



Multi-drug resistance in *Salmonella* serovars of zoonotic importance and detection of anti-microbial resistance genes*

CHANDRA SHEKHAR¹ and S P SINGH²

Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263 145 India

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ABSTRACT

Total 50 *Salmonella* isolates belonging to 10 different serovars comprising *Salmonella* 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 isolated from 1,132 different samples. Extreme variation of anti-microbial resistance in *Salmonella* isolates was observed in a range from 0–100%. Maximum resistance was observed against sulphamethizole and furazolidone (100% each) followed by kanamycin (50%), gentamicin (44%), nalidixic acid (14%), tobramycin (10%), amikacin, ampicillin, streptomycin and tetracycline (8% each), amoxicillin/clavulanic acid (6%), norfloxacin (4%), ciprofloxacin and cefotaxime (2% each) and chloramphenicol (0%). High level of sensitivity in *Salmonella* isolates was observed against chloramphenicol (100%) and tetracycline (92%). The anti-microbial resistance genes detected were *bla*_{TEM}, *tetA*, *aphAI*-LAB, *aadB* and *strA* in *Salmonella* serovars that showed resistance against ampicillin, tetracycline, kanamycin, gentamicin and streptomycin, respectively. The *bla*_{TEM} gene was present in all ampicillin resistant *Salmonella* isolates (100%) and *strA* gene in all streptomycin resistant *Salmonella* isolates (100%) followed by *tetA* gene in 75% tetracycline resistant *Salmonella* isolates, *aadB* gene in 14.28% gentamicin resistant *Salmonella* isolates and *aphAI*-LAB gene in 8% kanamycin resistant *Salmonella* isolates. All *Salmonella* isolates (100%) were multi-drug resistant (MDR), indicating injudicious and extensive use of anti-microbial agents in these areas that may pose great risks to animal health and production. Moreover, MDR *Salmonella* serovars may pose great public health problems through consumption of foods of animal origin. Multi-drug resistance genes exhibited by all *Salmonella* serovars also indicated serious threats to the animal health and production including great risks to the public health.

Key words: Animal faecal samples, Anti-microbial resistance genes, Foods of animal origin, Human stool, Multi-drug resistance, *Salmonella* serovars

The infections caused by *Salmonella* serovars are implicated as important public health problems world-wide. Foods of animal origin were identified as vehicles for transmission of *Salmonella* to human beings. Use of anti-microbial drugs in the animals for prophylaxis, treatment and growth promotion, has resulted in their exposure, irrespective of their health, to frequently sub-therapeutic concentrations of anti-microbials. Resistance to combination of several classes of anti-microbial agents has led to the emergence of multidrug-resistant strains that may pass from food animals to humans (O'Brien 2002). Resistance to 2 or more classes of drugs (MDR phenotype) is becoming increasingly widespread in *Salmonella* spp.

Multidrug-resistant (MDR) strains of *Salmonella* are now encountered frequently and rates of multidrug-resistance have increased considerably in recent years. Keeping in view the importance of *Salmonella* organism, present study was undertaken to isolate and identify *Salmonella* serovars from varied sources to know the resistance pattern as well as anti-microbial resistance genes of *Salmonella* serovars of zoonotic importance.

MATERIALS AND METHODS

Total 1,132 samples 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 aseptically from Pantnagar and nearby areas and processed for isolation of salmonellae using conventional culture method.

Isolation of *Salmonella* from human and animal faecal samples was carried out as per WHO/CDC (2003). Isolation of non-typhoidal *Salmonella* was done as per USDA/FSIS (2002) and of typhoidal *Salmonella* from meat and eggs

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Present address: ¹Associate Professor (cshekharyph@gmail.com), Department of Veterinary Public Health and Epidemiology. ²Head, Department of Veterinary Public Health and Epidemiology, College of Veterinary and Animal Sciences, N. D. University of Agriculture and Technology, Kumarganj, Faizabad.

Table 1. Primer profile of targeted anti-microbial resistance genes

Target gene	Resistance	Resistance mechanism	Primer sequence (5' to 3')	Amplicon/product size (bp)	Reference
<i>tetA</i>	Tetracycline	Efflux	F:GCTACATCCTGCTTGCCTTC R:CATAGATCGCCGTGAAGAGG	210	Fonseca <i>et al.</i> (2006)
<i>bla</i> _{TEM}	Ampicillin	β-Lactamase	F:CATTTCCTGTCGCCCCTTAT R:TCCATAGTTGCCTGACTCCC	794	Randall <i>et al.</i> (2004)
<i>aphAI</i> -LAB	Kanamycin	Aminoglycoside phosphoryltransferase	F:AAACGTCTTGCTCGAGGC R:CAAACCGTTATTTCATTCGTGA	461	Randall <i>et al.</i> (2004)
<i>aadB</i>	Gentamicin	Aminoglycoside adenylyltransferase	F:GAGCGAAATCTGCCGCTCTGG R:CTGTTACAACGGACTGGCCGC	320	Randall <i>et al.</i> (2004)
<i>strA</i>	Streptomycin	Aminoglycoside phosphoryltransferase	F:AGCAGAGCGCGCCTTCGCTC R:CAAAGCCCACCTTCACCGAC	704	Randall <i>et al.</i> (2004)

Table 2. PCR optimization conditions applied for different anti-microbial resistance genes

PCR steps	PCR conditions	Anti-microbial resistance genes				
		<i>bla</i> _{TEM}	<i>tetA</i>	<i>aphAI</i> -LAB	<i>aadB</i>	<i>strA</i>
Initial denaturation	Temperature (°C)	95	95	95	95	95
	Time (min)	5	5	5	5	5
Denaturation	Temperature (°C)	95	95	95	95	95
	Time (min)	1	1	1	1	1
Annealing	Temperature (°C)	55	55	55	61	59
	Time (min)	0.5	0.5	0.5	0.5	0.5
Extension	Temperature (°C)	72	72	72	72	72
	Time (min)	0.5	0.5	0.5	0.5	0.5
Final extension	Temperature (°C)	72	72	72	72	72
	Time (min)	10	10	10	10	10
Number of cycles		40	40	40	40	40

according to the manual of WHO/CDC (2003). *Salmonella* identified on the basis of cultural, morphological and biochemical reactions were subjected to agglutination test using O antiserum poly A-I and Vi. *Salmonella* showing positive agglutination test were sent for serotyping to National *Salmonella* Centre, Division of Bacteriology and Mycology, IVRI, India.

Anti-microbial susceptibility testing of *Salmonella* by disc diffusion on Mueller-Hinton agar was performed (CLSI 2011). Anti-microbial discs used were ampicillin (10 mcg), amoxycillin/clavulanic acid (20/10 that is 30 mcg), amikacin (30 mcg), norfloxacin (10 mcg), ciprofloxacin (5 mcg), cefotaxime (30 mcg), gentamicin (10 mcg), kanamycin (30 mcg), streptomycin (10 mcg), tobramycin ((10 mcg), tetracycline (30 mcg), chloramphenicol (30 mcg), nalidixic acid (30 mcg), furazolidone (50 mcg) and sulphonamethizole (300 mcg).

Plasmid DNA was isolated from drug resistant *Salmonella* by alkaline lysis with SDS as per Sambrook

and Russell (2001). Anti-microbial resistance genes were detected in anti-microbial resistant *Salmonella* serovars using PCR technique following Gebreyes and Altier (2002). Different PCR thermal cycling conditions were applied for amplification of different anti-microbial resistance genes (Table 2).

RESULTS AND DISCUSSION

Salmonella organisms were isolated from various samples using multiple selective enrichment as well as selective plating media to ensure better recovery of this organism. In the present study, Tetrathionate broth was found the most suitable selective enrichment medium for isolation of salmonellae from foods of animal origin and autopsied poultry tissues, while Rappaport-Vassiliadis broth and Selenite F broth were found suitable for isolation of salmonellae from faecal samples. BGA was found the most appropriate for isolation of salmonellae from foods of animal origin as well as autopsied poultry tissue samples,

while HEA and XLD agar were found more suitable for isolation of salmonellae from faecal/stool samples.

In the present study, total 50 *Salmonella* isolates belonging to 10 different serovars comprising *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. The multiple 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 single sample of 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*). Therefore, it may be assumed that multiple serovars of *Salmonella* recovered from 6 different single samples might have involved in the complication of pathogenesis in the animals from which the samples were collected. Moreover, in the present study, *S. Drogana* was recovered from human stool sample. As per reports available so far, this serovar appears to be the first isolate from human source in India.

In the present study, anti-microbial resistance ranged from 0–100%, showing extreme variation in the sensitivity of *Salmonella* isolates. Maximum resistance was observed against sulphamethizole and furazolidone (100% each) followed by kanamycin (50%), gentamicin (44%), nalidixic acid (14%), tobramycin (10%), amikacin, ampicillin, streptomycin and tetracycline (8% each), amoxicillin/clavulanic acid (6%), norfloxacin (4%), ciprofloxacin and cefotaxime (2% each) and chloramphenicol (0%). However, Musgrove *et al.* (2006) observed resistance in *Salmonella* isolates against tetracycline (63.4%), nalidixic acid (63.4%), and streptomycin (61.0%). Comparatively higher resistance was reported by above researchers which might be due to frequent exposure as well as sub-therapeutic doses of these anti-microbial agents used for prevention or control of infection. Moreover, resistance level also varies according to change in time and geographical areas.

A high level of sensitivity in *Salmonella* isolates was observed against chloramphenicol (100%) and tetracycline (92%). The high level of sensitivity in *Salmonella* isolates against these antibiotics could be attributed to the fact that over a long period of time, these antibiotics have not been in use due to availability of new classes of anti-microbial agents.

In the present study, all *Salmonella* isolates (100%) exhibited resistance against 2 or more anti-microbial agents. The prevalence of such a large number of MDR *Salmonella* isolates indicates injudicious and extensive use of anti-microbial agents in these areas showing great risk to the animal health and production as well as public health. Mammina *et al.* (2002) observed that 52% of all *S. Typhimurium* were multiple resistant to chloramphenicol, tetracycline, sulphonamides and β -lactamases. Carraminana

et al. (2004) observed multiple resistance in 65% *Salmonella* isolates against tetracycline, neomycin and streptomycin. As per the findings of these researchers, it may be concluded that there were wide variations in the prevalence of multi-drug resistance in the *Salmonella* which might be due to use of anti-microbial agents in different frequencies in different geographical areas. Moreover, use of anti-microbial agents in sub-therapeutic doses also results in the development of multi-drug resistance in organisms.

Anti-microbial resistance genes detected were *bla*_{TEM}, *tetA*, *aphAI*-LAB, *aadB* and *strA* in *Salmonella* serovars showing resistance against ampicillin, tetracycline, kanamycin, gentamicin and streptomycin, respectively. The *bla*_{TEM} gene was present in all ampicillin resistant *Salmonella* isolates (100%) and *strA* gene in all streptomycin resistant *Salmonella* isolates (100%) followed by *tetA* gene in 75% tetracycline resistant *Salmonella* isolates, *aadB* gene in 14.28% gentamicin resistant *Salmonella* isolates and *aphAI*-LAB gene in 8% kanamycin resistant *Salmonella* isolates.

Salmonella serovars exhibited amplification (794 bp) with primers targeting *bla*_{TEM} gene (Fig. 1). This gene was present in all 4 ampicillin resistant *Salmonella* serovars (100%) belonging to serotype *S. Typhimurium*, *S. Lagos* and *S. Drogana*. A higher prevalence of this gene was also reported by Gebreyes and Altier (2002) in which they recorded the presence of this gene in all *Salmonella* isolates belonging to serovar *S. Typhimurium*. However, Lynne *et al.* (2008) reported that out of 19 isolates (*S. Newport*), 5 isolates were positive for *bla*_{TEM} gene. The function of anti-microbial resistance against ampicillin in *Salmonella* isolates negative for *bla*_{TEM} gene might have been carried over by some other genes such as *bla*_{CMY} or *blapsE-i* genes.

Salmonella serovars exhibited amplification (210 bp) with primers targeting *tetA* gene (Fig. 2). This gene was present in 3 out of 4 tetracycline resistant *Salmonella* serovars (75%) belonging to *S. Typhimurium* and *S. Lagos*, while 1 isolate (*S. Typhimurium*) did not exhibit the presence of this gene. Almost similar findings in a study in Italy were recorded by Pezzella *et al.* (2004) where, prevalence of *tetA* gene was 68%. The function of anti-

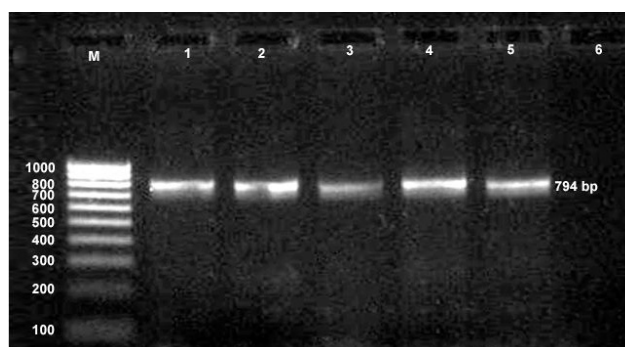


Fig. 1. Agarose gel electrophoresis showing amplified PCR products of *bla*_{TEM} gene- lane M: 100 bp marker; lane 1–4: *Salmonella* isolates; lane 5: positive control (ampicillin resistant *Salmonella* containing *bla*_{TEM} gene); lane 6: negative control.



Fig. 2. Agarose gel electrophoresis showing amplified PCR products of *tetA* gene- lane M: 100 bp marker; lane 1: negative control; Lane 2–4: *Salmonella* isolates; lane 5: positive control (tetracycline resistant *Salmonella* containing *tetA* gene).

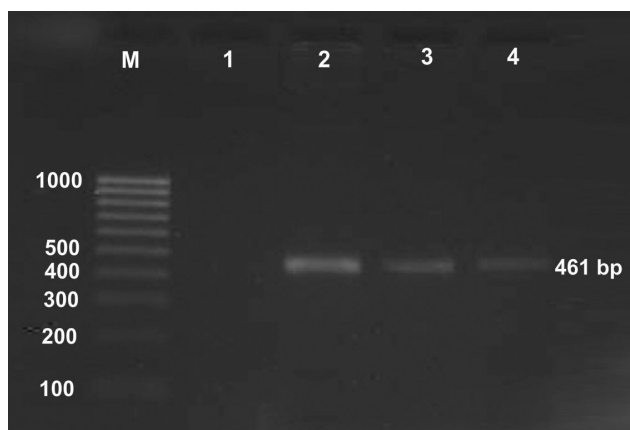


Fig. 3. Agarose gel electrophoresis showing amplified PCR products of *aphAI*-LAB gene- lane M: 100 bp marker. lane 1: negative control. lane 2–3: *Salmonella* isolates. lane 4: positive control (kanamycin resistant *Salmonella* containing *aphAI*-LAB gene).

microbial resistance against tetracycline in *Salmonella* isolates negative for *tetA* gene might have been carried over by some other genes such as *tetB*, *tetC* or *tetG* genes.

Salmonella serovars exhibited amplification (461 bp) with primers targeting *aphAI*-LAB gene (Fig. 3). The *aphAI*-LAB gene was present only in 2 of 25 kanamycin resistant *Salmonella* serovars (8%) belonging to serovars *S. Typhimurium* and *S. Ughelli* while, 23 isolates belonging to many serovars which did not exhibit the presence of this gene. Randall *et al.* (2004) reported that 1 of 14 kanamycin resistant *Salmonella* strain did not contain *aphAI*-LAB gene. In the present study, however, the lower prevalence of *aphAI*-LAB gene was observed, which indicated that the kanamycin resistance might have been conferred by the genes other than *aphAI*-LAB gene.

Salmonella serovars exhibited amplification (320 bp) with primers targeting *aadB* gene (Fig. 4). This gene was present in 3 out of 21 gentamicin resistant *Salmonella* serovars (14.28%) belonging to serovars *S. Typhimurium* and *S. Weltevreden* while, 18 isolates belonging to other serovars did not amplify with this gene. However, Randall *et al.* (2004) reported that 5 of 7 gentamicin resistant *S. Newport* contained *aacC* gene, while none of these



Fig. 4. Agarose gel electrophoresis showing amplified PCR products of *aadB* gene- lane M: 100 bp marker; lane 1: positive control (gentamicin resistant *Salmonella* containing *aadB* gene); lane 2–4: *Salmonella* isolates.



Fig. 5. Agarose gel electrophoresis showing amplified PCR products of *strA* gene- lane M: 100 bp marker; lane 1: negative control; lane 2: positive control (streptomycin resistant *Salmonella* containing *strA* gene); lane 3–5: *Salmonella* isolates.

possessed *aadB* gene. In the present study, lower prevalence of *aadB* gene was observed, which indicates gentamicin resistance might have been conferred by some other genes such as *aacC* or *aadC* genes.

Salmonella serovars exhibited amplification (704 bp) with primers targeting *strA* gene (Fig. 5). This gene was present in all 3 streptomycin resistant *Salmonella* serovars (100%) belonging to *S. Typhimurium* and *S. Lagos*, which is in accordance to the findings of Gebreyes and Altier (2002) as they also reported the presence of this gene in all *Salmonella* isolates belonging to *S. Typhimurium* serovar. However, Lynne *et al.* (2008) demonstrated resistance to streptomycin in *Salmonella* isolates and found that 91.3% isolates exhibited the presence of *strA* gene. The function of anti-microbial resistance against streptomycin in *Salmonella* isolates negative for *strA* gene might have been conferred by some other genes such as *strB*, *strAB*, *aadA1* or *aadA2* genes.

As per our findings it may be concluded that emerging *Salmonella* serovars, viz. *S. Elisabethville*, *S. Essen*, *S. Lagos*, *S. Ughelli* and *S. Drogana* were first time recovered in Pantnagar and nearby areas. Multiple serovars of *Salmonella* were recovered from 6 different samples which might have been involved in complication of the pathogenesis. *S. Drogana* was recovered from human stool

sample which is first isolate from human source in India. All *Salmonella* isolates were found multi-drug resistant (MDR), indicating injudicious and extensive use of anti-microbial agents in these areas, which may pose great public health problems. Moreover, high level of sensitivity in *Salmonella* isolates was observed against chloramphenicol (100%) and tetracycline (92%) indicating infrequent use of these antibiotics since last few years in these areas. The present study also indicated a wide variation in the level of anti-microbial resistance as well as prevalence of anti-microbial resistance genes.

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