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Full Length Article

Phenotypic Antimicrobial Resistance Patterns in *Salmonella typhimurium* and *Enteritidis* Strains Isolated from Human, Food, and Environmental Samples of Broiler Meat Production Chain in Punjab

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Abstract

The emergence of antimicrobial resistance is a growing public health issue worldwide due to extensive use in agriculture, food, and veterinary medicine. The rate of antimicrobial resistance varies with different antibiotics and serotypes but *Salmonella enterica* serovar *Enteritidis* is one of the most widespread serotypes which is comparatively more susceptible to antimicrobial resistance followed by *S. Typhimurium*. A total of 71 *Salmonella* strains (*S. Typhimurium*, n=45; *S. Enteritidis*, n=26) isolated from humans, food and the environment were used in the study. Both isolates were confirmed through PCR by targeting their specific *spy* and *sdf* genes respectively. Kirby-Bauer disc diffusion method was used to determine antimicrobial susceptibility against 10 antibiotics used. An exponentially high level of resistance was found in *S. Typhimurium* strains. The highest level of resistance was found against amoxicillin (97.78%) followed by tetracycline (95.56%), gentamicin (93.33%), trimethoprim (86.67%), streptomycin (84.44%), nalidixic acid (77.78%), sulphafurazole (64.44%), ampicillin (62.22%), chloramphenicol (46.67%) and least resistance was found against ciprofloxacin (31.11%). Four penta MDR (ACSSuT) and two tetra MDR (ASSuT) resistance patterns were found *S. Typhimurium* strains. In *S. Enteritidis* strains, a high level of resistance was found against sulphonamides and streptomycin (92.31%) with the least resistance against ciprofloxacin (11.54%). Two penta MDR (ACSSuT) and six tetra MDR (ASSuT) resistance patterns were found. The presence of high antimicrobial resistance in zoonotic *S. Typhimurium* and *Enteritidis* in the broiler meat production chain is alarming. Immediate action and appropriate measures are required to control over the counter and irrational use of antibiotics both in poultry and humans. © 2021 Friends Science Publishers

Keywords: *Salmonella Typhimurium*; *Salmonella Enteritidis*; Antimicrobial resistance; Punjab

Introduction

Non-typhoidal *Salmonella* is one of the important zoonotic pathogens affecting both humans and animals is also considered the leading cause of bacterial diarrhea in the entire world. An estimated 153 million cases and 57000 deaths of nontyphoidal human salmonellosis are reported every year worldwide (Brunette 2017; Sharma *et al*. 2019). The majority of human cases of non-typhoidal *Salmonella* are foodborne. Infection caused by non-typhoidal *Salmonella* is usually self-limiting and in most cases, antimicrobial treatment is not required.(Angulo *et al*. 2000). However antimicrobial resistance is relevant in 3–10% of cases where the infection is caused by invasive strains resulting in bacteremia and life-threatening conditions particularly in young and immunocompromised individuals (Okeke *et al*. 2005; Chen *et al*. 2013).

The rise of antimicrobial resistance is a growing public health issue worldwide due to extensive use in agriculture, food and veterinary medicine which need to be controlled at the international level (De Oliveira *et al*. 2005). The development of Multi-Drug Resistance among foodborne pathogens is increasing dramatically all over the world and among these AMR in *Salmonella* is a serious emerging issue (Bronzwaer *et al*. 2002; Chen *et al*. 2013; Eng *et al*. 2015). Zoonotic non-typhoidal *Salmonella* has the properties of acquiring antimicrobial resistance over the years (Michael and Schwarz 2016). However the rate of antimicrobial resistance varies with different antibiotics and serotypes and *Salmonella enterica* serovar *Enteritidis* followed by *Salmonella Typhimurium* is relatively more susceptible to antimicrobial resistance than others, is one of

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the most prevalent serotypes (Su *et al* 2004; Chen *et al.* 2013). A much higher rate of antibiotic resistance is also found in, another important serovar prevalent worldwide Centers for Disease Control and Prevention consider MDR *Salmonella* a serious public health threat that should be controlled efficiently on an urgent basis to avoid aggravated circumstances. Conventional antimicrobial agents such as sulphamethoxazole, chloramphenicol, ampicillin and trimethoprim are used as traditional first-line treatments for treating *Salmonella. Salmonella enterica* spp which are resistant to conventional antimicrobials are denoted as multidrug-resistant (MDR) *Salmonella* (Su *et al*. 2004; Eng *et al*. 2015; Bugarel *et al.* 2017). Resistance to the second-line treatment including fluoroquinolones which were used as a choice of treatment in MDR regions has also been reported (Chau *et al*. 2007; Klemm *et al*. 2018). Antimicrobial resistance both in typhoidal and non-typhoidal *Salmonella* is increasing in developing countries particularly the Indian subcontinent and Southeast Asia due to extensive use of antibiotics in public hospitals and communities (Threlfall 2002; Sharma *et al.* 2019). This situation is even grimmer in middle and low-income countries like Pakistan where there is no law enforcement to control the overwhelming sale of antibiotics. In Pakistan like our neighboring country, antibiotics are easily available in pharmacies and anyone can purchase over the counter without the prescription of health practitioners. (Sharma *et al*. 2019)

Until now in Pakistan, limited data is available on the prevalence of zoonotic nontyphoidal *Salmonella* and its susceptibility to different antimicrobial agents as compared to typhoidal *Salmonella*. A few studies have been reported on antimicrobial resistance of *Salmonella Enteritidis* and *Salmonella Typhimurium* in regions including Faisalabad (Akhtar *et al*. 2010; Wajid *et al*. 2018), in Sawat (Uddin *et al*. 2018) and Karachi (Shah and Korejo 2012).

The objective of this study was to check antimicrobial resistance in *Salmonella Enteritidis* and *Salmonella Typhimurium* isolated from different sources of the poultry food chain and humans in Lahore.

Materials and Methods

A cross-sectional study was conducted from 2017–2019 and samples were collected from different steps of the broiler meat production chain. Isolates from different sources including human, food and, the environment were used For the present study, a total of 71 *Salmonella* strains (*Salmonella Typhimurium*, n=45; *Salmonella Enteritidis*, n =26) were included for antimicrobial susceptibility testing (Table 1).

All *Salmonella* isolates were initially confirmed by biochemical tests. *Salmonella* serovar *Typhimurium* and *Enteritidis* and were confirmed through PCR by targeting their specific *spy* and, *sdf* genes, respectively (Alvarez *et al*. 2004).

Antimicrobial susceptibility testing by disc diffusion method

Antimicrobial susceptibility testing was performed by Kirby-Beur disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotics which are commonly used in poultry production were selected and purchased. The antibiotics disks (OXOID, Thermo Scientific Ltd). and their concentration (microgram) used were, ampicillin 25 (AMP), amoxicillin 10 (AML), gentamicin 10 (CN), streptomycin 10 (S), ciprofloxacin 5 (CIP), nalidixic acid 30 (NA), trimethoprim 5 (W), tetracycline 30 (TE), sulphafurazole 300 (SF) and chloramphenicol 30 (C).

Refreshing of isolates and preparation of inoculum

Purified preserved cultures of *Salmonella Typhimurium* and *Salmonella Enteritidis* were refreshed in 10 mL tryptone soy broth with an overnight incubation at 37ºC. A loopful of the bacterial isolate was then streaked on nutrient agar plates and incubated for 24 h at 37ºC. Four to five well isolated and purified colonies were picked from plates with sterilized loop and properly mixed in test tubes containing 10 mL phosphate buffer saline. The turbidity of bacterial culture was standardized at 0.5 McFarland (CLSI 2005). Another test tube containing 10 mL of PBS solution without inoculum was used as blank.

Approximately 20 mL of Mueller-Hinton agar (OXOID, Ltd) medium was poured into 90 mm diameter sterile Petri dishes to a depth of 4 mm with overnight incubation at 37°C to check for sterility (Kebede *et al.* 2016). After the adjustment of absorbance value, bacterial culture was spread on the Mueller-Hinton agar (OXOID, Ltd) plate within 15 min with the help of a sterilized cotton swab. Briefly, the swab was dipped in a culture mixture and excess liquid was removed by squeezing the swab on the walls of the test tube. The swab was then streaked over the entire surface of the agar three times by rotating the plate approximately 60º after each application to ensure an even distribution of the inoculum. The plates were then allowed to dry for 5 min before the application of antibiotic discs. A total of five discs were applied on a single plate for accurate measurement of the zones. The zones were measured with a calibrated scale according to Clinical & Laboratory Standards Institute (CLSI) guidelines.

Results

The zones of inhibitions were measured according to the CLSI guidelines as resistant and sensitive. Fig. 1 summarizes the resistance of all *S. Typhimurium* serovars to 10 antimicrobial agents (Fig. 1).

Table 2 shows the antimicrobial resistance percentages of different antimicrobials against *S. Typhimurium* isolated from human, food and environmental samples. All the

Table 1: *Salmonella Typhimurium* and *Enteritidis* isolated from different sources

Type of sample		*Salmonella Typhimurium*	*Salmonella Enteritidis*	Total
Humans	Hand swab	7	2	9
	Stool samples	2	0	2
Food samples	Chicken meat sample	10	4	14
	Egg samples	7	9	16
Environmental samples	Commercial broiler farms	6	4	10
	Transportation van samples	13	6	19
	Chopping board samples	0	1	1
Total		45	26	71

Table 2: Antimicrobial resistance (%) in *Salmonella Typhimurium* isolated from different sources

Sample Type (*Typhimurium*)	No. of isolates tested	AMP	AML	S	SF	CIP	C	NA	TE	CN	W
Human samples	9	88.89	100	77.78	88.89	44.44	11.11	66.67	88.89	100	88.89
Food samples	17	23.53	94.12	76.47	35.29	29.41	29.41	64.71	100	76.47	88.24

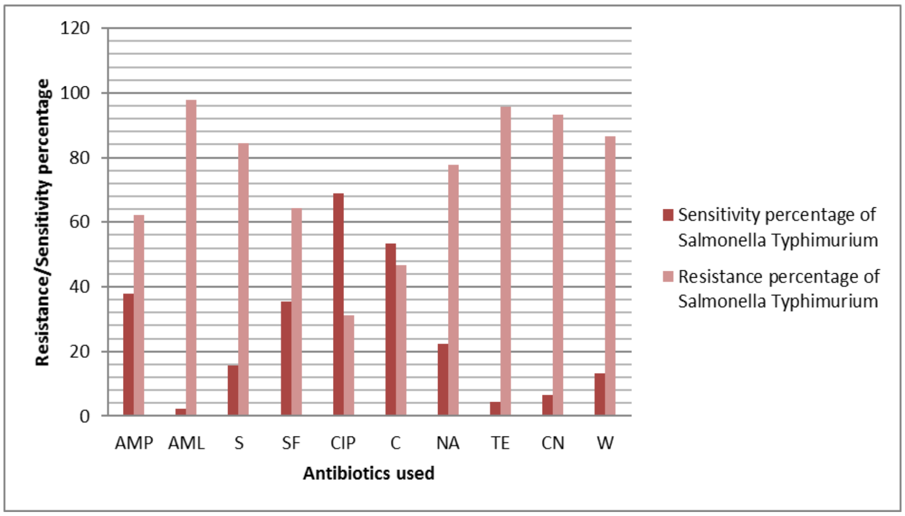

Fig. 1: Resistance (%) of *Salmonella Typhimurium* isolated from different sources against 10 antibiotics

strains (100%) were resistant to at least one antimicrobial agent. The highest level of resistance was found in amoxicillin (AML), gentamycin (CN), tetracycline (TE), and, trimethoprim (W). All (100%) of human and environmental samples while (94.12%) of food samples were resistant to AML. Similarly, (100%) of human and environmental samples and (76.64%) were resistant to gentamycin (CN). The higher level of resistance with (100%) of food samples, (94.74%) of environmental samples and, (88.89%) of human samples were resistant to tetracycline (TE). An equal level of high resistance was found (88%) in human food and environmental samples. A moderate level of resistance was found against AMP, SF, S, NA, C and CIP (in declining order) (Table 2).

A total of 25 resistance patterns were found (Table 3) with the most common (16%) resistant pattern was (AMP, AML, S, SF, C, NA, TE, CN, W). According to the patterns of antimicrobial resistance, the highest resistance was shown in *Salmonella Typhimurium* strains isolated from vehicles (13.33%) followed by chicken samples (28.8%), human samples (20%), egg samples (15.5%) and (13.3%) in broiler farms samples. Four penta MDR (ACSSuT) and two tetra MDR (ASSuT) pattern were found is *S. Typhimurium.* (Table 3).

Fig. 2 showed the resistance percentages of *Salmonella Enteritidis* isolated from different sources against 10 antibiotics with the highest resistance found against sulphonamides and streptomycin. (Fig. 2).

A great proportion of antimicrobial resistance was found in *Salmonella Enteritidis* strains with (96%) of the isolated strains showed resistance to at least one antimicrobial agent (Table 4). The highest level of resistance was found in streptomycin (S), tetracycline (TE), nalidixic acid (NA) and CN. All the human and environmental (100%) samples while (92.31%) of food samples were resistant to streptomycin (S). Similarly (100%) of human samples, (92.31%) of food samples and (91%) of environmental samples were resistant to tetracycline (TE). Similarly, (100%) of human and environmental samples and (76.64%) were resistant to gentamicin (CN). Again (100%) of human samples, (69.23%) and (61.54%) of food samples, (36%) and (64%)

Table 3: Antimicrobial resistance Patterns of *Salmonella Typhimurium*

Antibiotics pattern	Human samples	Chicken samples	Egg samples	Vehicle samples	Broiler farm samples	Total	Percentage
AMP,AML,S,CN	0	0	0	0	1	1	2%
AML,TE,CN,W	0	0	1	0	0	1	2%
AML,CIP,TE,W	0	0	1	0	0	1	2%
AML,S,SF,NA,TE,	0	2		0	0	2	4%
AML,S,TE,CN,W	0	0	1	0	0	1	2%
AML,S,SF,TE,CN,W	0	1	1	0	0	2	4%
AMP,AML,S,CIP,TE,W	0	0	1	0	0	1	2%
AMP,AML,S,NA,TE,CN	1	0	0	0	0	1	2%
AML,S,C,NA,TE,CN,W	0	1	1		1	3	7%
AMP,AML,C,NA,TE,CN,W	0	0	0	1	0	1	2%
AMP,AML,S,SF,NA,TE,CN	0	1	0	0	0	1	2%
AMP,AML,SF,NA,TE,CN,W	1	0	0	0	0	1	2%
AMP,AML,S,NA,TE,CN,W	0	2	0	0	0	2	4%
AMP,AML,S,SF,CIP,NA,TE,CN	1	0	0	1	0	2	4%
AMP,AML,SF,C,NA,TE,CN,W	1	0	0	1	0	2	4%
AML,S,CIP,C,NA,TE,CN,W	0	1	0	0	0	1	2%
AMP,AML,S,C,NA,TE,CN,W	0	0	0	2	0	2	4%
AML,S,SF,C,NA,TE,CN,W	0	0	0	0	1	1	2%
S,SF,CIP,C,NA,TE,CN,W	0	0	0	0	0	0	0%
AMP,AML,S,SF,NA,TE,CN,W	1	1	0	0	1	3	7%
AMP,AML,CIP,C,NA,TE,CN,W	0	1	0	0	0	1	2%
AML,S,SF,CIP,C,NA,TE,CN,W	1	0	1	0	2	4	9%
AMP,AML,S,SF,C,NA,TE,CN,W	1	0	0	6	0	7	16%
AMP,AML,S,SF,CIP,NA,TE,CN,W	2	0	0	1	0	3	7%
AMP,AML,S,SF,CIP,C,NA,TE, CN,W	0	0	0	1	0	1	2%
	9	0	1	8	2	45	100%

Table 4: Antimicrobial resistance (%) in *Salmonella Enteritidis* isolated from different sources

Samples (*Enteritidis*)	No. of isolates tested	AMP	AML	S	SF	CIP	C	NA	TE	CN	W
Human samples	2	50%	50%	100%	50%	50%	50%	100%	100%	100%	50%
Food samples	13	53.85	62%	92.31	61.54	0%	23.08	61.54	92.31	69.23	46.15
Environmental samples	11	45%	45%	100%	63.64	9.09	45%	64%	91%	36%	45%

of environmental samples were resistant to gentamicin (CN) and nalidixic acid (NA) respectively. A moderate level of resistance was found against AMP, AML, SF, W, C and CIP. (in declining order) (Table 4). A total of 22 resistance patterns were found for *Salmonella Enteritidis* with the most common pattern among all samples were (S, NA, AML, W, TE, SF, AMP) which was 16% (Table 5). According to the patterns of antimicrobial resistance highest resistance was shown in *S. Enteritidis* strains isolated from egg samples (34.6%) followed by transportation van samples (23.0%), chicken samples and broiler farms samples (15.3%), human samples (7.6%) and least in chopping board samples (3.6%). Two penta MDR (ACSSuT) and six tetra MDR (ASSuT) resistance patterns were found in *S. Enteritidis* (Table 5).

Discussion

In developing countries of Asia including Pakistan antimicrobial drugs are extensively used in food-producing animals and poultry for growth promotion and prophylaxis. Food-borne bacteria can attain resistance as a result of extensive use in food animals and can transmit these resistance genes to humans via the food chain (Bouchrif *et al*. 2009). The importance of antimicrobial resistance against zoonotic *Salmonella* serovar *Enteritidis* and *Typhimurium* has been shown in this study. In our study, exponentially high resistance was found in *Salmonella Typhimurium* isolates where all (100%) of the strains isolated from different sources were resistant to at least one antibiotic. One study from Minnesota reported (89%) of *Salmonella Typhimurium* isolated from animals and (44%) from humans were resistant to at least one antibiotic (Wedel *et al*. 2005). In two studies from Italy, one reported (75%) of *S. Typhimurium* were resistant to at least one antimicrobial agent (Busani *et al*. 2004), and the second reported (87%) of human isolates and (81%) from animal source showed resistance to at least one antibiotic (Graziani *et al*. 2008) The high level of resistance found in our study may be due to widespread and over the counter use of antimicrobials in humans and veterinary (poultry) medicine in Pakistan.

According to the source of isolates a high level of resistance was found (76.64–100%) among isolates of humans, environmental and, food against amoxicillin (94–100%), gentamicin (76.64–100%), tetracycline (88.89%) and trimethoprim (88%). Moderate level of resistance was found against (in declining order) ampicillin (45–50%), sulphonamides (35–88%), Streptomycin (76–89%), nalidixic acid (64–94%), chloramphenicol (11–73%) and ciprofloxacin (26–44%). Our results are inconsistent with reports from Italy that showed resistance

Table 5: Anti Microbial Resistance Patterns of *Salmonella Enteritidis*

AMR Pattern	Human samples	chicken samples	egg samples	transportation van samples	broiler farm samples	Chopping board	Total
C,S	0	0	1(11%)	0	0	0	1(4%)
S,TE	0	1(25%)	1(11%)	0	0	0	2(8%)
CN,S,TE	0	0	0	0	1(25%)	0	1(4%)
S,NA,TE,AMP	0	0	1(11%)	0	0	0	1(4%)
S,CIP,W,SF,C	0	0	0	0	1(25%)	0	1(4%)
CN,S,TE,SF,AMP	0	0	0	1(17%)	0	0	1(4%)
CN,NA,AML,W,TE	0	1(25%)	0	0	0	0	1(4%)
CN,S,NA,AML,TE	0	0	0	1(17%)	0	0	1(4%)
S,NA,TE,SF,AMP,C	0	0	0	1(17%)	0	0	1(4%)
S,NA,W,TE,SF,C	0	0	0	1(17%)	0	0	1(4%)
CN,S,NA,AML,TE,SF	0	0	0	1(17%)	0	0	1(4%)
CN,S,CIP,NA,W,TE	1(50%)	0	0	0	0	0	1(4%)
CN,S,NA,TE,SF,C	0	1(25%)	0	0	0	0	1(4%)
CN,S,AML,W,TE,SF	0	0	1(11%)	0	0	0	1(4%)
CN,S,NA,AML,TE,SF,AMP	0	0	1(11%)	0	0	0	1(4%)
S,NA,AML,W,TE,SF,AMP	0	0	1(11%)	0	2(50%)	1(100%)	4(16%)
S,NA,AML,W,TE,AMP,C	0	0	0	1(17%)	0	0	1(4%)
S,NA,AML,W,TE,SF,C	0	1(25%)	0	0	0	0	1(4%)
CN,S,AML,W,TE,SF,AMP	0	0	1(11%)	0	0	0	1(4%)
CN,S,NA,AML,AMP,TE,SF,C	1(50%)	0	0	0	0	0	1(4%)
CN,S,NA,AML,TE,SF,W,AMP	0	0	1(11%)	0	0	0	1(4%)
S,NA,AML.W,TE,AMP,SF,CN	0	0	1(11%)	0	0	0	1(4%)
	2	4	9	6	4	1	26

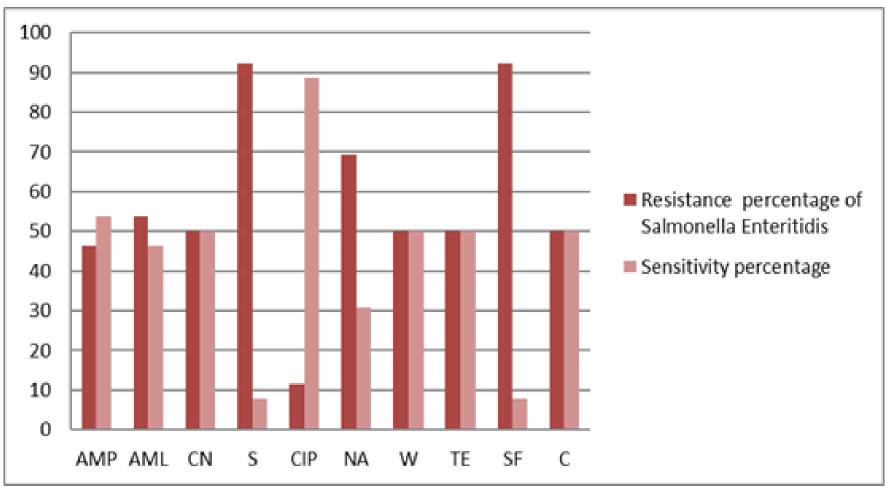

Fig. 2: Resistance of *Salmonella Enteritidis* isolated from different sources

to ampicillin (67.6%), tetracycline (73.6%), streptomycin (65.4%), sulfonamides (73.3%) and chloramphenicol (32.3%) (Graziani *et al*. 2008). Another study reported from the UK showed (82%) of *S. Typhimurium* were predominately resistant to, sulfonamides, streptomycin, tetracycline's, ampicillin, and chloramphenicol, while (20%) were resistant to trimethoprim (Threlfall *et al*. 2003). Our results are also nearly equal to those reported from Italy, showed (83.9%) of *S. Typhimurium* isolates showed resistance to tetracycline, sulfamethoxazole, streptomycin, chloramphenicol and ampicillin (De Vito *et al*. 2015). Another study from Ireland reported (77.6%) of *Typhimurium* isolates were resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Gorman and Adley 2004). In the present study, a very low level of resistance was found against ciprofloxacin as also reported from Italy where all *S. typhiurium* isolates were susceptible to ciprofloxacin (Busani *et al*. 2004).

A total of 26 resistance patterns were found in *Salmonella Typhimurium* isolates of which 4 (ACSSuT) and 2 (ASSuT) patterns were found. The highest prevalent pattern was ((ASSuT) AML, NA, CN, W) which was present in 16% of MDR *Typhimurium* isolates. Our results are inconsistent with reports from China, Italy where the most frequently observed patterns of resistance were (ACSSuT) and (ASSuT) (Graziani *et al*. 2008; Wang *et al*. 2019).

A great proportion of antimicrobial resistance was also found in *S. Enteritidis* strains with, (96%) of the isolated strains showed resistance to at least one antimicrobial agent. Maximum level (92.31%) of resistance was shown against sulphonamides and streptomycin and (69.23%) for nalidixic acid. Our results are supported by a study in Brazil where (91%) of *S. Enteritidis* showed resistance to at least one antimicrobial agent with the maximum level of resistance was found against sulphonamides (75.8%) (Dias de Oliveira *et al*. 2005). One study from Korea reported that (90%) of *S. Enteritidis* strains were resistant to sulphonamides and nalidixic acid (Hur *et al.* 2011) which is very close to our study. Another study from Taiwan reported (70%) resistance to streptomycin and (75%) to tetracyclin in two studies from Brazil reported (73.3%) and (28.12%) of S. enetritidis were resistant to nalidixic acid (Chu *et al*. 2009; Campioni *et al* 2012; Campioni *et al.* 2014). Nalidixic acid that targets DNA gyrase is one of the common antimicrobials used for the treatment of salmonellosis.

In the present study moderate level of resistance was found against ampicillin (46.2%), amoxicillin (53.8%), gentamicin (50%), trimethoprim (50%), tetracycline (50%), and chloramphenicol (50%). Lower level resistance was shown from a study in Brazil where a low level of resistance was found for tetracycline (15.4%), streptomycin (7.7%), gentamicin (5.5%), trimethoprim (3.3%), ampicillin (1.1%), and chloramphenicol (1.1%). One study from Iran in agreement with our study reported (100%) resistance of S. *Enteritidis* against ampicillin (70%) against streptomycin and 60% against gentamicin(Ghazaey and Mirmomeni 2012). The high level of resistance against different antibiotics may be due to without prescription over the counter use in human and veterinary medicine.

In the present study, a total of 22 resistance patterns were found and the most prevalent pattern (16%) was ((ASSuT) W, AML, NA). A total of two (ACSSuT) and four (ASSuT) were found in *S.Enteritidis* isolates. Our results are in agreement with the study reported from Spain where 23 different resistant patterns were found in *S. Enteritidis* (Carramiñana *et al*. 2004).

Conclusion

An alarmingly high level of resistance was found in both *Salmonella Typhimurium* and *Enteritidis* isolated from humans, food and environmental sample. In addition to this, our study isolates were resistant to sulphonamides, nalidixic acid, ampicillin, streptomycin and tetracycline is the indication of extensive use of these antibiotics in human and veterinary medicine. In our study low level of resistance and a high level of susceptibility were found against ciprofloxacin. So fluoroquinolones can be the choice for treating salmonellosis in our poultry industry.

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Author Contributions

MDA, MC and HA planned the study, SS conduct the whole research work and MHM, JS and SH help in formatting of manuscript.

Conflicts of Interest

Authors declare no conflict of interest.

Data Availability

Data are available from the first author on reasonable request.

Ethics Approval

Not applicable in this paper

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