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Research Article

Prevalence and Antibiotic Resistance of *Salmonella* Species Isolated from Chicken Liver in Peshawar

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Abstract

The study was conducted to analyze the prevalence and antibiotic resistance of *Salmonella* species in chicken liver. For this purpose, a total of 50 chicken liver samples were randomly collected from different areas of Peshawar and examined for the presence of *Salmonella*. The prevalence rate of *Salmonella* was 24%. In our study different antibiotics were used against *Salmonella*, Ofloxacin which is 83.33% susceptible and 16.66% resistant, Cefixime which is 100 % susceptible, Amoxil which is 33.33% susceptible and 66.66% resistant, Azithromycin which is 100% susceptible, Ceftriaxone which is 83.33% susceptible and 16.66% resistant. All the isolates showed the highest resistant to Amoxycillin and the highest sensitive/ susceptible antibiotic was Cefixime followed by Azithromycin. Similarly, Ofloxacin and Ceftriaxone also showed sensitivity against isolated *Salmonella* spp. Furthermore, it is one of the reasons that human infections of antimicrobial-resistant *Salmonella* may be due to chicken liver.

Keywords: *Salmonella*, Antimicrobial-resistant, Chicken liver, Amoxycillin resistant, Peshawar.

Abbreviations: µg: Microgram; µg/L: Microgram/liter; µl: Microliter; C_2H_6O: Ethanol; UV: Ultraviolet; Vol: Volume; CFU: Colony-forming units; MIC: Minimal Inhibitory concentration; %: Percentage; ^oC: Centigrade; GDP: Growth domestic product; $Bacl_2$: Barium chloride; H_2SO_4: Sulfuric Acid; $C_3H_8O_3$: Glycerin;MHA: Muller Hinton agar; Hrs: Hours; XDR: Extreme Drug Resistance.

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About author: Gulzar Ahmad is the former student of institute of integrative biosciences Cecos University Peshawar and now working as a researcher at S-khan computational lab Mardan KPK Pakistan since 2019. He is currently studying on computational study of post translation modifications and biological databases, his field of interest is biotechnology, microbiology, molecular biology and bioinformatics.

Introduction

Poultry sector is one of the most important subsectors of livestock in Pakistan. More than 700 billion investment is done in this sector during 2017-18 in Pakistan. About 1.163 million broilers are produced annually thus making Pakistan the 11th largest poultry industry in the world[1]. During

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2017-18, 1.39 million tons of poultry meat was produced which contributed 32.7 percent of the total meat production in the country. During 2017-18 the poultry has contributed 1.4 percent in the GDP, while its contribution in agriculture and livestock value added at 7.5 percent and 12.7 percent, respectively [2,3]. The poultry industry has made a major contribution to the food sector of Pakistan, and its products are largely consumed throughout the country. But due to poor poultry practices and feed, Poultry meat has lost its nutritional values. This is mainly due to the poor feed which leads to a weak immune system[4]. As a result, these birds become an easy target to the pathogenic microorganisms. Moreover, the substandard quality of chicken feed, in turn, leads to a poor mechanism of resistance towards pathogens in chicken. Besides this, the chickens are fed by antimicrobial agents that cause antibiotic resistance which is one of the main disadvantages of chicken.

In North America, poultry farms often used antibiotics such as tetracycline, bacitracin, tylosin, salinomycin, virginiamycin, and bambermycin [5]. In the United States, tetracyclines represent more than two-thirds of antimicrobials administered to animals [3]. In Canada 1.5 million kg of active antimicrobial agents were distributed during 2014 for use in animals which is 5% greater than from the previous year 2013. These antimicrobial agents were distributed 99% for farm animals and less than 1% for pets. In 2014, 81% of the antimicrobial agents were used in Canada on broiler farms for prevention purposes [6]. Due to these bad practices and substandard feed antimicrobial resistance developed in the normal flora of poultry and other pathogenic organisms. Among these pathogenic microorganisms, *Salmonella* and Campylobacter are the major inhabitants of the chicken liver [7]. *Salmonella* is a major causative agent of food-borne diseases due to *Salmonella*ingestion; a person is likely to develop symptoms of typhoid fever including diarrhea, abdominal pain, vomiting, and fever [8,9]. Due to the poor sanitation system, the rate of enteric fever increases up to 10-15%. In the developed countries the mortality rate in case of typhoid fever is 5 to 30 %. According to WHO 16 to 17 million people suffer annually from typhoid fever that leads to 600,000 deaths. The mortality rate is 1.3 billion in the case of non-typhoidal Salmonellosis [10]. In another report by the Central Veterinary Laboratory, the prevalence rate of *Salmonella* is continuously increasing in chicken since 1993 [11]. Most *Salmonella* diseases in humans result from ingestion of contaminated poultry products like beef, eggs, liver, etc.[12]. A scientific study showed that in Canada poultry meat may play a role in human infections [13].It is estimated that about 94 million cases of gastroenteritis due to *Salmonella* species occur annually worldwide, leading to 155,000 deaths every year[12].

Most antimicrobial-resistant *salmonella* infections are due to consuming contaminated food of animal origin. From 1 November 2016 through 9 December 2018, 5274 cases of XDR typhoid out of 8188 typhoid fever cases were reported by the provincial diseases surveillance and Response unit (PDSRU) in Sindh province, Pakistan, sixty-nine percent of cases were reported in Karachi, 27% in Hyderabad district, and 4% in other districts in the province. The circulating XDR strain of S.

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Typhi haplotype 58 was resistant to the first- and second-line antibiotics as well as third-generation cephalosporins[8]. Bacterial resistance to antibiotics has been the focus of several studies in recent years[14]. A Scientific study held in Canada showed that different stereotypes of *Salmonella* isolated from broiler farms, resistant and multi-resistant to antibiotics [15].

The aim of this research was to check antimicrobial susceptibility and antibiotic-resistant of *Salmonella* species isolated from chicken liver from the local market of Peshawar area. For this purpose, we have done the antimicrobial susceptibility analysis of screened bacteria and determination of MIC, for isolated antimicrobial-resistant *Salmonella*.

Materials and Methods

Sample collection

A total of 50 chicken liver samples were collected from the different areas of Peshawar and Nowshehra, 10 samples were collected from FaqirabadDalazak road Peshawar, 10 samples were collected from Momin town Dalazak road Peshawar, 10 samples were collected from Chargano chowk Peshawar, 10 samples were collected from Azar Khwani Peshawar, 10 samples were collected from AkbarpuraNowshehra. Each sample was collected in a separate plastic zipper bag so that the samples cannot mix, to avoid contamination and then directly transfer to the ice box.

Isolation

Isolation was performed by using nutrient agar media. Nutrient agar media was prepared by mixing 0.5% peptone, 0.3% yeast, 0.5% NaCl, 1.5 to 2% agar in 100 ml of distill water. The media was autoclaved at 121°C for 20 minutes Then media was shifted to the biosafety cabinet, and poring was done in Petri plates in the biosafety cabinet. Nutrient agar media plates were shifted to the incubator for 24 hours at 37° C to check the sterility. After that, the liver samples were homogenized, and then serial dilutions up to 2 folds were performed to reduce the number of bacterial cells. Samples were cultured on media plates through the spread plate technique. Culture plates were shifted to the incubator for 24 hours at 37°C to obtain bacterial isolates. After that, the culture plates were shifted to the fridge.

Purification

Purification was done by using the same nutrient agar media. Nutrient agar media was prepared by mixing 2.8 g nutrient agar media in 100 ml of distilled water and then autoclave at 121°C for 20 minutes. The media was shifted to the biosafety cabinet and poring was done in Petri plates and the plates were then shifted to the incubator for 24 hours at 37°C to check the sterility. Random discrete bacterial colonies were picked from previous plated and were streaked on nutrient agar media plates in a biosafety cabinet for purification of the single isolate from distinct colonies. After that, the cultured media plates were transferred to the fridge at 4°C.

Screening

Screening was done by using bismuth sulphite media. Bismuth sulphite is a selective media for the growth of *Salmonella* species. Bismuth sulphite media was prepared by mixing 4 g of bismuth sulphite in 100 ml of distilled water and

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was heated using a hot plate until bubbles started to appear. Media was shifted to biosafety cabinet and poring was done in Petri plates. Then media plates were transferred to the incubator for 24 hours at 37°C to check the sterility. The isolates from previous plates were cultured on Bismuth sulphite agar media plates by streak plate method and transfer to the incubator for 48 hours at 37°C and checked for typical *Salmonella* growth, and then transferred to fridge.

Disk Diffusion Test or Kirby Bauer Test

Disk diffusion test is used to check antibiotics susceptibility, for this purpose MHA (Muller Hinton agar) was prepared by mixing 3.8 g of MHA in 100 ml of distilled water and was autoclaved for 20 minutes at 121°C. After autoclaving media was shifted to the biosafety cabinet and poring was done in Petri plates. Then media was shifted to the incubator for 24 hours to check sterility.

To ensure the uniform number of bacterial cells in the inoculum, 0.5 McFarland standard was prepared by mixing 1.175 g of $BaCl_2$ ln 98.825 ml of water in a flask, and in another flask, 1 ml of H_2SO_4 was added to 99ml of water. The desired 0.5 McFarland standard was prepared by mixing 0.05 ml of 1% $BcCl_2$ solution with 9.95 ml of 1% of H_2SO_4 solution in a sterile test tube. Bacterial inoculum was prepared by using an overnight culture. Colonies were fresh in saline solution and were compared with McFarland standard to get uniform bacterial cells of each sample.

Freshly prepared bacterial inoculum was spread over the MHA media plate with the help of sterile cotton swabs in a biosafety cabinet, after bacterial lawn spreading, various antibiotic discs were placed on cultured Petri plates with the help of sterile forceps. The following antibiotics were used for disc diffusion assay; Cefixime (5 µg), Ofloxacin (5 µg), Azithromycin (30 µg), Ceftriaxone (30 µg), Amoxycillin (30 µg). The distance between each antibiotic was kept 24 cm. The plates were then kept in incubator for 24 hours at 37°C. The zone diameter around each antibiotic disc was then measured and plates were shifted to the fridge at 4°C. Larger the zone diameter means more susceptible the antibiotic discs, smaller the zone diameter means less susceptible the antibiotic discs.

MIC(MinimumInhibitoryConcentration)

Minimum inhibitory concentration is the lowest concentration of drug that stops the growth of bacteria. The previous culture was fresh for performing MIC determination using nutrient agar media and incubated for 24 hours to increase the growth of bacteria. Further, the bacterial sample was mixed with nutrient broth by inoculation loop with the help of vertex. The nutrient broth containing bacterial culture was compared with McFarland standard to ensure the uniform number of bacterial cells in each test tube and the cutoff values of each antibiotic were checked using the 2019 CLSI report.According to the cutoff values of each antibiotic, the antibiotic solutions of various concentrations were prepared.

Cefixime, Ofloxacin, Azithromycin, Ceftriaxone, and Amoxycillin were used, the bacterial culture and antibiotic solution were added to 96 well plate (195 µl antibiotic solution and 5 µl bacterial inoculum) Along with bacterial culture

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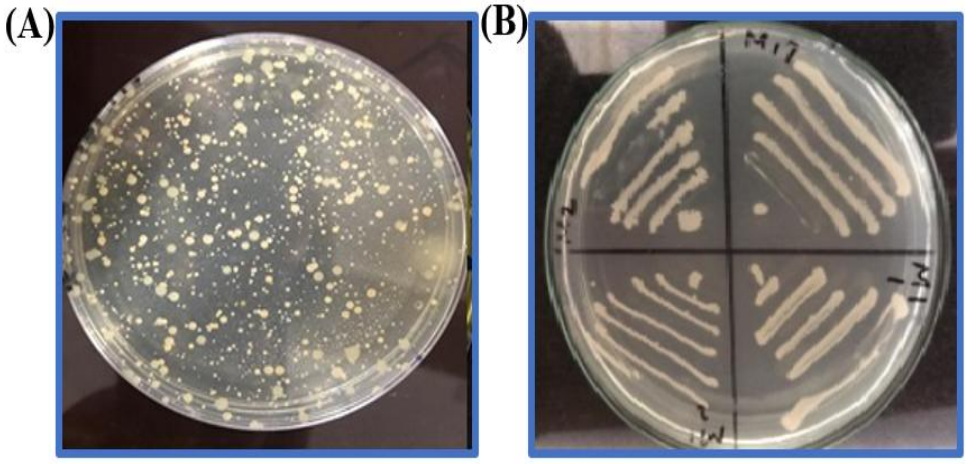
control and antibiotic control (200 µl). Spectrophotometry was performed for initial reading at 600 nm wavelength, at zero hour. The plate was then kept in the incubator for 24 hours at 37°C. After incubation, a second reading was taken using a spectrophotometer to analyze the susceptibility of antibiotics.

Results

Isolation and Purification of Bacteria

Isolation was done by using nutrient agar media. Samples were culture on nutrient agar plates through spread plate technique and shifted to the incubator for 24 hours at 37°C to obtain the following result as shown in Figure 1A and purification was done on the same nutrient agar media random colonies were picked and cultured via streak plate the method which is shown in Figure 1B.

Figure 1: (A) Isolated bacteria from chicken liver samples; (B) Random colonies on the nutrient agar plate.



Screening of Bacteria

Screening was done on Bismuth Sulphite agar media via the streak plate method.

After incubation, growth of the positive isolates was observed in Figure 2.

Figure 2: Positive isolates of *Salmonella*spp on Bismuth Sulphite agar media.

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Disc Diffusion Test or Kirby Bauer Test

Disk diffusion test is used to check antibiotic susceptibility. For this purpose, MHA (Muller Hinton agar) was prepared and isolates from previous plates were culture on media plates. After that antibiotic disc was applied to obtain the

following result as shown in Figure 3 andTable 1.

Figure 3: Zones of inhibition of different antibiotics on positive isolates.

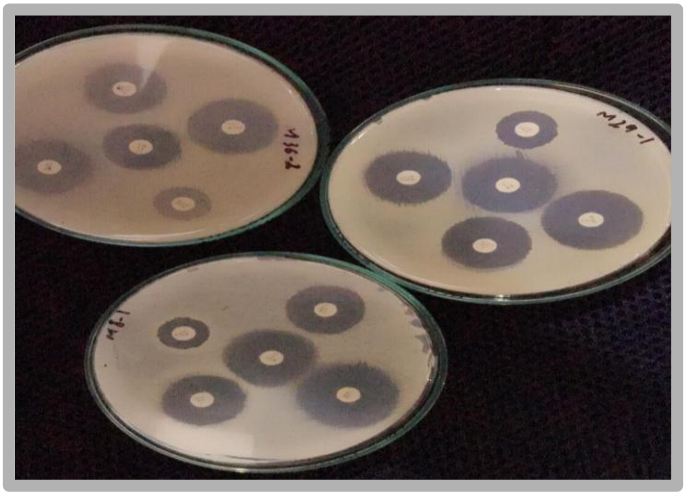

Table 1: Zone of inhibition for each strain of five different antibiotics.

Sample strain	Cefixime 5	Ofloxacin 5	Azithromycin 15	Ceftriaxone 30	Amoxycillin 30
Susceptible value	≥ 19	≥ 31	≥ 13	≥ 23	≥ 18
S44-1	19 mm	8 mm	14 mm	20 mm	17 mm
S44-2	20 mm	10 mm	23 mm	24 mm	16 mm
S47-1	20 mm	8 mm	23 mm	24 mm	17 mm
S47-2	21 mm	25 mm	20 mm	24 mm	15 mm
S44-2	22 mm	31 mm	24 mm	26 mm	16 mm
S41-2	19 mm	33 mm	22 mm	21 mm	17 mm
S40-1	22 mm	36 mm	26 mm	24 mm	13 mm
S29-2	20 mm	31 mm	24 mm	20 mm	12 mm
S22	20 mm	34 mm	21 mm	24 mm	20 mm
S23	21 mm	33 mm	26 mm	25 mm	21 mm
S24	19 mm	31 mm	24 mm	21 mm	22 mm
S48	19 mm	30 mm	14 mm	21 mm	17 mm

MIC (Minimum inhibitory concentration)

Minimum inhibitory concentration is the lowest concentration of drug that stops the growth of bacteria. The previous culture was fresh for performing MIC determination using nutrient agar media to find out the exact cutoff value Table 2. In our study, five antibiotics were used against *Salmonella*, Ofloxacin which is

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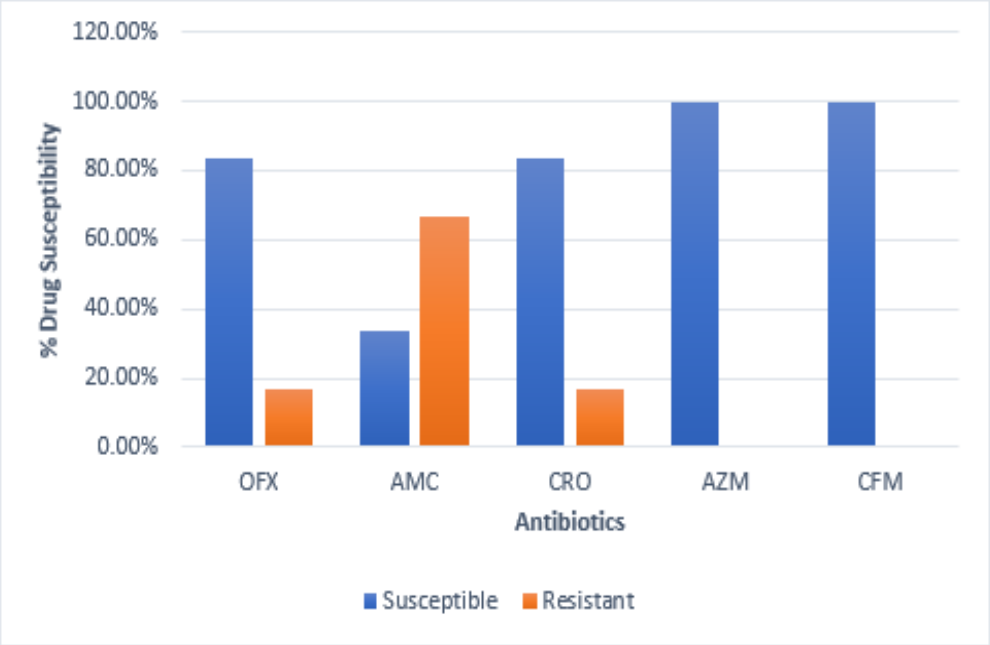
83.33% susceptible and 16.66% resistant, Cefixime which is 100% susceptible, Amoxil which is 33.33% susceptible and 66.66% resistant, Azithromycin which is 100% susceptible, Ceftriaxone which is 83.33% susceptible and 16.66% resistant Figure 4 shows resistance

and Susceptibility of the strains towards different antibiotics.

Table 2: Shows the antibiotic susceptibility of *Salmonella* determined on the basis of the T-test. If p value>0.05 means the bacteria are resistant. If p-value ≤ 0.05 means antibiotics are susceptible.

Antimicrobial susceptibility of *Salmonella*	**% Drug Susceptibility**				
	OFX	**AMC**	**CRO**	**AZM**	**CFM**
Susceptible	83.33%	33.33%	83.33%	100%	100%
Resistant	16.66%	66.66%	16.66%	0%	0%

Figure 4: Resistance and Susceptibility of the strains towards different antibiotics.



Statistical Analysis

Descriptive statistics allow investigators to summarize large amounts of data to more understandable levels using numerical descriptors (e.g., mean, mode, median, or standard deviation) or graphical methods.

Statistical analysis and data reconfiguration were done. Initial and final readings of bacterial growth in the presence of

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antibiotics were analyzed via T-test shown in Figure 5.

Figure 5: T-test for analyzing the growth of the strains (A) contains P value less than 0.05 no bacterial growth, and (B) contains P value greater than 0.05, means Significant bacteria growth.

(A)

	A	B	C	D
1	t-Test: Paired Two Sample for Means			
2				
3		*0.1011*	*0.4494*	
4	Mean	0.102675	0.552375	
5	Variance	4.22E-05	0.00412	
6	Observati	4	4	
7	Pearson C	0.973047		
8	Hypothesi	0		
9	df	3		
10	t Stat	-15.5374		
11	P(T<=t) or	0.00029		
12	t Critical o	2.353363		
13	P(T<=t) tw	0.000579		
14	t Critical t	3.182446		

(B)

	A	B	C	D
1	t-Test: Paired Two Sample for Means			
2				
3		*0.0979*	*0.4937*	
4	Mean	0.10275	0.639325	
5	Variance	2.92E-05	0.000519	
6	Observati	4	4	
7	Pearson C	0.230968		
8	Hypothesi	0		
9	df	3		
10	t Stat	-48.4193		
11	P(T<=t) or	9.7E-06		
12	t Critical o	2.353363		
13	P(T<=t) tw	1.94E-05		
14	t Critical t	3.182446		

Discussion

Salmonella is a main causing agent for salmonellosis which is a zoonotic disease and *Salmonella* involves in the contamination of food products. In humans, food poisoning or gastroenteritis occurs due to many pathogenic microorganisms such as Giardia lamblia, Escherichia coli, Shigella spp, Campylobacter, Bacillus cereus but the highest rate of food poisoning is due to the infections caused by *Salmonella*spp [16]. In 2012 the presence of *Salmonella* was reported in poultry products, especially meat. In the poultry industry, the main problem is the continuous spreading of *Salmonella* infection all over the world. The world is facing an economic problem in the form of a high death rate and low development of poultry chicken [17,18]. *S. Enteriditis* was the most commonly isolated serotype accountable for *Salmonella* infections around the world (65 percent). S. Typhimurium and S. Newport came in second and third, with 12 percent and 4% of the clinical isolates, respectively In Asia, Latin America, and Europe, *Salmonella*

Enteriditis was the most common serotype, accounting for 38 percent, 31 percent, and 87 percent of clinical isolates, respectively. In comparison to the countries listed previously, S. Typhimurium (29%) and S. Enteriditis (21%) were the most common bacteria found in clinical isolates in North America[19].

About 20 to 70 % of *Salmonella* is observed in broiler chicken in many countries of the world, there are many reasons due to which *Salmonella* are present in broiler meat, like the poor handling of the infected chicken, using of the contaminated tool and equipment in the industry [20, 21]. In the present study,*Salmonella* was detected in the chicken liver sample using Bismuth Sulphite media which is a selective media for the growth of *Salmonella*. In total 50 liver samples, the prevalence rate of *Salmonella* was 24%. This study revealed the prevalence rate less than that of the

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result of [22] and [23] which was 30% and 48.75% in Pakistan inthe Sindh area.

Further to investigate the susceptibility and resistance of the strains, a T-test was performed to calculate the exact P-value to find out whether the antibiotic is susceptible or not, or the bacteria is resistant or not.P-value>0.05 means the bacteria is resistant if P-value ≤ 0.05 means the antibiotic is susceptible. Furthermore, various antibiotics such as Cefixime, Ofloxacin, Azithromycin, Amoxycillin, Ceftriaxone were used, the antibiotic Amoxycillin has been observed to be less effective against *Salmonella*. The most effective drugs against *Salmonella* were Azithromycin and Cefixime which show 100 % susceptibility. The resistance of *Salmonella* towards antibiotics are shown in the various study[24,25] in Pakistan and some extreme drugs resistance (XDR) cases were also pointed out by some studies like in 2019 a study[26] elaborates the case of 2018 in which six cases of XDR typhoid in travelers from the United Kingdom and the United States have been linked to the Pakistan outbreak. With the ease of travel, this number is likely to rise, which will have consequences for clinicians caring for returning travelers from outbreak areas, especially in terms of empiric antibiotic selection[26]. And the other hand, the resistance was also increased due to the misuse of antibiotics in Pakistan. *Salmonella* shows high resistance to Amoxycillin antibiotic because bacteria have genes coding for Beta-lactamase that destroys antibiotic before entrance into the cell. Besides this, bacteria have efflux pumps through which they can expel antibiotics before they reach their target site [27].

Conclusion

It is concluded from the current study that *Salmonella* species were isolated from chicken liver samples and have high resistance against Amoxycillin and showed reduced action of *Salmonella* infection. It is one of the reasons that Human infections of antimicrobial-resistant *Salmonella* may be due to chicken liver. In the future, characterization of these *Salmonella* strains is required, need to calculate exact breakpoint for each antibiotic, and the genotypes of the observed resistance strains need to be investigated.

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Conflict of interest

All authors of this work have declared that there is no conflict of interest.

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Author's contribution

Humaira Akhtar, Khansa Ahsan, and Dr Shahid Ullah supervised the project. Mr. Azaz Ullah Khan, Mr. Gulzar Ahmad, Mr. Muhammad Ijaz, and Mr. Faheem Ali Khan perform and analyze the experiments, all the authors have written the manuscript, reviewed the manuscript, and agreed to submit it.

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