Journal of Population Therapeutics & Clinical Pharmacology

Prevalence of *Salmonella enteritidis* and *Salmonella typhimurium* in raw and minced beef of districts Attock and Chakwal regions, Pakistan

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Abstract

Salmonella. enteritidis and *Salmonella*. *typhimurium* is responsible for gastroenteritis, also called *Salmonella* influenza. *Salmonella* species have been discovered over wide range of environment including food chain. In order to isolate and characterize the *S. enteritidis* and *S. typhimurium* from raw beef and its products, a total 100 samples of raw beef and minced beef were aseptically collected from butcher's shops and marketplaces located across both the districts of Attock (n=50) and Chakwal (n=50). Xylose Lysine Deoxycholate and *Salmonella* Shigella agar media were utilized to culture and isolate *Salmonella spp*. Further molecular identification was done to identify *Salmonella enteritidis* and *Salmonella typhimurium*. Total 30 samples were found positive for *Salmonella spp* with 70% positive samples of S. *enteritidis* and 30% positive samples of *S. typhimurium*. At district level, out of 50 samples from Attock, 16 samples were positive for *Salmonella* with 68.75% positive samples of *S. enteritidis* and 5 31.25% positive samples of *S. typhimurium*. While out of 50 samples from Chakwal, 14 samples were positive for *Salmonella* with 71.4% positive samples of *S. enteritidis* and 28.6% positive samples of *S. typhimurium*. No sample was found positive for *Salmonella* collected from the super market in the frozen form from the both districts. Overall, the contamination of *S. enteritidis* (70%) was found higher than the *S. typhimurium* (30%) in both the districts. Antimicrobial susceptibility pattern of *S. enteritidis* and *S. typhimurium* revealed that resistance was showed to antibiotics like Ampicillin, Penicillin, Amoxicillin, Erythromycin, Streptomycin, and bacterial isolates found sensitive to cepahalosporins like Cephazolin and

Ceftazidime. The present study revealed that beef samples from both the districts of Attock and Chakwal were contaminated with *S. enteritidis* and *S. typhimurium*.

Keywords: Nontyphoidal Salmonella, Raw beef, Minced beef, PCR, Antimicrobial Resistance.

1. Introduction

Food is vital essentials for the continuously growing populations to exist. Though, owing to the truly mounting requirements it is becoming more tough to keep strict quality control measures in food supplies. Billions of people are in risk of food borne maladies, which is resulting into a major concern to global health (Fung et al., 2018). As per the Center of Disease Control and prevention (CDC), nontyphoidal *Salmonella* are considered as the highest reasons for looseness of the bowels alongside gastroenteritis worldwide by causing around 153 million deaths of gastroenteritis and in excess of 57,000 deaths internationally every year (Healy & Bruce, 2022). It is assessed that typhoidal *Salmonella* causes about 21.7 million intestinal or typhoid fever cases and 5.4 million paratyphoid fever cases, which results in with regards to 0.22 million deaths overall every year (Harish & Menezes, 2011). In 2017, about 535 000 cases of non-typhoidal salmonellosis were accounted for, which came about in around 77,500 deaths around the world (Stanaway et al., 2019). Both the serovars of Salmonella enterica, i.e. enteritidis and typhimurium, are the two predominant Salmonella enterica serovars which are the most leading causative agents of major food borne illnesses in humans globally (Afshari et al., 2018). The foodborne invasive Salmonellosis in humans is most significantly caused by consumption of foods from animal sources like poultry, pork, mutton, beef and its products contaminated via *S. enteritidis* and *S. typhimurium* (Antunes et al., 2016). Pakistan produces about 4.5 million tons of meat annually, out of which beef production comprises of about 2.3 million tons (Sohaib and Jamil, 2017). One of the major bases of foodborne ailments worldwide is raw meat as it facilitates the rapid growth of microorganisms including certain foodborne pathogens owing to its high nutrient content (Tan et al., 2019). Raw meat containing even low dose of 10^6 cells of pathogenic *Salmonella* spp. could be a possible source of foodborne invasive Salmonellosis in humans if it is not properly handled, stored or cooked (Obeid et al., 2019). The likelihood of contamination of meat by pathogenic *Salmonella* like *S. enteritidis* and *S. typhimurium* is due to cross-contamination at the slaughterhouse, marketplace, unhygienic packaging, malapropos storage and poor handling procedures (Obeid et al., 2019; Sohaib and Jamil, 2017). Due to lack of awareness regarding foodborne pathogens like *S. enteritidis* and *S. typhimurium,* enhanced mortality and morbidity in Pakistan has been reported (Huda et al., 2010). Raw food variety cleanliness and anti-microbial obstruction knowledge is

still in its infancy in many developing countries, including Pakistan, which has increased the risk of foodborne microbes having a range of resistant genes. By reason of the extensive tradition of antimicrobial medications to avoid disease and enhance research in cattle and other animals, MDR *Salmonella* strains have emerged (Monte et al., 2019). MDR *Salmonella* has contaminated food animals with the extensive usage of antimicrobials in veterinary treatment (Chuanchuen et al., 2010). The transfer of antimicrobial-resistant microorganisms originating from animals to the humans is probing a significant health risk to humans (Khemtong & Chuanchuen, 2008).

It was the need of hour to examine the quality of foods such as raw beef and enhance the food safety in order to prevent foodborne Salmonellosis in Pakistan and this study will be helpful to launch effective prophylactic program against nontyphoidal foodborne Salmonellosis.

The objectives of the current research study were to isolate *Salmonella enteritidis* and *Salmonella typhimurium* from raw beef and identify it via Molecular characterization as well as determination of the antibiotic resistance profile of isolated *Salmonella enteritidis* and *Salmonella typhimurium.*

2. Methodology

This study was a cross sectional study comprising of randomly selected butcher's shops and marketplaces located across each tehsil of both the districts Attock and Chakwal.

2.1 Sample collection and culturing

A total 100 samples (total n=100) of raw beef (n=50), and minced beef (n=50), each weighing roughly 50g from butcher's shops, including a total 10 samples (n=5 from each district) of minced beef in frozen form from super markets located in both the districts of Attock and Chakwal were collected aseptically in sterilized plastic-bags and labelled. The samples were conserved in icebox and transported to the laboratory for additional processing under cold circumstances within 6-8 hours. XLD (Xylose Lysine Deoxycholate) and SS (*Salmonella* Shigella) agars were utilized to isolate *Salmonella* after pre-enrichment and selective enrichment (Pal, 2012). Once isolates were placed on culture medium, plates incubated for 24 hrs at 37°C in an aerobic environment.

2.2 Microscopic determination of isolates

Isolates were identified by Gram reaction. Briefly, with the help of a dropper one droplet of distilled water was put on a glass-slide, a sterile platinum wire loop was used to pick bacterial colony from culture media and was mixed in order to make a thin smear on a glass slide. Smear was air-dried for few a minute and heat-fixed by slightly facing the slide on the oxidizing flame. After heat fixing glass slides were placed on staining racks and with the help of a dropper smear

were dipped in crystal violet stain and let aside for 30 secs. After 30 secs, the glass slide was rinsed for 5 secs with water. After that, Gram's iodine solution was added and set aside for 1 minute. After one min, the glass slide was cleaned for 5 secs by utilizing water. Ethanol (95%) was used to decolorize the smear for 15-30 seconds. Then glass slide was washed for 5 secs by water. Then, the glass-slide was flooded with safranin counter strain for 60 to 80 secs. The glass-slide was washed by water for 5 secs and the absorbent paper was used to clean and wipe back and the front side of the slide. The slides were observed microscopically using 40 X and 100 X (oil immersion) objective lens in order to examine the morphology and Gram reaction of bacteria (Cheesbrough, 2006).

2.3 Biochemical identification of isolates

Gram-negative rods identified by microscopic examination were characterized by the following biochemical tests.

2.3.1 Catalase test

On the basis of catalase enzyme production, the test was performed to distinguish catalase producing bacteria from catalase non-producing bacteria in the beef samples. Two drops of hydrogen peroxide (H_2O_2) solution on the slide were put. By utilizing a sterile glass rod or wooden stick, the test bacteria were mixed in one drop of H_2O_2 solution. The catalase enzyme was capable of breakdown the hydrogen peroxide into water and oxygen gas. Immediate bubble formation due to effervescence of oxygen indicated catalase-positive test while no bubble formation indicated a negative catalase test (Cheesbrough, 2006).

2.3.2 Methyl red test

To evaluate the fermentation behavior of all gram-negative bacteria, a methyl red test was used. It was utilized to distinguish between *Salmonella enteritidis* and *Salmonella typhimurium* colonies. Due to the generation of tiny organic acids by mixed acid fermenters, the medium became acidic. Butanediol and acetoin were generated by butanediol fermenters. Bacteria were injected into MR/VP broth media and incubated for 24 hrs at 37^{o}C. 0.2 mL of methyl red indicator was added after incubation and placed at room temperature for 15 mins and noted results as after the methyl red indicator turns into red then the production of red color indicated a positive test was confirmed while in case of production of the yellow color of methyl red indicator the test was negative was confirmed (Anokhe et al., 2021).

2.3.3 Voges Proskauer test

Voges Proskauer (VP) test was performed for the detection of all gram-negative rod-shaped bacteria. MR/VP broth contains buffered peptone, glucose and dipotassium phosphate (pH = 6.9). In this test glucose was metabolized by bacteria which produced 2, 3-butanediol, red color

is produced after reaction with peptone (Leboffe et al., 2021). VP medium bacteria were injected into test tubes and put in incubator at 37°C for 24 hrs. Followed by the addition of 0.6 ml of alpha naphthol (Barrett's solution) and 0.2 ml of potassium hydroxide in each tube and shake them and place the tubes at room temperature for 15 mins. The result was recorded by a change in the color in 15 minutes. Production of red color indicated a positive result. Negative Voges Proskauer culture produced copper-like color after 1 hour, so it should not read out (Anokhe et al., 2021).

2.4 Storage of bacterial isolates

Morphologically, biochemically and genetically identified *S. enteritidis* and *S. typhimurium* were stored in the form of glycerol stocks for long term storage purpose. Briefly, the purified bacterial colony was resuspended in 20 % glycerol containing nutrient broth and incubated for 8 hours in shaking incubator. Then glycerol stocks of *Salmonella enteritidis* and *Salmonella typhimurium* were frozen at -20 ºC freeze (Cody et al., 2008).

2.5 Molecular identification of *S. enteritidis* and *S. typhimurium*

Morphologically and bio-chemical characterized *Salmonella enteritidis* and *Salmonella typhimurium* were identified at the genetic level. Initially, bacterial chromosomal DNA was extracted, quantified and identified by PCR reaction using a species-specific primer.

2.5.1 DNA extraction

Genomic DNA-extraction was performed by boiling method. Briefly, sterile Eppendorf tubes of 2 ml were engaged and filled with 200 µl of nucleic acid-free double distilled water by utilizing a micropipette. Using a sterile toothpick single bacterial colony was picked off from culture and thoroughly resuspended in the Eppendorf. Then, Eppendorf tube was sealed with parafilm to prevent from the accidental opening of the lid during boiling. The microcentrifuge was placed in water both at 95 ºC for 10 mins. Finally, centrifuged at 14000 rpm for 5 minutes and 100 µl of supernatants were collected by micropipette for as extracted chromosomal DNA and cell debris was discarded (Jamshidi et al., 2010).

2.5.2 Polymerase chain reaction

Conventional PCR (Multiplex PCR) was performed for the detection of *S. enteritidis* and *S. typhimurium* by using primers to amplify the *prot6E* and *fliC* gene regions through thermocycler, creating an amplicon of size varying between 185 bp and 559 bp depending on the type of *Salmonella spp.* (Afshari et al., 2018).

Both *prot6e-6* and *prot6e-5* primers being specified for *prot6E*-gene were utilized for identification of *S. enteritidis*, whereas both *Fli15* and *Tym* primers being specified for *fliC*-gene were utilized for identification of *S. typhimurium*. Primer sequences are given in Table 1.

Table 1: Oligonucleotide sequence of *prot6e* and *fliC* primers for multiplex PCR.

Primer Name	Oligonucleotide sequence (5′–3′)	Target gene	Product size in bp	Reference
Prot6e-5-F *Prot6e-6-R*	ATATGGTCGTTGCTGCTTCC CATTGTCCACCGTCACTTTG	*Prot6E*	185	Afshari et al., 2018
Fli15-F *Tym-R*	CGGTGTTGCCCAGGTTGGTAAT ACTCTTGCTGGCGGTGCGACTT	*fliC*	559	Afshari et al., 2018

Purified extracted DNA (2 µL) and 0.5 µL each of all forward and reverse primers were added to 2X-DreamTaq green PCR master-mix and then nucleic-acid-free double distilled water was added to adjust its final volume. The order of amplification was: one cycle for initial denaturation (duplex-unwinding) of 5 minutes duration at 9 ºC; followed by 35 cycles each comprising of one minute for denaturation at 95ºC, 30 secs for annealing (primer-binding) at 56ºC, 90 secs for elongation (duplex amplification) at 72ºC; ending with one cycle of 10 mins for final elongation (DNA amplification) at 72ºC. (Afshari et al., 2018). The obtained amplicons were then analyzed using 1.5 percent agarose-gel and their sizes were assessed by DNA ladder.

2.6 Antibiogram

Disc diffusion assay was performed to assess the antibiotics sensitivity of the characterized *Salmonella enteritidis* and *Salmonella typhimurium* isolates on Muller Hinton agar media. The antibiotics ampicillin (10 µg), amoxicillin (30 µg), cephazolin (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin (10 µg), streptomycin (10 µg), trimethoprim (5 µg) and vancomycin (30 µg) were selected according to Clinical and Laboratory Standards Institute (CLSI), 2023 guidelines (Gaur et al., 2023). Results were obtained by measuring the zones of inhibition.

2.6.1 Inoculation && measurement zones of inhibition

The bacterial suspension was inoculated on already prepared MH-agar and all the petri plates were labelled accordingly. Then, the antibiotic discs were properly placed using disc-dispenser and all the inoculated plates were then incubated over-night at 37 ºC. On the following day, the measurement of zones of inhibition was done using a ruler by measuring the zone diameter up

to the boundary of growth in order to assume the results by following the CLSI, 2023 guidelines (Gaur et al., 2023).

3. Statistical analysis

All the data was processed via Microsoft excel, latest version of office 365.

4. Results

Out of 100, 30 (30%) samples were positive for *Salmonella* with 21 (21%) positive samples of *S. enteritidis* and 9 (9%) positive samples of *S. typhimurium*. At district level, out of 50 samples from Attock, 16 (32%) samples were positive for *Salmonella* with 11 (22%) positive samples of *S. enteritidis* and 5 (10%) positive samples of *S. typhimurium*. While out of 50 samples from Chakwal, 14 (28%) samples were positive for *Salmonella* with 10 (20%) positive samples of *S. enteritidis* and 4 (8%) positive samples of *S. typhimurium*. None of the total 10 samples was found positive for *Salmonella* collected from the super market in the frozen form from the both districts of Attock and Chakwal.

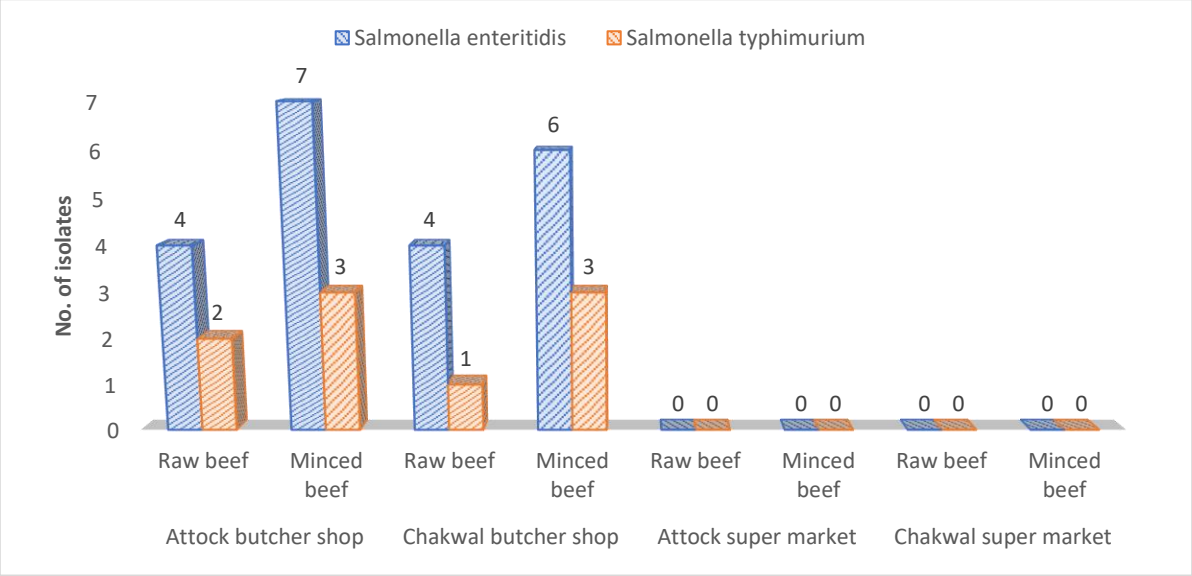

Figure 1. District wise isolation of *S. enteritidis* and *S. typhimurium* from samples of raw and minced beef

4.1 Morphological and biochemical examination

After culturing the samples, 30 positive samples were initially observed under microscope, found as rod shaped bacterial colonies and were gram negative rods. Similarly, all isolates were catalase, methyl red positive and negative for Voges Proskauer test.

4.2 Antibiogram

Anti-microbial susceptibility pattern of *S. enteritidis* isolated from district Attock beef samples revealed that 82% of *S. enteritidis* population showed resistance to penicillin, and 64% of the isolates were found with resistance to erythromycin, ampicillin, streptomycin, and vancomycin antibiotics. *S. enteritidis* (91%) were sensitive to ceftazidime and 82% to cephazolin as shown in figure 2. *S. typhimurium* isolated from district Attock revealed that 60% of the bacterial population were resistant to penicillin, erythromycin, ampicillin, streptomycin, and vancomycin antibiotics. All the *S. typhimurium* isolates (100%) were found sensitive to ceftazidime (Figure 3).

Similarly, anti-microbial susceptibility pattern of *S. enteritidis* isolated from district Chakwal beef samples showed that 80% of bacterial population were resistant to penicillin and 70% of the *S. enteritidis* isolates showed resistance to ampicillin, erythromycin, streptomycin, and vancomycin. *S. enteritidis* (90%) were sensitive to ceftazidime and 80% to cephazolin as shown in figure 4. *S. typhimurium* isolated from district Chakwal beef samples revealed that 75% of *S. typhimurium* population were found resistant to erythromycin, ampicillin, penicillin, streptomycin, and vancomycin antibiotics. All the *S. enteritidis* isolates (100%) were found sensitive to ceftazidime (Figure 5).

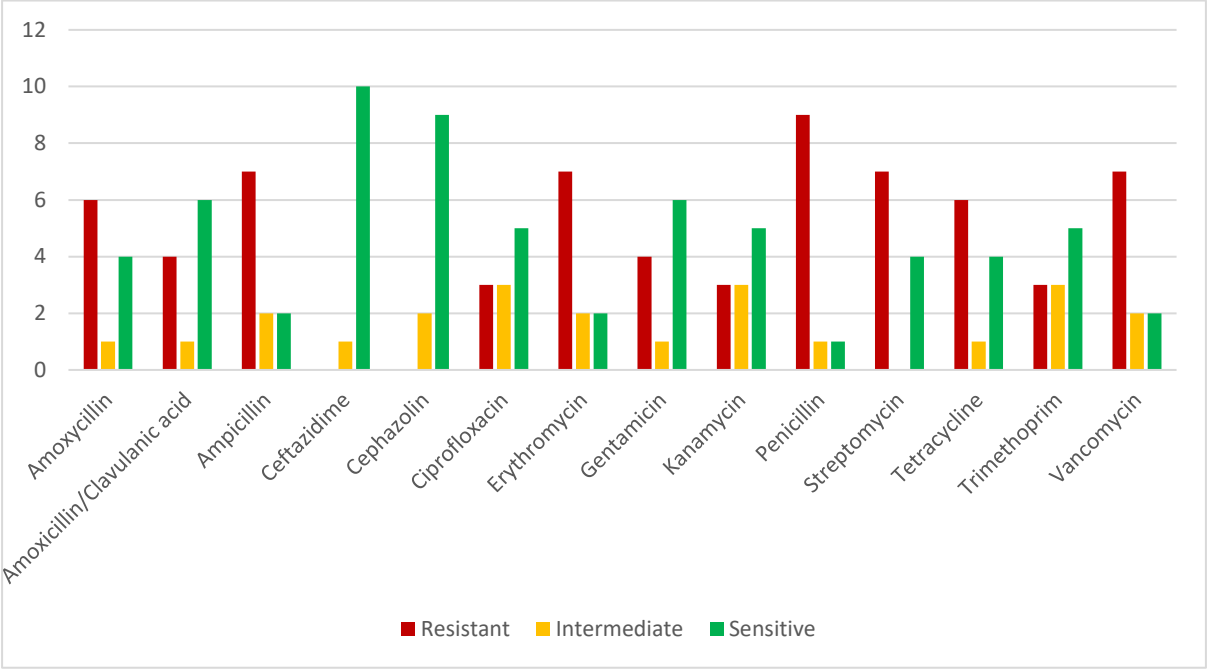

Figure 2: Anti-microbial susceptibility pattern of *S. enteritidis* (Attock)

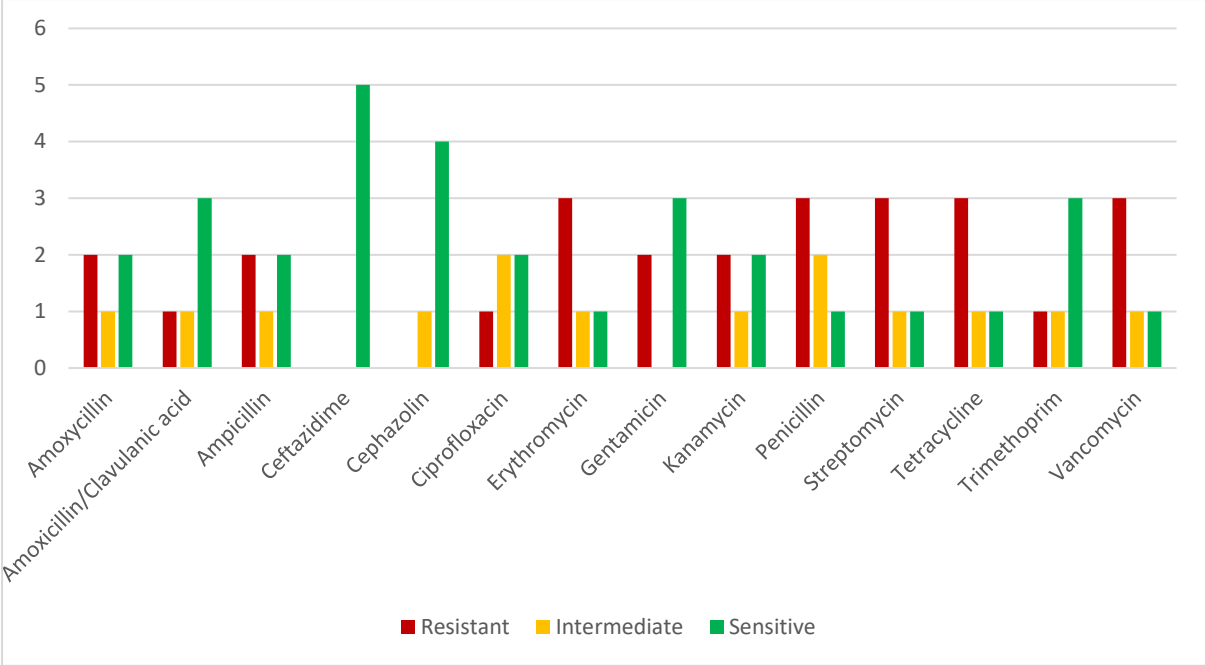

Figure 3: Anti-microbial susceptibility pattern of *S. typhimurium* (Attock)

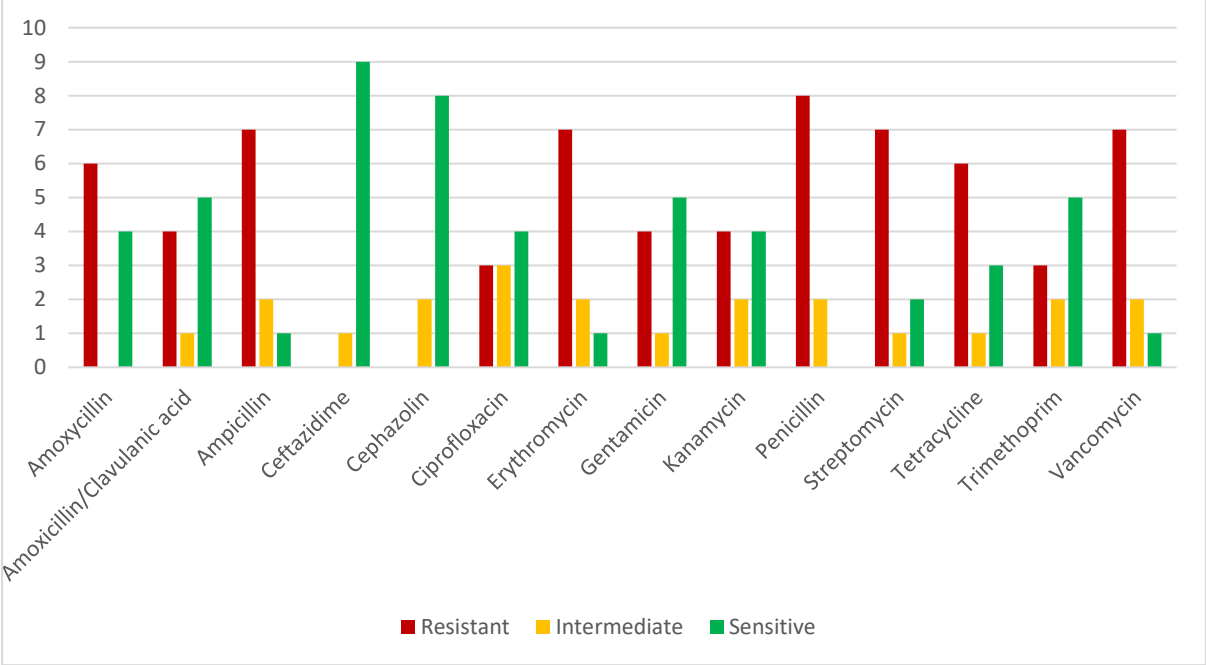

Figure 4: Anti-microbial susceptibility pattern of *S. enteritidis* (Chakwal)

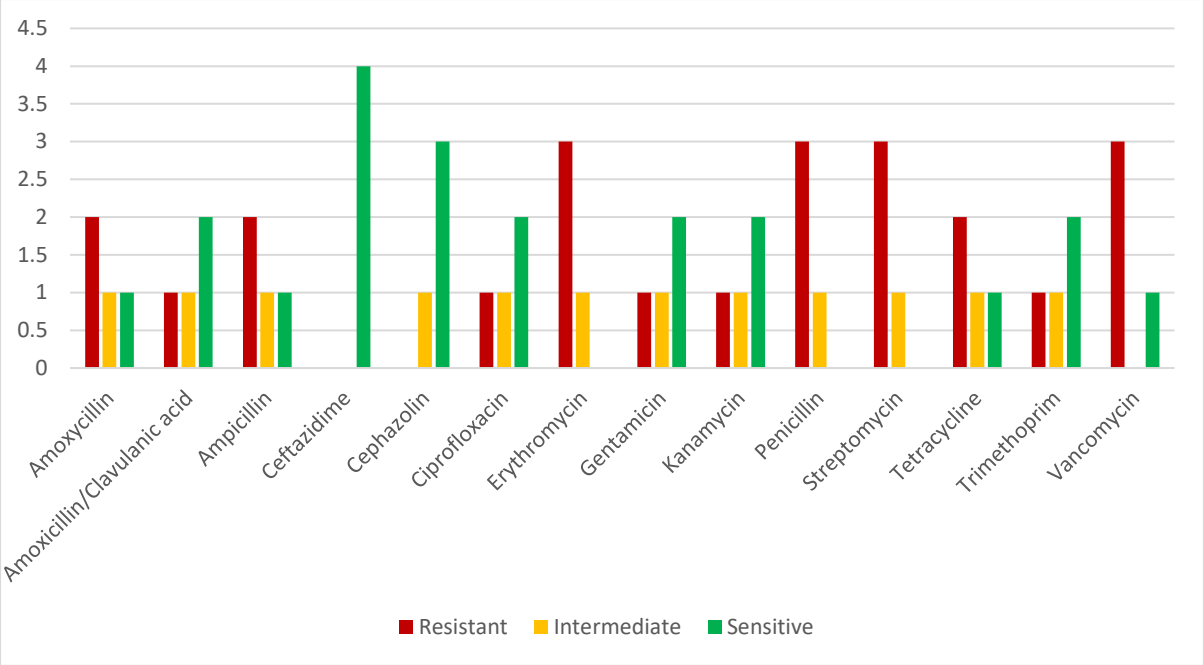

Figure 5: Anti-microbial susceptibility pattern of *S. typhimurium* (Chakwal)

4.3 Genetic Characterization of *Prot6e and fliC* genes

The primers of *prot6e-6* and *prot6e-5* specified for *prot6e*-gene were employed for identification of *S. enteritidis*. The amplified product size was about 185 bp and visualized on ethidium bromide-stained 1.5 percent agarose-gel as shown below in figures 6 and 7. The primers of *Fli15* and *Tym* specified for *fliC*-gene were employed for the identification of *Salmonella typhimurium*. The amplified product size was about 559 bp and visualized on ethidium bromide-stained 1.5 percent agarose gel as shown below.

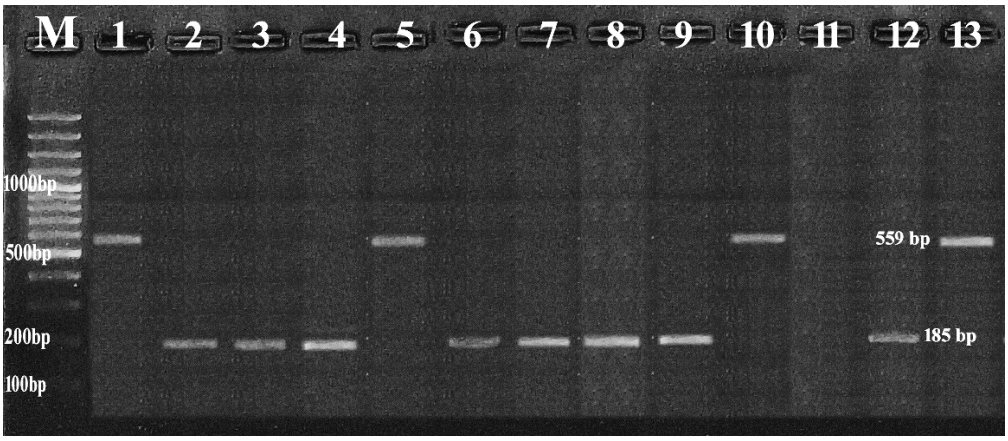

Figure 6: Gel Electrophoresis image showing positive samples for *S. enteritidis* (185bp) and *S. typhimurium* (559bp), M is 100bp DNA ladder while Lane 1 to 10 are samples from Attock district, Lane 11 is negative control, while Lanes 12 and 13 are positive controls.

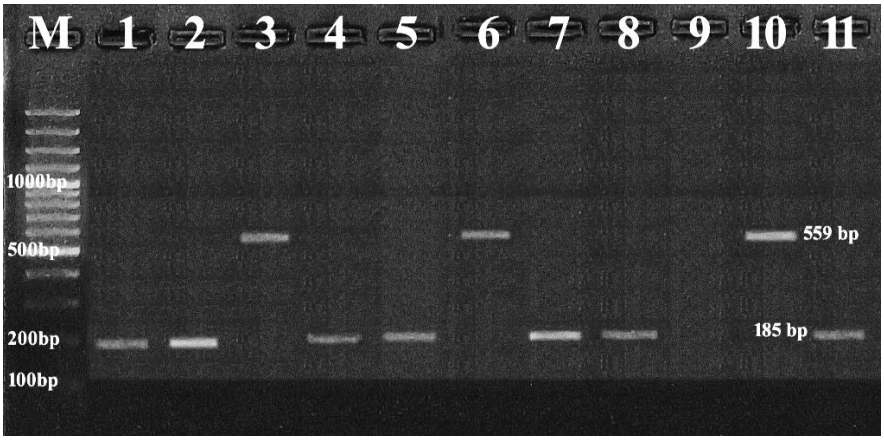

Figure 7: Gel Electrophoresis image showing positive samples for *S. enteritidis* (185bp) and *S. typhimurium* (559bp), M is 100bp DNA ladder while Lane 1 to 8 are samples from Chakwal district, Lane 9 is negative control, while Lanes 10 and 11 are positive controls.

5. Discussion

Food derived from animals, like beef is thought as a main vehicle for the spread of Salmonella to human-beings (Almeida et al., 2013). Salmonella infection in raw beef sold in butchers' shops as well as super-stores was the focus of this investigation. Salmonella isolates found in raw beef in Attock and Chakwal (Pakistan), have been characterized and shown to be somehow antibiotic-resistant, according to this article. Raw beef and minced beef samples from both the districts of Attock and Chakwal had a significant prevalence of Salmonella, which is consistent with prior findings (Barkocy et al., 2003). The further unhygienic handling resulted that minced beef samples were more contaminated as compared to raw beef samples. It's important to highlight that the samples from the supermarket in frozen-form had no *Salmonella*. When compared to studies of Hyeon et al. (2011), similar prevalence of *Salmonella* spp. found a in raw beef samples employed in recent study. In a study led in Karachi reported 21.6% Salmonella contamination in raw beef (Altaf et al., 2020), which correlated with current the results of current study. Significant population of *Salmonella* isolates were found resistant to ampicillin, penicillin, amoxicillin, erythromycin, streptomycin, tetracycline and vancomycin from raw beef and minced beef of both the districts Attock and Chakwal, which is consistent to the investigation of Pakistan's domesticated animal industry (Rahman & Mohsin, 2019). Antibiotics are very comprehensively employed in cows and other live-stock for prophylaxis and developmental advancement in addition to therapeutics, which eventually led to the formation of multi-drug-resistant (MDR) strains of *Salmonella*. This raised situation of resistance was noted in the current study as well as reported by Rahman & Mohsin (2019). in the same manner.

6. Conclusion

In order to protect the public, it is necessary to establish and follow a proper protocol for slaughtering, handling and management of meat at butcher's shops. It is essential for local government to begin to regular monitor beef and its products for Salmonella contamination. Future Salmonella contamination of beef can be effectively stopped and eliminated by proper handling of meat. The current detection of S. enteritidis and S. typhimurium in beef also support the current concept and necessity of the use of food safety procedures as an alternative and complementary indicator for improved meat quality and safety monitoring. In present study, the higher percentage of antimicrobial resistance indicated that the unnecessary usage of antibiotics may also be eliminated in cattle in order to cope with the elevating rates of antimicrobial resistance.

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