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Molecular characterization of *Salmonella* serovars of zoonotic importance*

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Received: 19 July 2014; Accepted: 2 September 2014

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. *inv*A, *sip*A, *sef*A, *fli*C, *stn* and *sop*B were detected in *Salmonella* isolates using PCR-based molecular technique. Among these virulence genes, *inv*A gene was the most prevalent one as is present in 98% *Salmonella* isolates followed by *sop*B, *stn*, *sip*A, *fli*C and *sef*A 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 (*inv*A) gene in nearly all *Salmonella* irrespective of serovar or source. The other *Salmonella* enterotoxin (s*tn*) gene has been consistently shown to correctly identify all *Salmonella* Enterica (Prager *et al.* 1995). *Salmonella* outer protein (Sop) encoded by *sop*B gene increased the virulence of bacteria (Rahman 2006). Cortez *et al*. (2006) identified *Salmonella* serovars by a multiplex PCR targeting *inv*A, *pef*A and *sef*A genes for *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis, respectively. The flagellin C (*fli*C) 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)

*Part of Ph. D. thesis, submitted to the G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263 145.

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

PCR steps	PCR conditions	Virulence genes					
		*inv*A **F:**ACAGTGC TCGTTTAC GACCTGAAT **R:**AGACGA CTGGTACT GATCTAT	*sip*A **F:**TTCGA CTAACAG CAGCA **R:**CCGTC GTACCGG CTTTATTA	*sef*A **F:**GATACTG CTGAACGTA GAAGG **R:**GCGTAAA TCAGCATCT GCAGTAGC	*fli*C **F:**CGGTGTTG CCCAGGTTG GTAAT **R:**ACTGGTA AAGATGGCT	*stn* **F:**TTGTGT CGCTATCA CTGGCAACC **R:**ATTCGTAA CCCGCTCT CGTCC	*sop*B **F:**CGGACC GGCCAGC AACAAAA CAAGAAG AAG **R:**TAGTGA TGCCCGTT ATGCGTG AGTGTATT
Initial denaturation	Temperature (°C)	94	95	94	94	95	95
	Time (min)	1	5	5	5	3	3
Denaturation	Temperature (°C)	94	95	94	94	95	95
	Time (min)	0.5	0.5	0.5	0.5	1	1
Annealing	Temperature (°C)	60	55	55	57	57	57
	Time (min)	0.5	0.5	0.5	1	1	1
Extension	Temperature (°C)	72	72	72	72	72	72
	Time (min)	2	2	1	1	2	2
Final extension	Temperature (°C)	72	72	72	72	72	72
	Time (min)	10	5	7	10	10	10
Number of cycles		30	30	30	35	35	35

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 *inv*A gene fragment (Chiu and Ou 1996). The PCR thermal cycling conditions were applied for amplification of *inv*A 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. *inv*A, *sip*A, *sef*A, *fli*C, *stn* and *sop*B 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

different virulence genes, viz. *inv*A, *sip*A, *sef*A, *fli*C, *stn* and *sop*B in *Salmonella* isolates using PCR-based molecular technique. The *inv*A gene was most prevalent (98%) and present in all *Salmonella* isolates except *S*. Drogana isolated from human stool followed by *sop*B, *stn*, *sip*A, *fli*C and *sef*A genes in 96, 86, 78, 32 and 10% *Salmonella* isolates, respectively.

The *inv*A 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, *inv*A gene can be targeted for confirmation of *Salmonella* organisms. However, one isolate, *S*. Drogana, showed the absence of *inv*A gene.

Hepfelmeier *et al*. (2004) established that the *sip*A 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 *sip*A 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 *sip*A 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

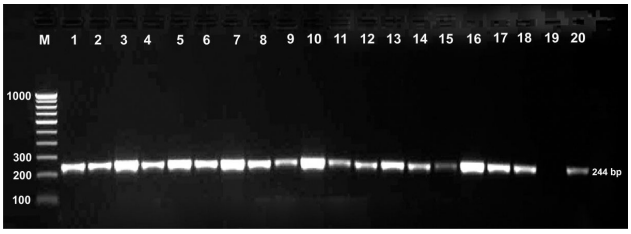

Fig. 1. Agarose gel electrophoresis showing amplified PCR products of *inv*A gene- lane M: 100 bp marker. Lane 1–18: *Salmonella* isolates; lane 19: negative control; lane 20: positive control (*Salmonella* Typhimurium).

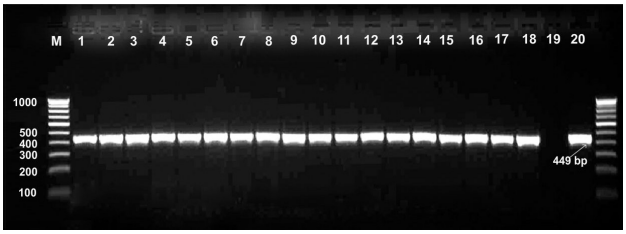

Fig. 2. Agarose gel electrophoresis showing amplified PCR products of *sip*A gene- lane M: 100 bp marker; lane 1–18: *Salmonella* isolates; lane 19: negative control; lane 20: positive control (*Salmonella* Typhimurium).

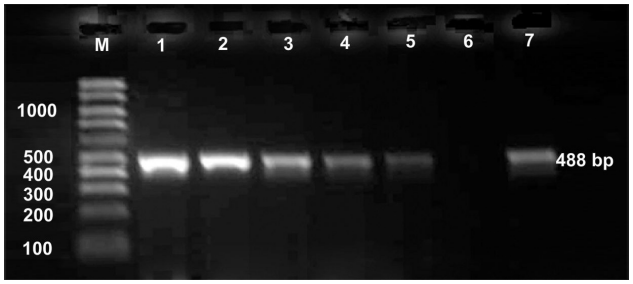

Fig. 3. Agarose gel electrophoresis showing amplified PCR products of *sef*A gene- lane M: 100bp marker; lane 1–5: *Salmonella* isolates; lane 6: negative control; lane 7: positive control (*Salmonella* Enteritidis).

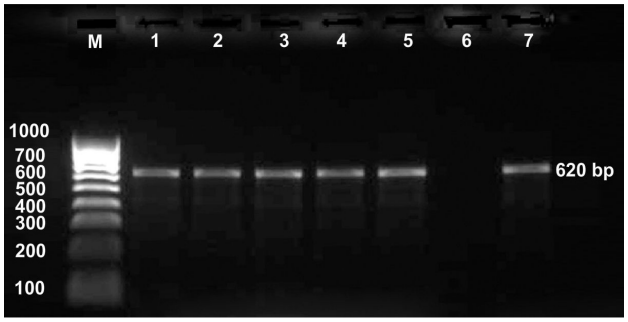

Fig. 4. Agarose gel electrophoresis showing amplified PCR products of *fli*C gene- lane M: 100bp marker; lane 1–5: *Salmonella* isolates; lane 6: negative control; lane 7: positive control (*Salmonella* Typhimurium).

*sef*A 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 *sef*A gene may not be the serovar specific.

Gene *fli*C 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 *fli*C 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 *sop*B gene (Fig. 6). It may be concluded that absence of *sop*B 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

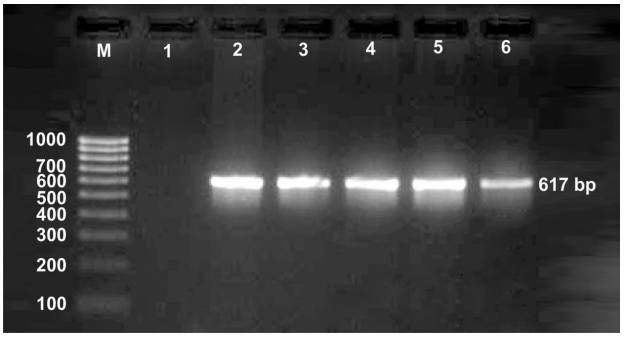

Fig. 5. Agarose gel electrophoresis showing amplified PCR products of *stn* gene. lane M: 100 bp marker. lane 1: negative control. lane 2: positive control (*Salmonella* Typhimurium). lane 3–6: *Salmonella* isolates.

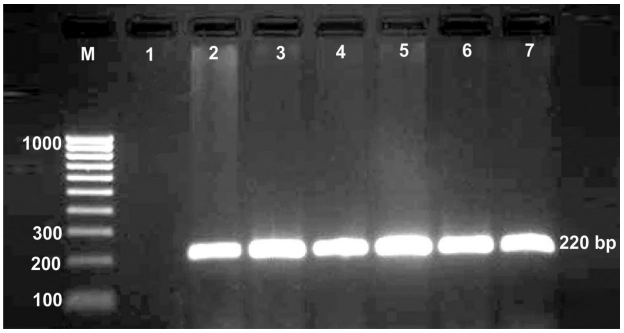

Fig. 6. Agarose gel electrophoresis showing amplified PCR products of *sop*B gene- lane M: 100 bp marker; lane 1: negative control; lane 2: positive control (*Salmonella* Typhimurium); lane 3–7: *Salmonella* isolates.

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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