Molecular characterization of *Salmonella* serovars of zoonotic importance*

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ABSTRACT

Isolates (50) of *Salmonella* were recovered from 1,132 samples from foods of animal origin and fecal samples from animals and human beings; belonging to 10 different serovars with most prevalent serovar *S. Typhimurium* (21) followed by *S. Weltevreden* (12), *S. Ughelli* (5), *S. Essen* (3), *S. Elisabethville* (2), *S. Lagos* (2), *S. Drogana* (2), *S. Enteritidis* (1), *S. London* (1) and un-typable *Salmonella* (1). Emerging *Salmonella* serovars, viz *S. Elisabethville*, *S. Essen*, *S. Lagos*, *S. Ughelli* and *S. Drogana* were first time recovered from Pantnagar and its vicinity. *S. Drogana* was recovered possibly for the first time from human source in India. Multiple *Salmonella* serovars (up to 3 serovars comprising *S. Typhimurium*, *S. Weltevreden* and *S. Essen*) were recovered from single cattle dung sample while, multiple serovars (up to 2 serovars) were also recorded in many single samples, viz cattle dung (*S. Weltevreden* and *S. Ughelli*), poultry droppings (*S. Essen* and *S. Ughelli*), pig faeces (*S. Weltevreden* and *S. London*), sheep faeces (*S. Typhimurium* and *S. Drogana*) and pig faeces (*S. Weltevreden* and *S. Ughelli*). Different virulence genes, viz. *invA*, *sipA*, *sefA*, *fliC*, *stn* and *sopB* were detected in *Salmonella* isolates using PCR-based molecular technique. Among these virulence genes, *invA* gene was the most prevalent one as is present in 98% *Salmonella* isolates followed by *sopB*, *stn*, *sipA*, *fliC* and *sefA* genes in 96, 86, 78, 32 and 10% *Salmonella* isolates, respectively. *Salmonella* serovars of zoonotic importance recovered from varied sources exhibited different virulence genes that may cause serious infections in animals as well as in human beings. Ultimately these virulent serovars may pose great risks to the health and production in the animals and serious health hazards in human.

**Key words:** Animal faecal samples, Foods of animal origin, Human stool, *Salmonella* serovars, Virulence genes

The dynamics of *Salmonella* infections is quite variable and affected by changes in human demographics and lifestyles, human behaviour, changes in industry and technology, changes in travel and commerce, microbial adaptation, breakdown in the public health infrastructure and lack of knowledge on food safety and handling practices among consumers. The most common non-typhoidal serovar isolated from human is *S. Typhimurium* followed by *S. Enteritidis*, whereas, the most common serovar of non-human origin is *S. Typhimurium* followed by *S. Newport* (CDC 2006).

Swamy *et al.* (1996) established the presence of invasive A (*invA*) gene in nearly all *Salmonella* irrespective of serovar or source. The other *Salmonella* enterotoxin (*stn*) gene has been consistently shown to correctly identify all *Salmonella* Enterica (Prager *et al.* 1995). *Salmonella* outer protein (Sop) encoded by *sopB* gene increased the virulence of bacteria (Rahman 2006). Cortez *et al.* (2006) identified *Salmonella* serovars by a multiplex PCR targeting *invA*, *pefA* and *sefA* genes for *Salmonella* spp., *S. Typhimurium* and *S. Enteritidis*, respectively. The flagellin C (*fliC*) gene encodes major component of flagellum in *S. Typhimurium* (Aldridge *et al.* 2006). In the present study, attempts were made to isolate and identify *Salmonella* organisms from samples of varied sources; to determine the prevalence of *Salmonella* serovars in Pantnagar and nearby areas considering that prevalence would change from time to time due to emergence of new serovars in the same geographical area; and to detect various virulence genes to know the virulence status of the organism.

MATERIALS AND METHODS

Samples (1,132) comprising poultry meat (212), poultry eggs (49), poultry droppings (60), autopsied poultry tissues (60), pork (156), pig faeces (189), cattle dung (105), buffalo dung (103), sheep faeces (11), goat faeces (31), deer faeces (2) and human stool (154) were collected from Pantnagar and nearby areas and processed for isolation of salmonellae using conventional culture method.

*Salmonella* organisms were isolated from human stool and animal faecal samples as per Standard ISO 6579: (2002)
and manual WHO/CDC (2003) for non-typhoidal and typhoidal Salmonella, respectively. Human stool samples were processed for both typhoidal and non-typhoidal Salmonella, whereas, animal faecal samples were subjected to only non-typhoidal Salmonella. Egg contents were screened for only non-typhoidal Salmonella organisms. The pork and poultry meat samples were processed for isolation of both the non-typhoidal and typhoidal Salmonella as per USDA/FSIS (2002) and manual WHO/CDC (2003), respectively.

Salmonella isolates identified on the basis of cultural, morphological and biochemical reactions were subjected to agglutination reaction using O antiserum poly A-I and Vi for serological identification of Salmonella organisms. Salmonella isolates showing positive agglutination test were sent for serotyping to Salmonella Typing Center, Division of Bacteriology and Mycology, IVRI, Izatnagar, India.

The genomic DNA was isolated as per Soumet et al. (1994). Salmonella isolates were confirmed using PCR technique by targeting invA gene fragment (Chiu and Ou 1996). The PCR thermal cycling conditions were applied for amplification of invA and other virulence genes (Table 1). The agarose gel electrophoresis of amplified PCR products was carried out as per Sambrook and Russell (2001).

Different virulence genes, viz. invA, sipA, sefA, flIC, stn and sopB were detected in Salmonella serovars using simplex PCR following the methods of Chiu and Ou (1996), Wang et al. (2009), Oliveira et al. (2003), Murugkar et al. (2003) and Skyberg et al. (2006), respectively.

### RESULTS AND DISCUSSION

In the present study, 50 Salmonella isolates belonging to 10 different serovars, viz. S. Typhimurium (21), S. Weltevreden (12), S. Ughelli (5), S. Essen (3), S. Elisabethville (2), S. Lagos (2), S. Drogana (2), S. Enteritidis (1), S. London (1) and un-typable Salmonella-isolate (1) were recovered from 1,132 different samples. It is worth mentioning that Salmonella serovars, viz. S. Elisabethville, S. Essen, S. Lagos, S. Ughelli and S. Drogana were possibly recovered for the first time from different sources from Pantnagar and its vicinity which were not reported earlier in these areas. The emergence of these serovars might be attributed to introduction of these organisms through contaminated poultry feeds or introduction of infected poultry.

In this study, there was another significant finding that the multiple serovars (up to 3 serovars, viz. S. Typhimurium, S. Weltevreden and S. Essen) were recovered from single cattle dung sample while, multiple serovars (up to 2 serovars) were recovered from many single source/samples. The isolation of multiple serovars of Salmonella was approached to know the involvement of number of serovars in the pathogenesis of disease. Therefore, it may be assumed that the multiple serovars recovered from 6 different single samples complicate the pathogenesis. Serovar S. Drogana recovered from human stool sample appears to be the first report from human source in India. However, Singh et al. (2007) reported Salmonella Drogana from non-human source that is in equines from equine farms in India.

In the present study, attempts were made to detect

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**Table 1. Oligonucleotide primer profile and PCR optimization conditions applied for different virulence genes**

<table>
<thead>
<tr>
<th>PCR steps</th>
<th>PCR conditions</th>
<th>Virulence genes</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>invA</td>
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<tr>
<td></td>
<td></td>
<td>sipA</td>
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<td>stn</td>
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<td></td>
<td></td>
<td>sopB</td>
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<tr>
<td><strong>Initial</strong></td>
<td><strong>Denaturation</strong></td>
<td></td>
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<tr>
<td>Temperature (°C)</td>
<td>Time (min)</td>
<td>94</td>
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<tr>
<td>Time (min)</td>
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<td></td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
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<td>94</td>
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<tr>
<td>Temperature (°C)</td>
<td>Time (min)</td>
<td>0.5</td>
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<tr>
<td>Time (min)</td>
<td>0.5</td>
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<tr>
<td><strong>Annealing</strong></td>
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<td>60</td>
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<td>Temperature (°C)</td>
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<tr>
<td>Time (min)</td>
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<tr>
<td><strong>Extension</strong></td>
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<td>72</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>Time (min)</td>
<td>2</td>
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<tr>
<td>Time (min)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Final extension</strong></td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>Time (min)</td>
<td>10</td>
</tr>
<tr>
<td>Time (min)</td>
<td>5</td>
<td></td>
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<tr>
<td><strong>Number of cycles</strong></td>
<td></td>
<td>30</td>
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</table>

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**F:** CATGTTAC
**R:** AAGACGA
**TCGTTTAC**
**CACGA**
**CGTGTA**
**CTTTATTA**

**F:** GATACGTG
**R:** GCCGTAAA
**CTGATC**
**GCAGTC**
**GCAGTAGC**

**F:** CGGTGTTG
**R:** CTGTTTAC
**CTGGTACT**
**GTACCGG**
**TCAGCATCT**

**F:** TTTTATTA
**R:** GCAGTAGC
**CGTCC**

**F:** GAGG
**R:** ACTGGTA
**AAGATGGCT**

**F:** 72
**R:** 72
**CGCCAGTCT**

**F:** 95
**R:** 95
**CGCTATCA**

**F:** 94
**R:** 94
**GTAAT**

**F:** 5
**R:** 5
**CTGGCAACC**

**F:** 94
**R:** 94
**CCACGGTTG**

**F:** 5
**R:** 5
**GAAG**

**F:** 95
**R:** 95
**CTGGTACT**

**F:** 57
**R:** 57
**GTACCGG**

**F:** 1
**R:** 1
**TCAGCATCT**

**F:** 72
**R:** 72
**TCAGCATCT**

**F:** 2
**R:** 2
**GCAGTAGC**

**F:** 72
**R:** 72
**GTAAT**

**F:** 10
**R:** 10
**CTGTA**

**F:** 35
**R:** 35
**GTACCGG**

**F:** 35
**R:** 35
**GCAGTAGC**
different virulence genes, viz. invA, sipA, sefA, fliC, stn and sopB in Salmonella isolates using PCR-based molecular technique. The invA gene was most prevalent (98%) and present in all Salmonella isolates except S. Drogana isolated from human stool followed by sopB, stn, sipA, fliC and sefA genes in 96, 86, 78, 32 and 10% Salmonella isolates, respectively.

The invA gene encodes for a protein in the inner and outer membrane, which is essential for invasion of epithelial cells (Darwin and Miller 1999). The genomic DNA of all serovars were subjected to the amplification, in which 49 out of 50 serovars (98%) got amplified and exhibited the amplified product of 244 bp (Fig. 1). As per our findings, invA gene can be targeted for confirmation of Salmonella organisms. However, one isolate, S. Drogana, showed the absence of invA gene.

Hepfelmeier et al. (2004) established that the sipA gene initiates intestinal infection and establishes systemic infection in the host, that is encodes the invasive character of Salmonella serovars. Therefore, in the present study, the sipA gene was targeted to determine the invasive character of Salmonella serovars. Out of 50 Salmonella isolates, 39 exhibited amplification (449 bp) with the primers targeting sipA gene (Fig. 2), while remaining 11 isolates could not show amplification. The higher prevalence of this gene in Salmonella serovars recovered in the present study indicated invasiveness of the organisms and involvement in the systemic infection in the hosts.

In the present study, S. Enteritidis exhibited amplification (488 bp) with primer fragment of this gene (Fig. 3). However, this gene also showed its presence in other Salmonella serovars such as S. Typhimurium, S. Weltevreden and S. Essen. Rahman et al. (2000) targeted sefA gene and found that amplification of this gene in S. Enteritidis, while the other serovars did not amplify with this gene. Our finding thus differs from the findings of the earlier researchers indicating that sefA gene may not be the serovar specific.

Gene fliC was exhibited by 76.19% S. Typhimurium and showed amplified PCR product of 620 bp (Fig. 4). However, Salmonella other than S. Typhimurium could not show amplification with this gene. The present finding concludes that the fliC gene is serovar specific.

In the present study, 43 (86.0%) out of 50 Salmonella isolates exhibited amplification (617 bp) with stn gene (Fig. 5). Our findings indicated that the stn gene is widely distributed among Salmonella irrespective of the serovars and source of isolation. However, some isolates could not exhibit the presence of stn gene which might be due to the mutational changes in the gene.

In the present study, 48 (96.0%) out of 50 Salmonella isolates, exhibited amplification (220 bp) with sopB gene (Fig. 6). It may be concluded that absence of sopB gene in some Salmonella isolates, the virulence function associated with enteritis and diarrhea might have been carried over by some other polymorphic Salmonella outer membrane proteins (sop) genes.

As per our findings, it may be concluded that emerging and multiple Salmonella serovars of zoonotic importance were recovered from varied sources that carrying different virulence genes may cause serious infections in animals as well as in human. Ultimately these serovars may pose great
one or more virulence genes. Where all the isolates obtained were found to possess either
the present study cannot be overlooked particularly in situations
animals, foods of animal origin and human in the area of
production. The recovery of Salmonella
risks to the health and production in animals and serious
health hazards in the human. Multi-virulent genes detected
in Salmonella serovars isolated from poultry and swine may
cause serious outbreaks of salmonellosis and losses to the
poultry and swine industries. Moreover, multi-virulent
Salmonella serovars isolated from cattle, buffalo, sheep and
goat may pose great risks to their health and loss of
production. The recovery of Salmonella organisms from
animals, foods of animal origin and human in the area of
present study cannot be overlooked particularly in situation
where all the isolates obtained were found to possess either
one or more virulence genes.

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laboratory identification of and anti-microbial susceptibility
testing of bacterial pathogens of public health importance
in the developing world.