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Original Article

Molecular detection and antimicrobial resistance profile of zoonotic *Salmonella enteritidis* isolated from broiler chickens in Kohat, Pakistan

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Abstract

Background: *Salmonella enteritidis* infection is a frequently encountered zoonotic problem, occurring with concerning regularity in recent years on a worldwide basis. The study that we undertook for the first time detected *S. enteritidis* and associated antimicrobial resistance pattern in broiler chickens.
Methods: A total of 150 different poultry samples were first enriched and grown on selective media, and then processed for molecular detection of *S. enteritidis* by amplification of the *spvb* gene.
Results: The overall detection rate of *S. enteritidis* was 23.3% ($n = 35$), while an increased detection rate of *S. enteritidis* was found in the chicken breast tissue ($n = 9$; 30%). When antibiogram was tested for *S. enteritidis* against common antibiotics, increased resistance to ampicillin ($n = 29$; 82.2%), tetracycline ($n = 28$; 80%), augmentin ($n = 27$; 77.14%), and chloramphenicol ($n = 19$; 54.2%) was observed. Multidrug resistance was reported in 54.8% ($n = 19$) of the *S. enteritidis* isolates, while 20% ($n = 07$) of isolates were extensively drug resistant.
Conclusion: The present study for the first time reports *S. enteritidis* on the basis of *spvb* gene detection. The increased drug resistance in *S. enteritidis* is an emerging problem that could negatively impact efforts to prevent and treat broiler-transmitted *S. enteritidis*.

Keywords: broiler chickens; drug resistance; *Salmonella enteritidis* detection

1. Introduction

The poultry industry has made a major contribution to the food sector of Pakistan, and its products are largely consumed throughout the country in order to meet important protein dietary requirements. However, there is a potential threat of bacterial infection to poultry that can result in a huge economic loss.[1] *Salmonella* is the most commonly reported cause of foodborne disease among bacterial infections.[2] It is estimated that about 94 million cases of gastroenteritis due to *Salmonella* species occur annually worldwide, leading to 155,000 deaths every year.[3] Among *Salmonella* species, *Salmonella enteritidis* is isolated predominantly from poultry and is the most frequent cause of human nontyphoidal salmonellosis.[4] In recent years, *S. enteritidis* has been reported as a major causative agent of foodborne gastroenteritis in humans.[5]

The current emergence of drug resistance in *S. enteritidis* is a major challenge due to the nonjudicious use of antimicrobial agents in the food and livestock sector.[6] Poultry, especially broiler chickens, can harbor antimicrobial-resistant strains and

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function as a vehicle for dissemination of these pathogens to humans.[7] Standard culture and serological methods for the detection of *S. enteritidis* are employed as disease control measures; however, polymerase chain reaction (PCR) is a preferred diagnostic method due to its reliable sensitivity, specificity, and detection speed.[8] The *spvb* gene, commonly involved in bacterial virulence, is routinely used for the detection of *S. enteritidis*.[9] As far as literature mining is concerned, no data exist on the molecular detection and drug resistance pattern of *S. enteritidis* from the Kohat region of Pakistan.

The present study has documented for the first time molecular detection of *S. enteritidis spvb* gene and its associated drug resistance pattern against commonly used antibiotics. The finding of this study will be efficacious in better controlling antibiotic resistance among *S. enteritidis* isolates from broiler chicken samples.

2. Methods

2.1. Sample collection and processing

The present study was conducted at the Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan, during the period from December 2014 to August 2015. A total of 150 different broiler chicken samples (30 samples each of heart, liver, kidney, breast tissue, and leg piece) were collected from different retail markets of three main areas of Kohat. Random samples were collected from individual chickens (1 sample from 1 chicken, which means 150 samples from 150 chickens). All the samples were processed separately and washed thoroughly with autoclaved water to avoid any cross contamination between two samples. The samples were collected in peptone water-filled sterile plastic bags and immediately transported on ice to the laboratory for inoculation on enriched medium.

2.2. Isolation and identification of Salmonella species

The culturing method to detect *Salmonella* species involved selective enrichment followed by plating on selective agar. One gram of poultry sample was added to 9 mL of tetrathionate broth and was incubated at 37°C for 24 hours. A loop full of broth culture from tetrathionate was streaked onto a plate of bismuth sulfite agar. Bismuth sulfite agar is the selective medium for the growth of *Salmonella* species. The plates were incubated at 37°C for 48 hours and checked for the growth of typical black *Salmonella* species colonies.

The presumptive colonies of *Salmonella* species were taken for further confirmation by biochemical testing, including oxidase, catalase, triple sugar iron slant reaction, motility, indole, urease, and citrate utilization tests, as described earlier.[10]

2.3. DNA extraction from bacterial culture

DNA was extracted by genomic DNA purification kit (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's protocol. Briefly, bacterial cells were resuspended in Tris-EDTA buffer. The sample was mixed with lysis solution and incubated at 65°C for 5 minutes. Subsequent to incubation, absolute chloroform was added and centrifuged at 12,880 *g* for 2−3 minutes. Following centrifugation, the upper aqueous phase was mixed with the precipitation solution and centrifuged at 10,000 rpm for 2−3 minutes. The pallet was dissolved in NaCl solution and processed for ethanol precipitation step. After incubation at −20°C for 10 minutes, the supernatant was centrifuged at 10,000 rpm for 5 minutes. The ethanol was removed and the DNA pallet was dissolved in TE buffer. The concentration and quality of DNA was checked using the Nano-drop equipment (Thermo Scientific).

2.4. Molecular detection of S. enteritidis

S. enteritidis, and *S1* and *S4* genes were detected using PCR (Gradient Thermal Cycler; Eppendorf, Hamburg, Germany). *S. enteritidis* was detected by the amplification of the *spvb* gene using specific primers.[9] Briefly for PCR, 3 μL of DNA was added to 25 μL of the reaction mixture containing 4 μL prepared master mix (Deoxynucleotide Triphosphate (dNTPs), 10× PCR buffer, Taq polymerase, and $MgCl_2$), and 1 μL of forward and reverse primer, while the remaining 16 μL was equalized by nuclease-free water. The prepared PCR tubes with the master mixture were placed in a gradient thermal cycler. Amplification was carried out with initial denaturation at 95°C for 5 minutes, followed by 34 cycles of denaturation (94°C for 1 minute), annealing (53°C for 1 minute), and extension (72°C for 1 minute). A final extension step was carried out at 72°C for 5 minutes. The amplified DNA product from *S. enteritidis*-specific PCR along with the positive control (*S. enteritidis*) and negative control (*Escherichia coli*) were analyzed with 1.3% agarose gels stained with ethidium bromide and visualized by UV transillumination.

2.5. Antimicrobial susceptibility testing of S. enteritidis

All the isolates that were identified as *S. enteritidis* on PCR were tested for antimicrobial susceptibility on Mueller−Hinton agar using Kirby−Bauer disk diffusion assay.[11] The antibiotics tested were ampicillin (30 μg), augmentin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), aztreonam (30 μg), tetracycline (30 μg), azithromycin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), and levofloxacin (5 μg) (Oxoid, Basingstoke, UK). The results were interpreted as resistant, intermediate, and susceptible, as described by Clinical and Laboratory Standard Institute guidelines.[12] Multidrug resistant (MDR) is defined as a microorganism resistant to at least one antibiotic in three or more antimicrobial categories, while extensively drug resistant (XDR) is defined as a microorganism resistant to at least one agent, but sensitive to two or equal categories. These MDR and XDR were reported as per criteria.[13]

3. Results

A total of 150 broiler chicken samples were obtained from Kohat and processed for molecular detection, and the total

number of *Salmonella* species identified by culture and biochemical techniques was 51 (34%). *S. enteritidis* was detected in 35 (23.33%) samples among the biochemically identified *Salmonella* species. The size of the amplified *spvb* gene was 250 bp (Fig. 1).

In specimen-wise distribution of *S. enteritidis* that included different parts (heart, kidney, liver, breast tissue, and leg piece) of poultry, a higher isolation rate of *S. enteritidis* was noticed in breast tissue ($n = 9$, 30%), while the lowest detection rate was observed in heart samples ($n = 4$; 13.3%; Table 1).

When antibiotic susceptibility was checked, most *S. enteritidis* isolates were resistant to ampicillin ($n = 29$; 82.8%), tetracycline ($n = 28$; 80%), and augmentin ($n = 27$; 77.1%). *S. enteritidis* showed resistance to ciprofloxacin (42.8%) and levofloxacin (40%); however, it showed less resistance against third-generation cephalosporins (including ceftazidime, cefotaxime, and ceftriaxone; Table 2). MDR and XDR patterns were also reported among 35 *S. enteritidis* isolates, in which 25.7%

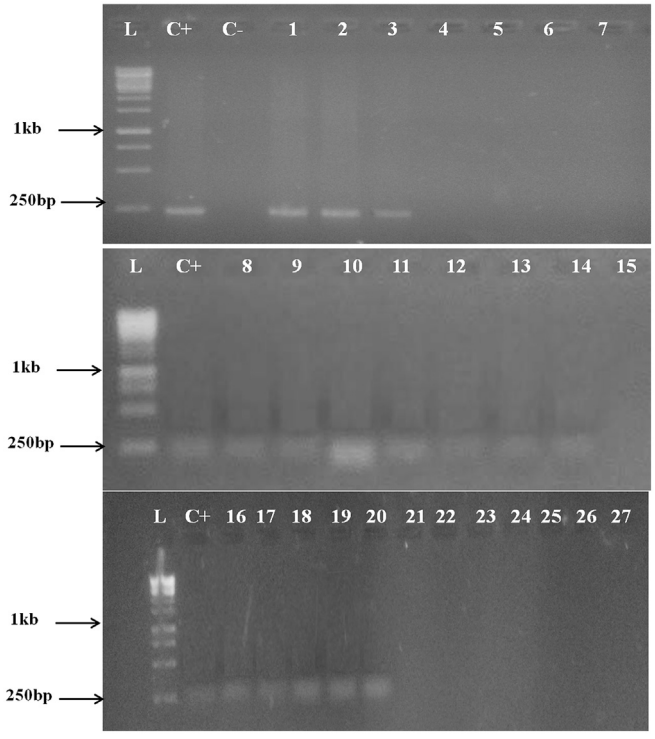

Fig. 1. Molecular detection of *Salmonella enteritidis*. Amplified *spvb* gene of *S. enteritidis* product size = 250 bp. L = ladder (1 kb); C+ = positive control (*S. enteritidis*); C− = negative control (*Escherichia coli*); 1, 2, 3, 8, 9, 10, 14, 16, 17, 18, 19, 20 = samples positive for *S. enteritidis*; 4, 5, 6, 7, 15, 21, 22, 23, 24, 25, 26, 27 = samples negative for *S. enteritidis*.

Table 1
Specimen-wise distribution of *Salmonella enteritidis*.

Specimen	Sample *N*	*S. enteritidis* positive *N* (%)	*S. enteritidis* negative *N* (%)
Heart	30	4 (13.3)	26 (86.6)
Kidney	30	6 (20)	24 (80)
Liver	30	8 (26.6)	22 (73.3)
Leg piece	30	8 (26.6)	22 (73.3)
Breast tissue	30	9 (30)	21 (70)

Table 2
Drug resistance pattern of *Salmonella enteritidis*.

Antimicrobial drug category	Code	*S. enteritidis*, *N* (%)		
		R	I	S
Penicillins				
Ampicillin	AMP	29 (82.85)	04 (11.42)	02 (5.71)
Augmentin	AUG	27 (77.14)	04 (11.42)	04 (11.42)
Cephalosporins				
Ceftazidime	CAZ	08 (22.85)	02 (5.71)	25 (71.42)
Cefotaxime	CTX	07 (20)	00 (00)	28 (80)
Ceftriaxone	CRO	05 (14.42)	00 (00)	30 (85.71)
Monobactam				
Aztreonam	ATM	04 (11.42)	01(2.85)	30 (85.71)
Phenicols				
Chloramphenicol	C	19 (54.2)	03 (8.5)	13 (37.1)
Tetracycline	TET	28 (80)	04 (11.42)	03 (8.57)
Macrolides				
Azithromycin	AZM	10 (28.57)	11 (31.42)	14 (40)
Fluoroquinolones				
Ciprofloxacin	CIP	15 (42.85)	09 (25.71)	11 (31.4)
Levofloxacin	LEV	14 (40)	12 (34.28)	09 (25.71)

I = intermediate sensitive; R = resistant; S = sensitive (Clinical and Laboratory Standard Institute 2014).

($n = 9$) were non-MDR. Additionally, 54.8% ($n = 19$) were MDR, while 20% ($n = 7$) were the XDR isolates (Table 3).

4. Discussion

Salmonellosis is the primary cause of foodborne diseases globally.[14] A broad range of foodstuff has been associated with such diseases. However, food from animal sources, especially if poultry derived, has been implicated in periodic cases and outbreaks of human salmonellosis.[15]

In the present study, *S. enteritidis* was detected in poultry samples. Moreover, their antimicrobial susceptibility pattern was also reported. In a total of 150 broiler chicken samples, the prevalence of *S. enteritidis* was 23.3%, while an increased detection rate (30%) was observed in breast tissue. The results of our investigation are compatible with those of a study conducted in Faisalabad, Pakistan.[4] Another study in Iran also reported 25% prevalence of *S. enteritidis* from broiler poultry farms.[7]

Resistance of *Salmonella* to antimicrobials is an emerging problem in developing and developed countries.[16] In our study, *S. enteritidis* isolates were resistant to commonly used antibiotics, i.e., ampicillin (82.85%), tetracycline (80%), augmentin (77.14%), and chloramphenicol (54.2%), which is in line with the findings of Beyene et al,[17] in which resistance to ampicillin, amoxicillin, and chloramphenicol were 75%, 75%, and 50%, respectively. However, our results are in contrast with the

Table 3
MDR and XDR patterns of *Salmonella enteritidis*.

Drug resistance pattern	*S. enteritidis*, *N*	*S. enteritidis*, %
Nonmultidrug resistant	09	25.7
Multidrug resistant	19	54.8
Extensively drug resistant	07	20

MDR = multidrug resistant (nonsusceptible to ≥1 agent in ≥3 antimicrobial categories); XDR = extensively drug resistant (nonsusceptible to ≥1 agent in all but sensitive to ≥2 categories).

findings of the 2010 study of Akhtar et al,[4] in which isolates of *S. enteritidis* were mostly sensitive to ampicillin, tetracycline, and chloramphenicol. The notably high rate of antimicrobial-resistant *S. enteritidis* strains in this study is probably due to the early introduction and subsequent widespread use of these antibiotics in human and veterinary medicine in our area.

The fluoroquinolone-class antibiotics ciprofloxacin and levofloxacin showed moderate resistance to *S. enteritidis* isolates (42% and 40%, respectively); however, a previous study reported that *S. enteritidis* isolates were sensitive to ciprofloxacin.[18] Fluoroquinolone resistance among *S. enteritidis* isolates might designate the common use of these antibiotics.

In the current study, *S. enteritidis* was least resistant to third-generation cephalosporin. Lower rates of cephalosporin resistance in this study are consistent with the results of Abdel-Maksoud et al's[19] study in 2015, who reported a low prevalence of cephalosporin resistance among *S. enteritidis* isolates from poultry sources. Lower resistance of *S. enteritidis* to cephalosporin is valuable to the community as cephalosporin resistance is a noteworthy public health concern.

Overall, MDR was observed among 54.2% *S. enteritidis* isolates. Our findings are in line with the results of a study by Hur et al[20] in 2011, in which 65.2% *Salmonella* isolates were multiple drug resistant. Another study from Brazil also reported 63.9% multidrug-resistant *S. enteritidis* isolates from chicken carcass samples.[18] However, another study reported a high prevalence of MDR of 90.9% to *Salmonella enterica* serovars *Indiana* and *enteritidis*.[21] In one recent study, 35.5% MDR *Salmonella* species isolates were reported.[22] The increased MDR isolates can be due to the use of antimicrobial drugs in poultry food at a subtherapeutic level, which can promote antimicrobial-resistant strains.[23,24]

In the current study, elevated levels of *S. enteritidis* were detected in broiler chickens. Increased drug resistance was observed to commonly used antibiotics, which suggests an emerging problem and could negatively impact an effort to prevent and treat broiler-transmitted zoonotic *S. enteritidis*.

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