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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

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