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Antimicrobial sensitivity and multidrug-resistance for *Salmonella* species isolated from broilers

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Abstract
Antibiotics are used as chemotherapeutic, prophylactic purposes and also as feed additives to promote growth and improve feed efficiency in poultry industry. The present study was conducted to evaluate the antimicrobial sensitivity and multidrug-resistance for *Salmonella* species isolated from broilers of Hoshagabad districts of Madhya Pradesh. A total of 153 samples consisting of cloacal swab (99), dry faeces (18), feed (18) and water (18) of all the three seasons were collected. Phenotypically 13 isolates were confirmed as *Salmonella* species. Genotypically all the isolates were positive for 16S rRNA and *inv*A gene in polymerase chain reaction. Out of the 13 isolates 69.23 isolates were positive for *Salmonella enterica* ser. Typhimurium gene. In antibiogram the isolates showing resistant pattern in decreasing order were gentamicin (11, 84.61%), norfloxacin and ampicillin / sulbactum (10, 76.92%), ofloxacin and ciprofloxacin (09, 69.23%), streptomycin and ampicillin (08, 61.53%). Antibiotics vancomycin and co-trimoxazole were sensitive for *Salmonella* organism. It was found that the maximum isolates were showing resistance to more than 06 antibiotics.

Keywords: Antimicrobial sensitivity, multidrug-resistance, *Salmonella*, broilers

1. Introduction
Poultry are the important reservoir of many zoonotically important pathogens, of which *Salmonella* is of prime importance [1]. Salmonellosis is one of the most important global poultry diseases which are caused by different *Salmonella* species [2]. The Salmonella organism comes under genus *Salmonella*, family *Enterobacteriacea.* Except the poultry-specific serovars of *Salmonella* Gallinarium and *Salmonella* Pullorum all the *salmonella* are motile. *Salmonella* is one of the most important pathogens responsible for human food poisoning in the developed world, where chicken and chicken products are widely considered to be significant sources for this organism.

In the past few decades, emergence of antibiotic resistance among different species of bacteria was on hike. Irrational use of antibiotics as growth promoters in poultry is an important factor that has favoured the selection of resistant bacteria in faecal microflora of poultry. Over five million people are directly or indirectly engaged in poultry sector, apart from numerous small poultry keepers in rural and tribal areas of the country. 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 *salmonella*. The most characteristic feature of *Salmonella* is its wide host range, which comprises most animal species including all mammals, birds and cold-blooded animals in addition to human.

Over five million people are directly or indirectly engaged in poultry sector, apart from numerous small poultry keepers in rural and tribal areas of the country. Hosangabad district of Madhya Pradesh occupies an important place in tribal population. The people of 'Gond' community keep poultry as supplementary income source through producer collective in Hosangabad district. The resistant *salmonella* strains can easily passed to human through food chains resulting in serious consequences in terms of treatment failure and rapid outbreaks of *salmonella*. Hence, avian salmonellosis is of large economic concern in all phases of the poultry industry from production to marketing. So, the present experiment was designed to identify the invasive *salmonella* organism from cloacal swab, dry faeces, feed and water of layers in tribal area of Hoshangabad district in all the three seasons.

2. Materials and methods

2.1 Ethical approval

All the procedures have been carried out in accordance with the guidelines laid down by the Institutional Ethics Committee and in accordance with local laws and regulations. The faecal samples of clinically ill broiler poultry birds were collected from private farms after having proper consent from the respective owners.

2.2 Collection of samples

A total of 153 samples consisting of cloacal swab (99), dry faeces (18), feed (18) and water (18) were in rainy, winter and summer season from individual clinically ill birds of Hoshangabad district of Madhya Pradesh.

2.3 Isolation of *Salmonella* organism

The *Salmonella* organism was isolated by conventional methods following the approved methodology [3] with some modifications. Pre-enrichment was done in buffered peptone water incubating at 37°C for 18 hrs. Selective enrichment was performed in Rappaport Vassilidis media incubation at 42°C for 24 hrs. and observed for turbidity for positive reaction. Subsequently the selective plating was done in MacConkey, brilliant green and Xylose Lysine Deoxycholate agar (XLD) agar and incubated at 37°C for 18 hrs. For the processing of feed samples the pre-enrichment was done in lactose broth at 37°C for 24 hrs.

a. Phenotypic identification

Phenotypically, for primary diagnosis Gram's staining and motility test was performed. Further identification was done by biochemical tests like oxidase, catalase, indole, methyl red, Voges Proskauer, triple sugar iron and urease test. Later they were confirmed by latex agglutination test.

b. Genotypic identification

All the phenotypically confirmed isolates were subjected to polymerase chain reaction (PCR) for 16S rRNA, *inv*A and *Salmonella enterica* serovar Typhimurium gene using already published primers having annealing temperature 58°C, 60°C and 63°C according to standard methodology [4] with some modifications. The initial denaturation was done at 94°C for 2 min, extension at 72°C for 40s and final extension at 72°C for 5min for 40 cycles. PCR reaction mix was 25 µL consisting of nuclease free water-6.5 µL, primer mix 1, 2 and template-2.0µL each and dreamTaq™ Green PCR Master mix-12.5µL.

2.4 Antibiotic sensitivity of *Salmonella* species

The multidrug resistance profile of *Salmonella* species isolates against 09 antibacterial agents was performed by disc diffusion assay [5].

2.4.1 Kirby-Bauer disk diffusion method for antibacterial susceptibility test (CLSI, 2013)

a. Preparation of Inoculum-Direct colony suspension method

Using a sterile loop a single colony from an 18-24 hrs. old XLD plate was transferred to a tube with sterile physiological saline to make a direct colony suspension. The inoculums were adjusted to 0.5 McFarland standards.

b. Kirby-Bauer disk diffusion method

A sterile non-toxic cotton swab on a wooden applicator (HiMedia) was soaked by dipping into the standardized inoculums. The soaked swab was rotated firmly against the upper inside wall of the tube to express excess fluid. The entire agar surface of the Mueller Hinton agar plate was streaked three times by turning the plate at 60◦ angle between each streaking. The inoculum was allowed to dry for 5-10 minutes with lid in place. Using the aseptic technique the antibacterial discs were applied to the agar plates at least 24mm apart with the help of template under the MH agar plate. The plates were incubated at 37◦C for 24 hrs. The zones of inhibition were measured using a transparent scale and diameters of the zones to the nearest millimeter were recorded.

Table 1: Zone size interpretative criteria for the disk diffusion assay

S. No	Antibacterial agent	Symbol	Concentration mcg/disc	Interpretative Criteria		
				Susceptible Mm or more	Intermediate Mm	Resistant Mm or less
1	Norfloxacin	NX	10	17	13-16	12
2	Ampicillin	AMP	2	18-21	17	13
3	Ampicillin/Sulbactum	A/S	10/10	15	12-14	11
4	Ciprofloxacin	CIP	5	21	16-20	15
5	Co-Trimoxazole	COT	25	16	11-15	10
6	Vancomycin	VA	30	12	-	12
7	Gentamicin	GEN	10	15	13-14	12
8	Ofloxacin	OF	5	2	4	8
9	Streptomycin	S	10	15	12-14	11

3. Results

3.1 Antibiogram of *Salmonella* species in broilers

A total of 13 *salmonella* isolates were isolated by conventional and molecular method from 153 samples consisting of (cloacal swab, dry faeces, feed and water). All the *Salmonella* positive isolates from broiler were subjected to antibacterial sensitivity test by inoculating in to Muller–Hinton agar plates for 09 different antibiotics. Genotypically all the isolates were positive for 16S rRNA and *inv*A gene. The isolates were showing the percentage of *Salmonella enterica* ser. Typhimurium gene was (09/13) 69.23%. The resistant pattern towards different antibiotics in decreasing order were gentamicin (11, 84.61%), norfloxacin and ampicillin / sulbactum (10, 76.92%), ofloxacin and ciprofloxacin (09, 69.23%) and streptomycin and ampicillin (08, 61.53%) as shown in table 2.

Table 2: Antibiogram of *Salmonella* species

S. No.	Antibacterial agent	Susceptible	Intermediate	Resistant	Total
1	Norfloxacin	03 (23.07%)	0 (0%)	10 (76.92%)	13
2	Ampicillin	02 (15.38%)	03 (23.07%)	08 (61.53%)	13
3	Ampicillin/ Sulbactum	01 (7.69%)	02 (15.38%)	10 (76.92%)	13
4	Ciprofloxacin	04 (30.76%)	0 (0%)	09 (69.23%)	13
5	Co-Trimoxazole	07 (53.84%)	03 (23.07%)	03 (23.07%)	13
6	Vancomycin	10 (76.92%)	0 (0%)	03 (23.07%)	13
7	Gentamicin	02 (15.38%)	0 (0%)	11 (84.61%)	13
8	Ofloxacin	04 (13.76%)	0 (0%)	09 (69.23%)	13
9	Streptomycin	02 (15.38%)	03 (23.07%)	08 (61.53%)	13

The sensitivity of vancomycin and co-trimoxazole for *Salmonella* organism were 76.92% and 53.84%.

Table 3: Antibiogram of different isolates

S. No.	Sample no	Norfloxacin	Ampicillin	Ampicillin/ Sulbactum	Ciprofloxacin	Co-Trimoxazole	Vancomycin	Gentamicin	Ofloxacin	Streptomycin
1	11	R	S	R	R	S	R	R	R	S
2	62	S	S	R	S	S	S	R	S	R
3	64	R	R	R	R	R	S	S	R	R
4	71	R	R	R	S	S	S	R	S	R
5	79	R	R	I	R	S	S	R	R	I
6	85	R	I	R	S	I	S	R	R	I
7	90	S	I	I	R	I	S	R	R	R
8	92	R	R	R	S	I	S	R	S	S
9	121	R	R	R	R	S	R	S	R	I
10	143	R	I	R	R	S	S	R	R	R
11	163	R	R	R	R	R	S	R	R	R
12	165	S	R	R	R	S	S	R	S	R
13	171	R	R	S	R	R	R	R	R	R

S= Sensitive, I= Intermediate, R= Resistant

3.2 Multidrug resistance of *Salmonella* isolates

In our study all the isolates from broiler were showing multidrug resistance pattern, in which, single isolate was resistance for 03 antibiotics, 03 isolates were showing resistance for 04, 05 and 06 antibiotics, one isolate was showing resistance for 07 antibiotics and two isolates were resistance for 08 antibiotics.

Likewise single isolate was showing sensitive for 06 antibiotics, 06 isolates were showing sensitive for 02 antibiotics, 02 isolates were showing sensitive for 03 antibiotics, 02 isolates were showing sensitive for 01 antibiotic and 02 isolates were sensitive for 04 antibiotics.

Our isolate no 171 showing resistance for norfloxacin, ampicillin, ciprofloxacin, Co-trimoxazole, vancomycin, ofloxacin and streptomycin. Isolate no 62 showing sensitive for norfloxacin, ampicillin, ciprofloxacin, co-trimoxazole, vancomycin and ofloxacin.

4. Discussion

Antibiotics as growth promoters are of utmost concern because its usage in sub therapeutic levels, stimulate survival of resistant bacteria in the ecosystem [6]. The WHO observed an alarming rate increment of resistant *Salmonella* strains due to the abusive use of antibiotics in intensive animal raising [7]. According to CDC's National Antibacterial Resistance Monitoring System (NARMS), the serovar with greater resistance to antibacterials are *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, *S.* Heidelberg and *S.* Dublin. In terms of multidrug resistance the most prevalent serovar of epidemiological importance are *S.* Typhimurium, *S.* Heidelberg, *S.* Dublin and *S.* Paratyphi [8].

The horizontal transmission of virulence genes in multidrug resistant *Salmonella* strains can increase virulence, invasiveness and it causes multidrug resistance. In north India, in a study it was observed that all the isolates were resistant to bacitracin, polymyxin-B and colistin [9].The isolates of *S.* Enteritidis reported from Namakkal of South India were resistant to erythromycin, ampicillin, kanamycin, cephalothin and tetracycline [10]. Some of the investigators reported that all the isolates of *S.* Gallinarum were found resistant to erythromycin, while 86.7% were resistant to nalidixic acid and 53% were resistant to kanamycin and tetracycline [11]. Moreover, *S.* Typhimurium showed maximum resistance against antibacterials followed by *S.* kastrup. *Salmonella* isolates (*S.* Gallinarum, *S.* Pullorum, *S.* Enteritidis, *S.* Typhimurium) recovered from disease outbreaks in broilers from different regions of Haryana were resistant to multiple drugs [12]. Researchers stated that *S.* Gallinarum (84%), *S.* Enteritidis (10%) and *S.* Typhimurium (6%) isolates from different parts of Haryana of poultry and were resistant to nalidixic acid and carbenicillin [13]. From the environmental samples of layer farms situated in Bareilly, Uttar Pradesh 3.3% *Salmonella* isolates were recovered and all the isolates were found resistant to clindamycin, oxacillin, penicillin and vancomycin [14]. It was found that multidrug resistance of *salmonella* isolates from poultry showing highly sensitive to Ciprofloxacin and moderately sensitive to Gentamycin, kanamycin, Erythromycin and Nalidixic acid [15]. However, the positive isolates were resistant to Azithromycin. In the present study it was found that salmonella isolates were

resistant to ampicillin. Some of the research worker studied that antimicrobial sensitivity results of salmonella isolates from fecal sample of broiler were resistant to sulfamethoxazole, ampicillin, tetracycline, doxycycline and trimethoprim, where 95% of the isolates sensitive to amikacin and polymyxin [16]. In an earlier investigation revealed that the sensitivity of *Salmonella* strains varies towards different antibiotics and found *S.* Gallinarum developed high resistance towards ampicillin, chloramphenicol, nalidixic acid and amoxicillin. Least resistance was developed towards norfloxacin, enrofloxacin and ciprofloxacin [17].

In West Bengal, the *Salmonella* species isolated from environmental samples was 6.1% and the isolates were found to resistant to chloramphenicol, ciprofloxacin, gentamicin, levofloxacin, norfloxacin and oxytetracycline [18]. In a study it was reported that 21 different resistance patterns from 38 isolates which indicates a very heterogeneous *S.* Typhimurium population which reflects a relatively serious public health problem and need strict regulation for the use of antibacterials in the agriculture and human health care sectors [19]. Fluoroquinolones and third generation cephalosporins are among the relatively effective drugs used for treatment of salmonellosis [20].

5. Conclusions

In our study the *Salmonella* isolates were showing maximum resistance towards higher antibiotics gentamicin, norfloxacin, ampicillin/sulbactum, ofloxacin, ciprofloxacin, streptomycin and ampicillin. Maximum isolates were showing resistance to more than 06 antibiotics. Antibiotics vancomycin and co-trimoxazole were sensitive for *Salmonella* organism. It can be concluded that the sensitivity and resistant to antibiotics vary from farm to farm and place to place. Multidrug resistant *Salmonella* organisms are threat to public health.

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