry water samples of Georgia. Barros *et al.* (2001) \[10\] and Traore *et al.* (2015) \[57\] also reported the non-existence of *Salmonella* in water samples of different broiler chicken farms of Finland. While some authors like Poppe *et al.* (1991) \[43\] and Samanta *et al.* (2014) \[50\] reported a higher prevalence rate of 12.3% and 20% of *Salmonella* serovars in poultry farm water samples of Canada and West Bengal, respectively. Absence of *Salmonella* in poultry farm water samples may be due to the supply of *Salmonella* free water to the birds and proper disinfection and treatment of water or may be due to the presence of *Salmonella* at a very low level, below the detection limit (10 CFU/ml) of the isolation/cultural methods used in the present study (Alali *et al.*, 2010) \[2\].

The PCR based approach has been successfully used for the detection of specific virulence genes in *Salmonella* spp. (Swamy *et al.*, 1996) \[54\]. The different virulence genes detected by PCR were *invA, invH, sopB, sopE, stn, pefA* and *sefC*. All the isolates were found to be carrying *invA, invH, sopB, sopE* and *stn* (100%) genes while variability was observed among *pefA* (33.33%) and *sefC* (16.66%) genes. *pefA* gene was found only in *S. Typhimurium* and *sefC* only in *S. Enteriditis*. The results of present study were in correlation with the work done by Choudhury *et al.* (2016) \[18\], who reported that 100% *Salmonella* isolates collected from different parts of India carried *invA, invH, sopB* and *stn* genes.

Muthu *et al.* (2014) \[38\] reported 79.5% prevalence of *stn* gene among different human clinical *Salmonella* serovars collected in and around Chennai, India and reported absence of *pefA* among screened *Salmonella Enteritidis* isolates.

**Fig 1:** Latex agglutination test kit results for *Salmonella* spp.
Well 1-3 Negative reaction (1- E. coli ATCC 25922, 2 and 3–Salmonella negative samples), Well 4 Positive control showing clear agglutination (*S. Typhimurium* ATCC14028), Well 5 & 6 *Salmonella* isolates showing clear agglutination reaction

**Fig 2:** Gel photograph of m-PCR showing bands for *Salmonella* genus, *S. Typhimurium* & *S. Enteritidis*, Lane M Molecular weight marker (100bp), Lane 1 Positive control of *S. Enteritidis* ATCC 13076 (312 bp and 429 bp), Lane 2 Positive control of *S. Typhimurium* ATCC14028 (429 bp and 559 bp), Lane 3 Negative control, Lane 4–7 *S. Typhimurium* positive isolates from different samples (chicken, mutton and pork), Lane 8 & 9 *S. Enteritidis* positive isolates from poultry cloacal swabs, Lane 10&11 *Salmonella* genus positive isolate obtained from poultry cloacal swabs (429bp)
P. H. Alternative antigens reduce Thum C,acteriology. RO
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Ss, 1996.

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Reference

University, Tirupati, Andhra Pradesh, for providing pati, Andhra Pradesh, for providing.

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Conclusion

The high frequency of presence of virulence genes that were investigated highlights the pathogenic potential of the studied Salmonella serovars which have been causing disease in humans due to consumption of contaminated foods of animal origin.

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