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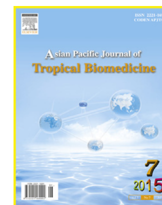

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Isolation, serotype diversity and antibiogram of *Salmonella enterica* isolated from different species of poultry in India

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ABSTRACT

Objective: To study the occurrence and serotype diversity of *Salmonella* isolates in different species of poultry (chicken, emu and duck) and determine their resistance pattern against various antibiotics of different classes.

Methods: About 507 samples comprising 202 caecal contents and 305 fecal samples from chicken, emu and duck were processed for isolation of *Salmonella enterica*. Salmonellae were isolated and detected by standard protocol of ISO 6579 Amendment 1: Annex D. Genetic confirmation was also made by using *16S rRNA* genus specific PCR. Serotype specific PCR was also done to detect the most common serovars *viz. Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Gallinarum. All obtained isolates were subjected to a set of 25 antibiotics to study their antibiogram by using Baeur–Kirby disk diffusion method.

Results: Out of 507 samples processed, 32 isolates of *Salmonella enterica* (18 from caecal contents and 14 from faecal samples) were obtained, of which 24 belonged to 6 different serovars, 6 were untypeable and 2 were rough strains. *Salmonella* Enteritidis was the most predominant serotype (9), followed by *Salmonella* Typhimurium (5), *Salmonella* Virchow (4), *Salmonella* Gallinarum (3), *Salmonella* Reading (2) and *Salmonella* Altona (1). Antibiotic resistance pattern was maximum (100%) to oxacillin, penicillin and clindamycin, followed by ampicillin (68.75%), tetracycline (65.62%), nalidixic acid (56.25%) and colistin (46.87%). High sensitivity of isolates was recorded for chloramphenicol (96.87%) followed by meropenem (84.37%).

Conclusions: Occurrence of high proportion of serovars in our study which can cause serious gastroenteritis in humans is a matter of concern. *Salmonella* Altona has been detected for the first time in India from poultry. This serotype is known to cause serious outbreaks of gastroenteritis in humans. Multidrug resistant isolates were recovered at high percentage which can be attributed to non-judicious use of antibiotics both in prophylaxis and treatment regimen. This observation draws serious attention as poultry serves as an important source of transmission of these multidrug resistant *Salmonella* serovars to humans.

1. Introduction

Salmonellosis is one of the important bacterial diseases which affect diverse number of hosts worldwide [1]. Poultry are the important reservoir of many zoonotically important pathogens, of which *Salmonella* is of prime importance [2]. Salmonellosis in poultry is an important area of study as it not only affects the poultry industry but can also occur in humans by consumption of contaminated poultry meat and eggs [3]. Poultry comprises a number of species which include chickens, ducks and emus. Salmonellosis has been endemic in poultry industry of India [4]. Several researchers have reported variable prevalence rates of *Salmonella* infection in different parts of India [5,6]. Diverse number of serovars of *Salmonella* has been reported from poultry worldwide. More than 53 serovars have been reported from India and this number is on ever increasing [7]. Various serovars like *Salmonella* Enteritidis (*S.* Enteritidis), *Salmonella* Typhimurium (*S.*

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Typhimurium), *Salmonella* Virchow (*S.* Virchow) and *Salmonella* Newport are important nontyphoidal causes of human salmonellosis caused by consumption of contaminated poultry products. *Salmonella* Gallinarum (*S.* Gallinarum) and *Salmonella* Pullorum are the only two host-specific true pathogens of poultry birds and they affect the poultry industry to a great extent resulting in huge economic losses in terms of morbidity and mortality. Isolation and identification of *Salmonella* are very tedious and take several days before coming to the final conclusion. There has been great demand in terms of quick and sensitive detection of *Salmonella* from poultry in order to take timely therapeutic and prophylactic measures. Several PCR-based assays have been developed for rapid detection of *Salmonella* sp. [8,9]. Various serotype-specific PCR have also been developed for some common serovars to reduce time and cost in processing isolates by conventional serotyping which is very much labor intensive and time-consuming [10].

In the past few decades, emergence of antibiotic resistance among different species of bacteria was on the rise [11]. This problem poses great threat to public health in case of zoonotically important bacteria transmitted from food animals. In this context, contaminated poultry products serve as an important threat to public health as it is an important reservoir of salmonellae. Irrational use of antibiotics as growth promoters in poultry is an important factor that has favored the selection of resistant bacteria in fecal microflora of poultry [12].These resistant strains are easily passed to human through food chains resulting in serious consequences in terms of treatment failure and rapid outbreaks of resistant salmonellae.

The present study was conducted to detect and determine the diversity of various serovars prevalent in poultry birds and associated public health risk in various regions of Rajasthan, India. The work will also help to know the status of antibiotic resistance pattern among various *Salmonella* isolates so as to aid in suggesting proper and effective therapeutic measures.

2. Materials and methods

2.1. Sampling

A total of 507 samples comprising 305 fecal samples and 202 caecal contents from different species of poultry (Table 1) were collected from March 2013 to August 2014. Freshly voided fecal samples were collected in sufficient amount in sterile test tubes by cotton swabs while caecal contents were taken from various slaughtered birds and transferred to laboratory as soon as possible on ice.

2.2. Isolation

Samples were homogenized in sterile phosphate buffer solution (pH 7.2) by stirrer to avoid contamination. Homogenized samples were centrifuged at 1500 r/min for 15 min to settle the coarse fecal matter. Supernatant was taken in fresh sterile tube to process according to guidelines of standard revised protocol for *Salmonella* isolation ISO 6579 Amendment 1: Annex D [13]. However, due to the limitation of this protocol in detection of only motile serovars, we also processed samples in less inhibitory selective broth of selenite cystine for recovery of nonmotile serovars. The protocol involved initial enrichment of supernatant in buffered peptone water (1:10) for 16 h at 37 °C. Three drops of each pre-enriched samples were placed separately on modified semi solid Rappaport-Vassiliadis (MSRV) agar and incubated at 41.5 °C for 24 h. After incubation, plates were observed for production of grey-white, turbid zone extending from point of inoculation (Figure 1). A loopful of culture was taken from the border of the opaque zone formed on MSRV and streaked on xylose lysine deoxycholate agar and Hektoen enteric agar. Plates were incubated at 37 °C for 24 h and observed for typical colonies of *Salmonella*. For detection of nonmotile *Salmonella*, pre-enriched samples were inoculated in selenite cystine broth and incubated at 37 °C for 24 h. Selective plating was done similarly to above. All suspected colonies were purified and preserved on nutrient agar slants.

Table 1
Detail of samples collected from different poultry species.

Type of samples	Poultry species			Total
	Chicken	Duck	Emu	
Fecal samples	232	38	35	305
Caecal contents	202	–	–	202
Total	434	38	35	507

2.3. Biochemical characterisation

All suspected colonies were subjected to different biochemical tests by HiSalmonella™ identification kit (Himedia, Mumbai, India). The kit contained 12 biochemical tests *viz.* methyl red, Voges–Proskauer, urease, hydrogen sulphide production, citrate utilization, lysine, o-nitrophenyl β-galactoside, lactose, arabinose, maltose, sorbitol and dulcitol. Also, isolates were inoculated in triple sugar iron agar slants to observe the triple sugar iron reaction.

2.4. Latex agglutination test

All suspected colonies were subjected to polyvalent latex agglutination test for preliminary identification by using

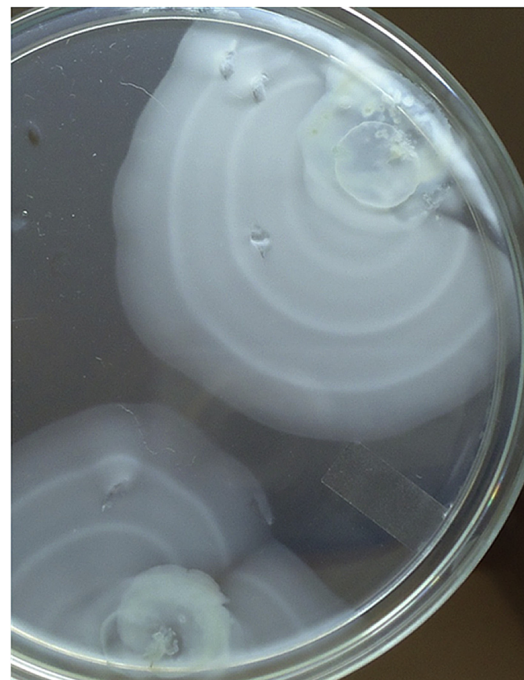

Figure 1. Grey-white, turbid opaque zone growth of tentatively positive sample of *Salmonella* sp. extending from point of inoculation on MSRV medium.

HiSalmonella™ latex agglutination kit (Himedia, Mumbai, India) according to manufacturer's instructions.

2.5. 16S rRNA gene specific PCR, serotyping and serotype-specific PCR

Primers targeting genus specific region of *16S rRNA* gene of *Salmonella enterica* (*S. enterica*) were used following protocol of Lin and Tsen [14]. All the isolates of *Salmonella* were referred to the National Centre on Serotyping of *Salmonella*, Indian Veterinary Research Institute, Uttar Pradesh, India, for final confirmation and serotyping. Serotype-specific PCR was also used for specific identification of *S.* Enteritidis and *S.* Typhimurium by following protocol of Alvarez *et al*. [15] while *S.* Gallinarum was detected by using allele-specific PCR developed by Shah *et al*. [16] with little modifications. The PCR conditions of *S.* Enteritidis and *S.* Typhimurium consisted of initial denaturation at 94 °C for 2 min, followed by 30 cycles at 95 °C for 1 min, 57 °C for 1 min and 72 °C for 2 min. The final extension was carried out at 72 °C for 5 min. The *S.* Gallinarum cycling conditions were at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min followed by final extension at 72 °C for 5 min. The amplified products were analyzed by electrophoresis on 1.5% (w/v) agarose gel and visualized in UV transilluminator. The primer sequences used in this study are given in Table 2.

2.6. Antibiogram

All confirmed isolates were subjected to *in vitro* antibiotic susceptibility testing against 25 antibiotics of different classes. Disk diffusion method of Bauer and Kirby was used following the guidelines of Clinical and Laboratory Standards Institute [17,18]. Antibiotics used in the study included oxacillin, cotrimoxazole, cefuroxime, penicillin, chloramphenicol, gemifloxacin, levofloxacin, colistin, nalidixic acid, ampicillin, trimethoprim, cephazolin, clindamycin, ciprofloxacin, cefotaxime, tetracycline, kanamycin, ticarcillin, meropenem, ceftriaxone/sulbactam, aztreonam, amikacin, piperacillin, gentamicin and cefepime. All antibiotic disks were procured from Himedia laboratories (Mumbai, India).

Table 2
List of primers used in the study.

Target genes	Primer sequence (5′→3′)
16S rRNA	TGT TGT GGT TAA TAA CCG CA
	CAC AAA TCC ATC TCT GGA
Enteritidis	TGT GTT TTA TCT GAT GCA AGA GG
	TGA ACT ACG TTC GTT CTT CTG G
Typhimurium	TTG TTC ACT TTT TAC CCC TGA A
	CCC TGA CAG CCG TTA GAT ATT
Gallinarum	GTA TGG TTA TTA GAC GTT GTT
	TAT TCA CGA ATT GAATA CTC

3. Results

Out of 507 samples processed, 32 samples were found positive for *S. enterica*. Among 32 isolates, 18 were recovered from caecal contents and 14 from fecal samples. Out of 32 isolates, 24 belonged to six different serovars while 6 were untypable and 2 rough strains. Serotyping report showed that 9 isolates belonged to *S.* Enteritidis, 5 of *S.* Typhimurium, 4 of *S.* Virchow, 3 of *S.* Gallinarum, 2 of *Salmonella* Reading (*S.* Reading) and 1 isolate belonged to *Salmonella* Altona (*S.* Altona). All the isolates showed agglutination reaction in latex agglutination test except rough and *S.* Gallinarum strains (Figure 2).

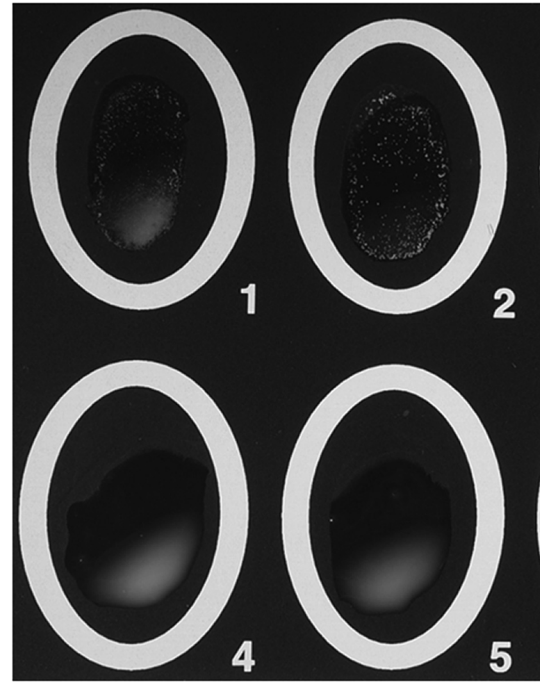

Figure 2. Agglutination reaction in latex agglutination test. Samples 1 and 2: Positive reaction; Samples 4 and 5: Negative reaction.

The detailed distribution of isolates from different poultry species is presented in Table 3. The biochemical reactions showed similar results for all serovars except for *S.* Gallinarum which was negative for citrate production and *S.* Virchow isolates which were exclusively negative for dulcitol fermentation. Besides, there was considerable variability in citrate utilization, sugar fermentation and hydrogen sulphide production in untypable strains. Also, *S.* Gallinarum isolates were found weakly positive for production of hydrogen sulphide and it was observed that rough strains produced initially little amount of hydrogen sulphide after 24 h of incubation which increased considerably after 48 h. All the isolates showed alkaline slant, acidic butt with blackish discolouration, pale colonies on MacConkey's agar and pink round with black centered colonies on

Table 3
Details of different serotypes of *S. enterica* obtained from different species of poultry.

Serotypes	Antigenic formula	Isolates (*n*)			Total
		Chicken	Duck	Emu	
S. Enteritidis	1,9,12:g,m:[1,7]	9	–	–	9
Untypeable	–	4	1	1	6
S. Typhimurium	4,5,12: i:1,2	4	1	–	5
S. Virchow	6,7:r:1,2	4	–	–	4
S. Gallinarum	9,12:-:-	3	–	–	3
S. Reading	1,4, [5], 12:eh:1,5	2	–	–	2
Rough	–	2	–	–	2
S. Altona	8, 20:r(i):z6	1	–	–	1
Total		29	2	1	32

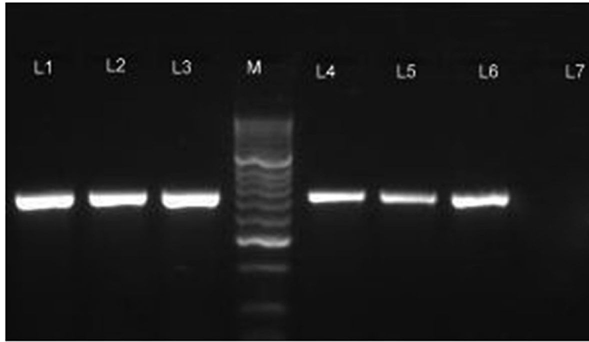

Figure 3. PCR amplified product (574 bp) of *S. enterica* isolates on 1% agarose gel.
L1-L6: Positive sample; Lane 7: Negative control. M: 100 base pair DNA ladder.

Table 4
Results of biochemical reactions of various serotypes of *S. enterica*.

Biochemical test	*S.* Ent.	*S.* Typ.	*S.* Vir.	*S.* Gal.	*S.* Read.	Untypable	Rough
Methyl red	+	+	+	+	+	+	+
Voges proskauer	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–
Hydrogen sulphide	+	+	+	Weakly positive	+	V	Late positive
Citrate utilisation	+	+	+	–	+	V	+
Lysine	+	+	+	+	+	+	+
O-nitrophenyl β-galactoside	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	–
Arabinose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	V	+
Dulcitol	+	+	–	+	+	V	+

S. Ent.: *S.* Enteritidis; *S.* Typ.: *S.* Typhimurium; *S.* Vir.: *S.* Virchow; *S.* Gal.: *S.* Gallinarum; *S.* Read.: *S.* Reading. V: Variable; +: Positive for test; –: Negative for test.

xylose lysine deoxycholate agar. The detailed results of various biochemical reactions of different serovars of *S. enterica* are presented in Table 4. All isolates amplified 574 bp product in *16S rDNA* genus specific PCR (Figure 3). In serotype specific PCR, *S. Enteritidis*, *S.* Typhimurium and *S.* Gallinarum showed amplified products of 304, 401 and 187 bp respectively (Figure 4).

In vitro antibiotic susceptibility assay showed that all isolates were resistant to oxacillin, penicillin and clindamycin followed by ampicillin, tetracycline while majority were sensitive to

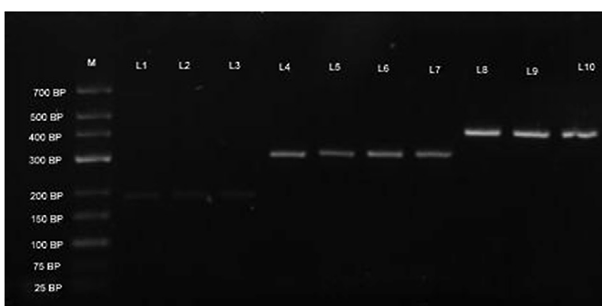

Figure 4. Amplified product of serotype-specific PCR.
M: Biolit ProxiO Low DNA ladder; L1-L3: 187 bp PCR amplified product specific of *S.* Gallinarum; L4-L7: 304 bp PCR amplified product of *S.* Enteritidis; L8-L10: 401 bp PCR amplified product of *S.* Typhimurium.

Table 5
Antibiogram results of *S. enterica* isolates (Total isolates = 32).

Antibiotic	Resistant [*n* (%)]	Sensitive [*n* (%)]	Intermediate [*n* (%)]
Oxacillin	32 (100.00)	0 (0.00)	0 (0.00)
Cotrimoxazole	6 (18.75)	24 (75.00)	2 (6.25)
Cefuroxime	14 (43.75)	13 (40.60)	5 (15.62)
Penicillin	32 (100.00)	0 (0.00)	0 (0.00)
Chloramphenicol	0 (0.00)	31 (96.87)	1 (3.12)
Gemifloxacin	10 (31.25)	17 (53.12)	5 (15.62)
Levofloxacin	3 (9.37)	24 (75.00)	5 (15.62)
Colistin	15 (46.87)	17 (53.12)	0 (0.00)
Nalidixic acid	18 (56.25)	9 (28.12)	5 (15.62)
Ampicillin	22 (68.75)	5 (15.62)	5 (15.62)
Trimethoprim	8 (25.00)	18 (56.25)	6 (18.75)
Cephazolin	9 (28.12)	19 (59.37)	4 (12.50)
Clindamycin	32 (100.00)	0 (0.00)	0 (0.00)
Ciprofloxacin	5 (15.62)	15 (46.87)	12 (37.50)
Cefotaxime	6 (18.75)	12 (37.50)	14 (43.75)
Tetracycline	21 (65.62)	5 (15.62)	6 (18.75)
Kanamycin	13 (40.62)	13 (40.62)	6 (18.75)
Ticarcillin	12 (37.50)	13 (40.62)	7 (21.87)
Meropenem	3 (9.37)	27 (84.37)	2 (6.25)
Ceftriaxone/Sulbactam	3 (9.37)	24 (75.00)	5 (15.62)
Aztreonam	4 (12.50)	17 (53.12)	11 (34.37)
Amikacin	12 (37.50)	12 (37.50)	8 (25.00)
Piperacillin	18 (56.25)	6 (18.75)	8 (25.00)
Gentamicin	6 (18.75)	14 (43.75)	12 (37.50)
Cefepime	4 (12.50)	20 (62.50)	8 (25.00)

chloramphenicol followed by meropenem, cotrimoxazole, levofloxacin and ceftriaxone/sulbactam. Susceptibility to other antimicrobials was variable and is given in Table 5.

4. Discussion

Salmonellosis is one of the major bacterial diseases transmitted from food animals. Every year, millions of salmonellosis cases are reported worldwide [19]. In US alone, salmonellosis is one of the most common diseases among food-borne diseases accounting for 800 000 to 4 000 000 human infections annually [20]. *Salmonella* not only poses serious threat to public health but also causes huge economic losses by generating mortality and morbidity to poultry industry. Monitoring and control are two important aspects to reduce the prevalence at farm level of this zoonotic disease. In the present study, a prevalence rate of 6.31% was recorded which is very similar to the findings of Mir *et al.* who reported an overall prevalence of 6.88% in Kashmir Valley, India [21]. However, the prevalence rate was lower than that in other studies conducted in other parts of India [22,23]. This could be due to bias in sample taking in their studies while we collected samples randomly rather than sampling from only suspected ill birds. It is worth to mention here that the success of detection depends not only on choice of sampling but also on the sensitivity of culture method. Besides, intermittent shedding and non-uniform distribution in poultry houses may also be responsible for variability in results [24]. Therefore, there are always possibilities for the high variability in results of detection rates by different workers. The present study used ISO 6539 Annex D protocol and found it highly accurate and specific in detection without any false positives. Many false positives were encountered while nonmotile sensitive serovars were isolated by direct enrichment in selenite cystine broth. This may be either due to the development of resistance against inhibitory

effect of selenite by competing bacteria or more contaminated samples. This draws attention in improving method for better isolation of sensitive serovars without extra workload in processing false positives [25].

The serotyping results showed that majority of isolates belonged to *S*. Enteritidis. Infections due to *S*. Enteritidis have been a major cause of food-borne salmonellosis over the last few decades worldwide [26,27]. However, few reports are available of human infections in India due to *S*. Enteritidis [28]. Yet, the high occurrence of *S*. Enteritidis in our study has raised a serious public health concern and needs strict monitoring and surveillance. Similar observations had been reported by Suresh *et al*. who recovered *S*. Enteritidis in high proportion compared to other serovars from various poultry products [29]. The other serovars isolated in the study of *S*. Typhimurium, *S*. Virchow, *S*. Reading and *S*. Altona have also been implicated in non-typhoidal salmonellosis and have important public health significance. *S*. Reading and *S*. Altona have been associated with several sporadic outbreaks of food-borne salmonellosis in humans [30,31]. To the best of author's knowledge and records available in literature, *S*. Altona has not been reported earlier in India. *S*. Gallinarum, a host-adapted serotype, was only recovered from caecal contents. This observation suggests that the serotype is poorly shed in fecal matter compared to other serotypes. *S*. Gallinarum has been responsible for severe economic losses in terms of morbidity and mortality. Majority of the serotypes recovered in the study are capable to cause serious gastroenteritis in humans except for *S*. Gallinarum [32]. Poultry act as an important source in transmission of various zoonotically important serotypes of *Salmonella* through food chains to humans [32]. Therefore, this study shows a serious need of quality check and surveillance programmes in order to reduce the risk of salmonellosis.

Non-judicious usage of antibiotics for therapeutic purpose or as growth promoters in poultry industry has led to selective pressure on various bacteria (*Escherichia coli*, *Salmonella* serovars; *Enterococcus* spp., *Clostridium perfringens*) resulting in emergence of multidrug resistant strains which is a matter of serious concern for public health [33,34]. Infections due to such strains are very difficult to treat. In the present study, antibiogram results revealed high resistance to beta-lactam antibiotics (oxacillin, penicillin, ampicillin) and clindamycin followed by colistin, nalidixic acid and tetracycline. Diarra *et al*. reported in their study the similar pattern of resistance against beta-lactam antibiotics like ampicillin, amoxicillin-clavulanic acid, ceftiofur, cefoxitin and ceftriaxone [35]. Growing resistance towards beta-lactam antibiotics has been prevalent worldwide among members of Enterobacteriaceae from animal origin, especially in *Salmonella* sp. [36]. This has been associated with various antibiotic resistant gene determinants like *ampC*, bla_{CMY-1}, bla_{CMY-2}, bla_{CTX-M}, and bla_{TEM} [37,38]. Resistance to cephalosporins was variable in contrast to the finding of Elhadi who did not found any resistance to any cephalosporins used in the study [39]. This can be due to variation in source as they isolated *Salmonella* sp. from freshwater fish which are not exposed to antibiotic pressure compared to poultry. The resistance pattern of clindamycin was similar to the observation of Cossi *et al*. who also found all isolates in their study resistant to clindamycin [40]. The results of colistin are in disagreement with Osman *et al*. who found most of their isolates sensitive to colistin while we recorded a higher percentage of resistance [41]. Increase in the trend of colistin resistance has been reported due to mis-sense mutations in two genes, *pmrA* and *pmrB* genes, which encode a regulator and sensor of a two-component regulatory system of outer membrane [42]. Level of resistance against nalidixic acid was very much in agreement to the findings of Halimi *et al*., who found 53% of their *Salmonella* isolates resistant to nalidixic acid [43]. However, Campioni *et al*. have reported more resistance to nalidixic acid compared to our observation which can be explained due to high level of exposure of poultry to drug used in study [44]. Nalidixic acid is a quinolone drug and resistance associated with it has been due to various point mutations in DNA gyrase enzyme where the drug acts [45]. Resistance to tetracycline was comparable to findings of Akbar and Ana [46] but less than that of Ellerboek *et al*. [47] who reported 100% resistance in their study. Resistance to tetracycline has been attributed to irrational usage of it as growth promoter in poultry feed. In the recent past years, the use of tetracycline has been limited in food animals which explain the change in pattern of resistance. High sensitivity to chloramphenicol was similar to that of Elmadiena *et al*. who also found majority of their *Salmonella* isolates sensitive to chloramphenicol [48]. Also, high susceptibility of isolates to meropenem was in agreement to results of Tang *et al*. who found meropenem a good therapeutic option in testing various multidrug resistant *Salmonella* isolates [49].

Thus, it is mandatory to implement strict control over abuse of antibiotics particularly in food animals. Proper scientific and public health regulations are needed to scrutinize non-judicial usage of antibiotics. Also, any treatment regimen should be followed after conducting *in vitro* antibiotic susceptibility testing. That will reduce the emergence of microbial bugs which are spreading worldwide and responsible for fatal disease outcome in different parts of world [50].

Conflict of interest statement

We declare that we have no conflict of interest.

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