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Antimicrobial Resistance Pattern of *Salmonella* Species Recovered from Livestock and Poultry

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ABSTRACT

Background: Non-typhoidal *Salmonella*e (NTS) are the predominant source of foodborne Salmonellosis in humans. Livestock and poultry are important reservoirs of NTS. In the context of food safety, this study was undertaken to characterize *Salmonella* species isolated from livestock, poultry and fish for antimicrobial resistance.

Methods: A total of 620 fecal and food samples were randomly collected from different places. Isolation and identification of *Salmonella* species were done employing microbial and molecular tests. Phenotypic AMR pattern was studied as per the CLSI guidelines and AMR genes were detected by PCR.

Result: *Salmonella* spp. were detected in 39(6.29%) out of 620 samples. *Salmonella* isolates were 100% sensitive to aztreonam, cefepime, cefpodoxime, followed by cefoxitin (97.43%), ceftazidime (94.43%), piperacillin/tazobactam (94.87%) and ciprofloxacin (71.79%). However, resistance to ampicillin (53.84%), colistin (66.66%), tetracycline (58.57%), amoxyclav (33.33%) and sulfafurazole (23.07%) was observed. Out of 39 isolates, 7(17.94%) were ESBL positive strains and bla_{TEM} was detected in the two ESBL positive isolates. Quinolone- resistant *QnrS* gene was present in 3(7.69%) isolates. KPC, AmpC and MBL positivity and genes encoding mobile colistin resistance (*mcr*) were not detected. The presence of virulent and drug-resistant strains of *Salmonella* in livestock and poultry may poses environmental, food safety and public health risks.

Key words: Antimicrobial resistance, Esbl, Livestock, Poultry, *Salmonella spp.*

INTRODUCTION

Among the food-borne pathogens, cases associated with non-typhoidal salmonellosis (NTS) are predominant. Several environmental reservoirs of NTS exist and these organisms are present in the intestinal tract of domesticated and wild animals. The immediate risk of transmission to humans is through contaminated food and water. Animal origin foods like contaminated raw poultry meat are considered an important source of *Salmonella* spp. Most of the studies on *Salmonella* spp. have targeted poultry and poultry meat however, *Salmonella* spp. were also detected in other foods (Makwana *et al.,* 2015; Prabhakar *et al.,* 2020). In India, Salmonella enterica subspecies enterica has been detected in the feces of cattle and buffalo suffering from chronic diarrhoea (Hassan *et al.,* 2020).

Salmonella species are responsible for a wide range of food- and water-borne diseases. Fecal shedding of *Salmonella* by humans and animals increases the risk of environmental contamination. It can enter the agricultural environment and contaminate the plants and surface water. The emergence of antimicrobial resistance (AMR) in foodborne pathogens including *Salmonella* species is a matter of concern. Resistance to extended-spectrum cephalosporins and fluoroquinolones has been reported in *Salmonella enterica* (Yan *et al.,* 2005). Monitoring of AMR traits is necessary to understand the trends and magnitude of food borne pathogens like *Salmonella* spp. Present study was aimed to determine the prevalence of resistant *Salmonella* in the fecal samples of livestock and poultry and raw foods like milk, meat, fish and water.

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MATERIALS AND METHODS

Sampling and bacterial culturing

A total of 620 samples (Table 1) were collected from different locations from August to December 2020. The work was carried out in the department of VPH, KNP College of Veterinary Science, Shirwal, Maharashtra. Fecal samples of livestock and poultry were directly collected using rectal/ cloaca swabs. Fecal droppings of wild pigeons were collected from their roosting places without touching or handling them. Water samples were collected from poultry farms and duck ponds. Raw food samples *viz.* chevon, mutton, milk and fish were collected from the local market. Isolation of *Salmonella* spp. was done as per the standard protocols. All the fecal swabs were pre-enriched with 10 ml

buffered peptone water and incubated at 37°C for 18-24 h. Later, 1 ml of pre-enriched culture was inoculated with 9 ml tetrathionate broth and after incubation at 37°C for 24 h, a loopful of culture was streaked on Xylose Lysine Desoxycholate (XLD) and Brilliant Green agar (BGA). All the plates were incubated at 37°C for 24 h and presumptive colonies were further subcultured and processed for identification of *Salmonella* species through Gram's staining, catalase, oxidase, IMViC test and H_2S production.

Extraction of DNA and PCR conditions

Genomic DNA from pure bacterial colonies was extracted by boiling and snap chilling method. All the PCR conditions were performed in 25 µl volume containing 12.5 µl 2 × PCR master mix, 1 µl each forward and reverse primers, DNA and nuclease free water to make final volume. Detection of *invA* gene for invasiveness was done by PCR as per the method described previously (Rahn *et al.,* 1992). Primers used for amplification of *invA* gene (284 bp) were F- GTG AAA TTA TCG CCA CGT TCG GGC AA and R-TCA TCG CAC CGT CAA AGG AAC C. In this PCR 3 µl DNA and 7.5 µl nuclease-free water was used. PCR conditions were set as initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 60 s. The final extension was carried out at 72°C for 7 min. PCR products were run in 1.5% agarose gel stained with ethidium bromide.

PCR for the detection of quinolone-resistant genes *viz., QnrA, QnrB* and *QnrS* PCR was performed as per the method of Cattoir *et al.* (2007). Annealing temperatures were 60, 56, 57 degrees Celsius, respectively for *QnrA, QnrB and QnrS* genes. Other PCR conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing as above for 30 s and extension at 72°C for 1 min and a final extension of 72°C for 10 min. The expected amplicon size for *QnrA, QnrB* and *QnrS* was 550, 264 and 428 base pairs, respectively.

Attempts were also made to detect mobile colistin-resistant genes (*mcr1-mcr5*) by the multiplex PCR developed by Lescat *et al.* (2018). Reactions were set at denaturation at 94°C for 4 min; followed by 30 cycles of 94°C for 5 s, 59°C for 20 s and a single, final, elongation step at 72°C for 5 min. Gel electrophoresis was carried out in 2.5% agarose gel stained with ethidium bromide. Detection of major beta-lactam encoding genes *viz.,* bla_{SHV}, bla_{TEM}, bla_{CTX-M} and bla_{OXA} was also performed by multiplex PCR as per the method described earlier (Fang *et al.,* 2008). The cycling conditions were set as initial denaturation (95°C/15 min^{-1} cycle) followed by 30 cycles of denaturation (94°C/30 sec), annealing (62°C/90 sec) and extension (72°C/60 sec). The final extension was set at 72°C for 10 min and at 4°C. Five microliters amplified product was further separated by electrophoresis in 1.5% agarose gel dissolved in 0.5x TBE stained by ethidium bromide.

Antibiogram

Antimicrobial resistance and sensitivity patterns of *Salmonella* species were studied by Kirby Bauer disk diffusion method results were interpreted using criteria suggested by the Clinical and Laboratory Standards Institute (2017). A total of 15 antimicrobial agents were used (Table 2). ESBL production was evaluated using double-disk synergy test (DDST). The procedure involved two steps, initial screening followed ESBL confirmation. Cefotaxime (30 µg) and ceftazidime (30 µg) antimicrobial discs were used for initial screening followed by ESBL confirmation using cefotaxime/clavulanic acid (30/10 µg) and ceftazidime/

Table 1: Sample-wise prevalence of *Salmonella* species.

Sampling source	Number of samples	Salmonella species detected	
		Number	%
Fecal samples			
Cattle	50	3	6
Buffalo	50	1	2
Sheep	50	-	-
Goat	50	2	4
Pigs	80	7	8.75
Broilers	50	10	20
Layers	50	8	16
Quail	10	1	2
Wild pigeon	15	-	-
Food and water samples			
Raw milk	50	-	-
Chevon	50	-	-
Chicken	50	3	6
Fish	50	4	8
Water (Farm waste and duck pond)	15	-	-
Total	620	39	6.29

clavulanic acid (30/10 µg) discs. Isolates showing the difference in a zone of inhibition of ≥5 mm of cephalosporin discs and cephalosporin plus clavulanic acid containing disc were considered as potential ESBL producers. Detection of carbapenemase (KPC), Metallo-beta-lactamase (MBL), ampC and colistin resistance was also assessed by epsilon test using EzyMIC strips (HiMedia) and results were interpreted accordingly.

RESULTS AND DISCUSSION

Prevalence

During this investigation, 39 *Salmonella* spp. were recovered from 620 samples with a prevalence rate of 6.29%. Fecal prevalence of *Salmonella* species was observed more in the broiler (20%) and layers (16%) followed by pigs (8.75%). Similarly, *Salmonella* species were detected in raw chicken (6%) and fish (8%) samples. Present findings affirm that healthy food animals and poultry birds harbor *Salmonella* species in their intestine. Foods of animal origin especially chicken and chicken products have been studied extensively for the detection of *Salmonella* spp. Some researchers also highlighted its presence in other foods like mutton, chevon, milk and fish (Makwana *et al.,* 2015; Singh *et al.,* 2018; Prabhakar *et al.,* 2020). An outbreak of Salmonellosis was also detected in the poultry farms in India (Rajagopal and Mini, 2013), indicating that food animals and poultry are the predominant reservoirs of NTS.

Table 2: Antimicrobial resistance and sensitivity pattern of *Salmonella* species.

Antimicrobials	Disc contents (mcg)	Sensitive (S)		Intermediate (I)		Resistant (R)	
		No.	%	No.	No.	%	No.
Ampicillin (AMP)	10	18	46.15	0.00	0.00	21	53.84
Amoxyclav (AMC)	30	26	66.66	0.00	0.00	13	33.33
Aztreonam (AT)	30	39	100.00	0.00	0.00	0.00	0.00
Ciprofloxacin (CIP)	5	28	71.79	05	12.82	06	15.38
Colistin (CL)	10	13	33.33	0.00	0.00	26	66.66
Trimethoprim (TR)	5	34	87.17	0.00	0.00	05	12.82
Cefepime (CPM)	30	39	100.00	0.00	0.00	0.00	0.00
Piperacillin/Tazobactam (PIT)	100/10	37	94.87	02	5.12	0.00	0.00
Sulfafurazole (SF)	30	30	76.92	0.00	0.00	09	23.07
Cefoxitin (CX)	30	38	97.43	0.00	0.00	01	2.56
Gentamicin (GEN)	10	37	94.87	0.00	0.00	02	5.12
Ceftriaxone (CTR)	30	33	84.61	04	10.25	02	5.12
Cefpodoxime (CPD)	10	39	100	0.00	0.00	0.00	0.00
Ceftazidime (CAZ)	30	37	94.43	0.00	0.00	02	5.12
Tetracycline (TE)	30	13	33.33	03	7.69	23	58.57

Table 3: Prevalence of ESBL positive *Salmonella* species.

Sampling source	Number of samples	No. of Salmonella detected	*ESBL strains*		*QnrS* +ve
			ESBL +ve	bla_{TEM} +ve	
Fecal samples					
Cattle	50	3	2	bla_{TEM}	
Buffalo	50	1	-	-	
Sheep	50	-	-	-	
Goat	50	2	-	-	
Pigs	80	7	2	-	
Broilers	50	10	3	bla_{TEM}	2
Layers	50	8	-	-	1
Quail	10	1	-	-	
Wild pigeon	15	-	-	-	
Food and water samples					
Raw milk	50	-	-	-	
Chevon	50	-	-	-	
Chicken	50	3	-	-	
Fish	50	4	-	-	
Water (Farm waste and duck pond)	15	-	-	-	
Total	620	39	7 (17.94%)	2 (5.12%)	3 (7.69%)

As observed in the present study, *Salmonella* spp. were detected in raw chicken meat (9%) and chevon (7%) samples collected from different districts of Chhattisgarh by Naik *et al.* (2015). From a food safety point, all the foods intended for human consumption either in the raw or processed form must be negative for *Salmonella* species. Thus, its presence in some of the animal-origin raw foods warrants the application of good hygienic practices at retail meat shops and vendors selling fish.

Invasiveness

Salmonella species isolated during this investigation were virulent strains as they have exhibited the presence of *invA* gene (Fig 1) which encodes for a protein on the inner membrane that is necessary for the invasion of epithelial cells. The presence of *invA* gene in *Salmonella* spp. is indicative of its virulence and generally virulent strains express the same along with other genes like *fimA, spvR and spvC* gene. The presence of *invA* has been reported by several researchers across the globe in *Salmonella* spp. isolated from foods and present findings are in agreement with previous studies (Mthembu *et al.,* 2019).

Antibiogram

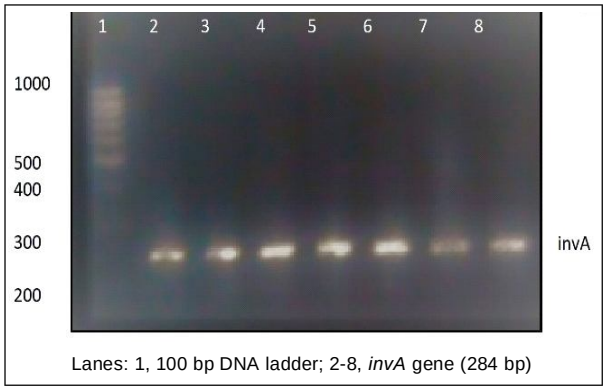
Salmonella species under study were mostly sensitive to monobactam and cephalosporin groups of antimicrobials. They have shown a high degree of sensitivity to aztreonam, cefepime, cefoxitin, ceftazidime, cefpodoxime and piperacillin-tazobactam. Over 71.79% of isolates were also sensitive to ciprofloxacin which belongs to the fluoroquinolone group. However, isolates showed resistance to ampicillin (53.84%), amoxyclav (33.33%), colistin (66.66%), tetracycline (58.57%) and sulfafurazole (23.07%). Mostly pig and poultry origin isolates shown resistance to more than two groups of antimicrobials indicating their multidrug resistance status. A total of 11 (28.20%) isolates were multidrug-resistant and MAR index varies from 0.2 to 0.6 which is suggestive of high-risk source. A study from Ethiopia also recorded a very high prevalence (86%) of multidrug-resistant *Salmonella* species isolated from caeca of slaughtered broiler birds (Asfaw *et al.,* 2020). In India, some of the *Salmonella* strains resistant to amoxicillin and cephalosporins were isolated from ready-to-eat street foods (Anukampa *et al.,* 2017). Another study from India also recorded moderate resistance in *Salmonella* Newport isolated from poultry and animal origin foods. However, a high degree of sensitivity was observed against ciprofloxacin, chloramphenicol and cefuroxime (Kumar *et al.,* 2016). Isolates of our study were mostly sensitive to cephalosporins but they have shown resistance to colistin, tetracycline and certain penicillin group of antimicrobials. About 17.94% (7/39) isolates of this study were ESBL producing strains (Table 3). ESBL producing and colistin-resistant *Salmonella* spp. was detected in pigs and pork in Thailand recently (Lay *et al.,* 2021). We have also screened all the *Salmonella* isolates for carbapenem, MBL and ampC production by epsilon test but all the isolates were negative.

Colistin resistance

We could not detect mobile colistin-resistant *mcr* genes in *Salmonella* spp. Detection of *mcr* genes is rare in the foodborne *Salmonella* spp. and there are no reports from India. Globally, researchers have detected *mcr* genes in *Salmonella enterica* (Sia *et al.,* 2020). Polymyxins, including polymyxin B and E (colistins), is regarded as the last-resort antibiotics for the treatment of infections caused by multidrug-resistant Gram-negative organisms, especially carbapenem-resistant *Enterobacteriaceae*. We have phenotypically detected colistin resistance using the epsilon test (Fig 2) and disk diffusion method. Phenotypically, about 66.66% *Salmonella* spp. were resistant to colistin.

Quinolone resistance

Out of 39 isolates, 3 (7.69%) showed the presence of *QnrS* gene and all were resistant to ciprofloxacin which is a second-generation quinolone (Fig 3). They were isolated from poultry. Fluoroquinolones are the most widely used antibiotics for treating salmonellosis in both humans and animals because of their broad spectrum in antimicrobial activity. The *Qnr* genes provide a low resistance level to quinolones in the *Enterobacteriaceae*. The rate of detection of *Qnr* genes is relatively low among *Salmonella* species, however, risk of spread of plasmid-mediated *Qnr* genes through foods is speculated. Quinolone resistant genes were detected in *Salmonella* species isolated from raw meat products from China (Zhou *et al*., 2019).



Lanes: 1, 100 bp DNA ladder; 2-8, *invA* gene (284 bp)

Fig 1: Detection of *invA* gene in *Salmonella* spp.

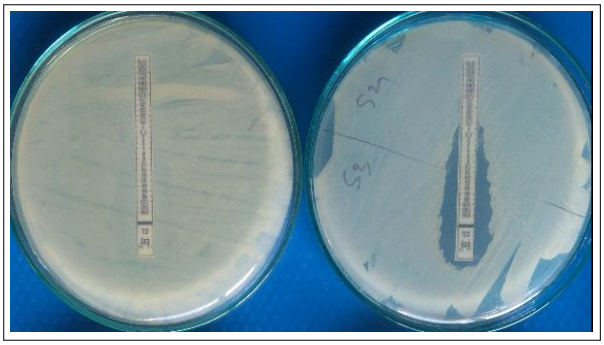
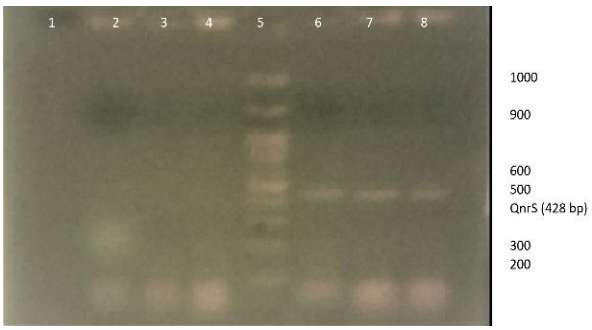
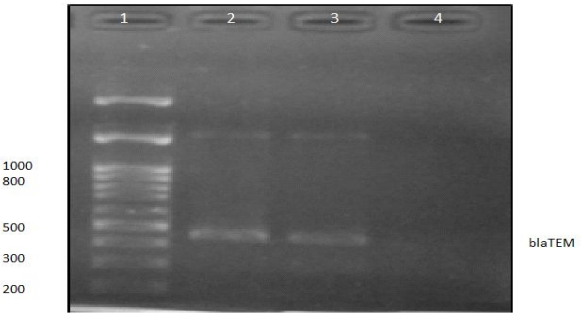


Fig 2: Detection of *colistin resistance* in *Salmonella* spp.



Lanes: 5, 100 bp DNA ladder; 6,7, 8, *QnrS* gene (428 bp).

Fig 3: Detection of *QnrS* gene in *Salmonella* spp.



Lanes: 1, 100 bp DNA ladder; 2 and 3 bla_{TEM} gene (445 bp).

Fig 4: Detection of bla_{TEM} gene in *Salmonella* spp.

Beta-lactam genes

All the ESBL positive strains were studied by multiplex PCR for the presence of genes encoding for beta-lactamases. One isolate each from cattle and broiler feces was positive for bla_{TEM} gene (Fig 4). In contrast to our findings, high prevalence of bla_{TEM} and bla_{CTXM} was recorded in the *Salmonella* spp. isolated from retail meat samples by Zhou *et al.* (2019). The worldwide incidence of resistance in *Salmonella* spp. isolated from the poultry and other foods of animal origin are varying, including ESBL productivity and rate of detection of AMR genes. However, in the context of AMR and One Health, epidemiological investigations need to be conducted to examine the local situation of prevalence and antimicrobial resistance in priority food-borne pathogens like *Salmonella* species.

CONCLUSION

Present study highlighted the importance of detecting *Salmonella* species in fecal samples and raw foods of animal origin obtained from the retail market. Detection of virulent, ESBL positive and multidrug-resistant strains in food samples and feces of livestock and poultry poses public health, food safety and environmental risk and therefore AMR traits and spread of animal origin NTS should be critically monitored.

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Conflict of interest

All authors declare that they have no conflict of interest.

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