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ANTIMICROBIAL RESISTANCE PROFILING OF *ESCHERICHIA COLI* ISOLATED FROM RETAILER POULTRY SAMPLES IN AZAD JAMMU AND KASHMIR

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Abstract:
Antimicrobial resistance (AMR) is a serious threat to animal and human health. Globally, a rapid and alarming increase in antimicrobial resistance (AMR) has been reported in past few years. *Escherichia coli* is one of the major bacterial enteropathogen of public health concern that causes food borne diseases. As a frequent commensal and zoonotic bacterium, *E. coli* can be a potential candidate as a pathogenic source for dissemination of antimicrobial resistance. Present study was designed to evaluate the magnitude of antimicrobial resistance in *E. coli* isolated from poultry samples collected from Bagh, Azad Jammu and Kashmir. For this purpose, 120 samples (liver, spleen, intestines and fecal wastes) were collected from different retailer's shops of chicken meat in district Bagh during the month of December, 2022 to March, 2023 to evaluate the frequency of *E. coli* in samples. All the collected samples were processed using established microbiological techniques and isolated *E. coli* were subjected to antimicrobial profiling by disc diffusion method against 15 different antibiotics. Out of 120 collected samples, 65 (54.17%) samples were found to be positive for *E.coli*. The prevalence of *E.coli* in different poultry samples ranged from 43.33 to 70%. Antibiogram results showed significant difference in antibiotic resistance pattern against selected antibiotics ($p<0.05$) and the highest resistance was observed against nalidixic acid (100%) followed by erythromycin, tetracycline, amoxycilin (95.38%), penicillin G (93.84%) and kanamycin (90.77%). Lower level of resistance was

observed against colistin-sulphate (16.92%) and cefipeme (9.23%). Whereas, all isolates were found to be susceptible to Cefoxitin. However, none of *E.coli* isolate was found susceptible to all antimicrobials tested. Furthermore, all the isolated *E.coli* were found to be multidrug resistant and showed simultaneous resistant against four or more classes of tested antimicrobials. Maximum number of *E.coli* isolates (n=28, 43.08%) showed resistance against seven different classes of antimicrobial agents. The current study delineated the incidence of multi drug resistant (MDR) *E.coli* in poultry samples which endorsed the requirement of proper attention towards regular monitoring and surveillance of exploitation of antimicrobials as chemotherapeutic agents in human and veterinary health care sector in Pakistan.

Keywords: Antimicrobial Resistance; Multi drug Resistance; *Escherichia coli,* Poultry samples; Azad Jammu and Kashmir; Pakistan

1. INTRODUCTION

Antimicrobial resistance is considered as a major global concern associated with usage of chemotherapeutic agents for treatment of human and animal diseases (Maqsood, 2012). Globally, antimicrobial chemotherapeutic agents are intensively used in animal husbandry as non-nutritive additives for infectious disease prevention, treatment (Denli et al., 2018; Mehdi et al., 2018; Lammie and Hughes, 2016; Diaz- Sanchez et al., 2015; Sattar et al., 2014; Akinbowale et al., 2006) and growth promotion (Ezenduka et al., 2014). However, benefits of antimicrobials in animal husbandry have been affected by emergence of resistance in microorganisms against all commonly used classes of antibiotics (WHO, 2014) due to indiscriminate use of antimicrobials (Tahir et al., 2018). The process is further expedited due to dissemination of antimicrobial resistance genes (ARGs) into the different environmental reservoirs from livestock and human wastes and effluents containing huge quantity of residual antibiotics from hospitals, livestock farms and pharmaceutical industry (Sumanth et al., 2017; Robinson et al., 2016; O'Neill, 2016).

Although, mostly *E. coli* is a commensal bacterium found as intestinal microflora in humans and animals, but some strains have developed a potential to cause serious intestinal and extra-intestinal infections in humans and animals (Vila et al., 2016; Kaper et al., 2004). The pathogenic strains of *E.coli* have acquired resistance against commonly used antimicrobials agents (Olorunmola et al., 2013). *E.coli* is a well- established zoonotic bacterium (García et al., 2010) and their strains are ubiquitous in the environment, food, animals and human. Therefore, occurrence of antimicrobial- resistant strains of *E. coli* pose a risk to public and animal health as well as food safety and became a matter of serious health concern globally (Pormohammad et al., 2019; WHO 2017; Van den Bogaard et al., 2001). Previous studies had reported the prevalence of *E. coli* in meat (Nedbalcova et al., 2023; Kiiti et al., 2021; Majewski et al., 2021; Makarov et al., 2020) and feed of fowls (Paredes et al., 2023; de Mesquita Souza Saraiva et al., 2022; Ngai et al., 2021; Ge et al., 2020; Kenneth et al., 2017). Moreover, high level of antimicrobial resistance (AMR) and multi drug resistance (MDR) have been reported in *E.coli* isolated from poultry samples (Nedbalcova et al., 2023; Koju et al., 2022; Kiiti et al., 2021; Majewski et al., 2021; Makarov et al., 2020). However, from Pakistan, few recent reports are available regarding antimicrobial resistance profiling of *E.coli* isolated from poultry samples (Liaqat et al., 2022; Amir et al., 2021; Iram et al., 2020; Nawaz et al., 2020; Rafique et al., 2020; Waseem et al., 2019). Therefore, it necessitates the regular surveillance of antimicrobial resistance in poultry meat, feed and fecal droppings from different geographical regions of Pakistan. This study is the first report to the best of our knowledge regarding prevalence and antimicrobial resistance in *E.coli* isolated from poultry samples collected from retailer shops located in Bagh, Azad Jammu and Kashmir.

2. MATERIALS AND METHODS

2.1 Sample Collection and Isolation of Bacteria

Chickens that were about to enter the food chain were sampled in this study. A total of 120 samples of liver, spleen, intestine and fecal waste were collected from different retailer shops located in Bagh, Azad Jammu and Kashmir during December, 2022 to March, 2023. The total sample group is comprised of 40 samples each of liver, spleen, intestine and fecal waste. The samples were aseptically collected and kept in ice storage (4ºC) during transportation to the laboratory. Ten grams of each sample was homogenized in 90 ml of sterile buffer peptone broth (BPB) followed by incubation under aerobic conditions at 37ºC for 24 hrs. After 24 hrs of incubation, an aliquot of 100 µl of each sample was poured and spread onto Eosin methylene blue agar and McCkonkey agar (Oxoid, UK) and subsequently incubated at 37ºC for 24 hrs. The distinct *E. coli* colonies were subcultured on Eosin Methylene Blue agar and further confirmed based on established microbiological methods of colony morphology, Gram's staining and conventional biochemical testing according to procedures outlined in Bergey's Manual of Determinative Bacteriology (Bergey, 1934). The *E.coli* were further biochemically characterized by using Citrate utilization, Indole, Motility, Oxidase, Triple sugar iron, Urease, Methyl red and Voges-Proskauer test. One well characterized colony of *E.coli* per sample was used for further processing. All the isolates were glycerol preserved at -20ºC.

2.2 Antimicrobial Susceptibility Testing

All selected strains of *E. coli* were subjected to antimicrobial susceptibility profiling against fifteen different antimicrobials of nine different classes. The antimicrobial discs used along with their classes are presented in table 2.1.The discs were kept in refrigerator at 4ºC to prevent potency loss. Before use, the working stock of disc was kept at room temperature to minimize condensation of moisture. Antibiotic susceptibility testing was carried out on Muller Hinton agar plates (Oxoid, UK) by disc diffusion assay as described by Bauer *et al*., (1966) for each isolate. *E. coli* ATCC 25922 was used as a reference strain. The results of antibiotic profiling were recorded by measuring the zone of inhibition around each disc and isolates were interpreted as sensitive, intermediate and resistant by following the Clinical and Laboratory Standards Institute guidelines (CLSI 2022).

Table 2.1: Antimicrobial categories and antimicrobial agents tested

Antimicrobial categories	Antimicrobial agents	Abbreviation and potency
Macrolides	Azithromycin	AZM(15µg)
	Erythromycin	E (10µg)
Quinolones	Ciprofloxacin	CIP (5µg)
	Nalidixic acid	NA (30 µg)
Aminoglycosides	Gentamicin	CN (30µg)
	Kanamycin	K (30µg)
	Amikacin	AK (30µg)
Monobactam	Aztreonam	ATM (30µg)
Phenicols	Chloramphenicol	C (30µg)
Tetracyclines	Tetracycline	TE (30µg)
Cephalosporin	Ceftriaxone	CRO (30µg)
	Cefepime	FEP (30µg)
Penicillins	Amoxycilin	AMX (30µg)
	Penicillin G	P (10µg)
Polymixin	Colistin Sulphate	CT (10µg)

2.3 Statistical Analysis

Pearson Chi-Square tests (χ^2) was performed using Graph Pad Prism (version 5.0) for statistical analysis.

3. RESULTS

3.1 Prevalence of *E. coli* in Different Sample Sources

Out of the 120 samples, 65 (54.17%) were found to contain *E. coli*. The prevalence of *E.coli* in different poultry samples ranged from 43.33 to 70% (Fig. 3.1). Maximum load of *E. coli* (70%) was observed in fecal waste samples (21/65). However, intestinal samples were found to be least contaminated (43.33%) with *E. coli* (13/30) (Table 3.1). However, no significant difference was observed among different sample sources (p>0.05). The isolates other than *E. coli* were excluded from this study.

3.2 Antimicrobial Resistance Profile of *E.coli*

Antibiotic resistance profile for all the 65 *E. coli* isolates was determined. A significantly highest resistance was observed against nalidixic acid (100%) followed by erythromycin, tetracycline, amoxycilin (95.38%), penicillin G (93.84%) and kanamycin (90.77%) (p<0.05) (Fig. 3.2). Lower level of resistance was observed against colistin-sulphate (16.92%) and cefipeme (9.23%). The frequencies and percentage of resistance against other antimicrobials tested are presented in table 3.2. However, none of *E.coli* isolate was found susceptible to all antimicrobials tested.

Table 3.1: Isolation frequency of *E. coli* from different samples

Type of sample	Total number of samples n (%)	Positive samples n (%)
Liver	30 (25)	15 (50.00)
Spleen	30 (25)	16 (53.33)
Intestine	30 (25)	13 (43.33)
Fecal Waste	30 (25)	21 (70.00)
Total	100	65 (54.17)

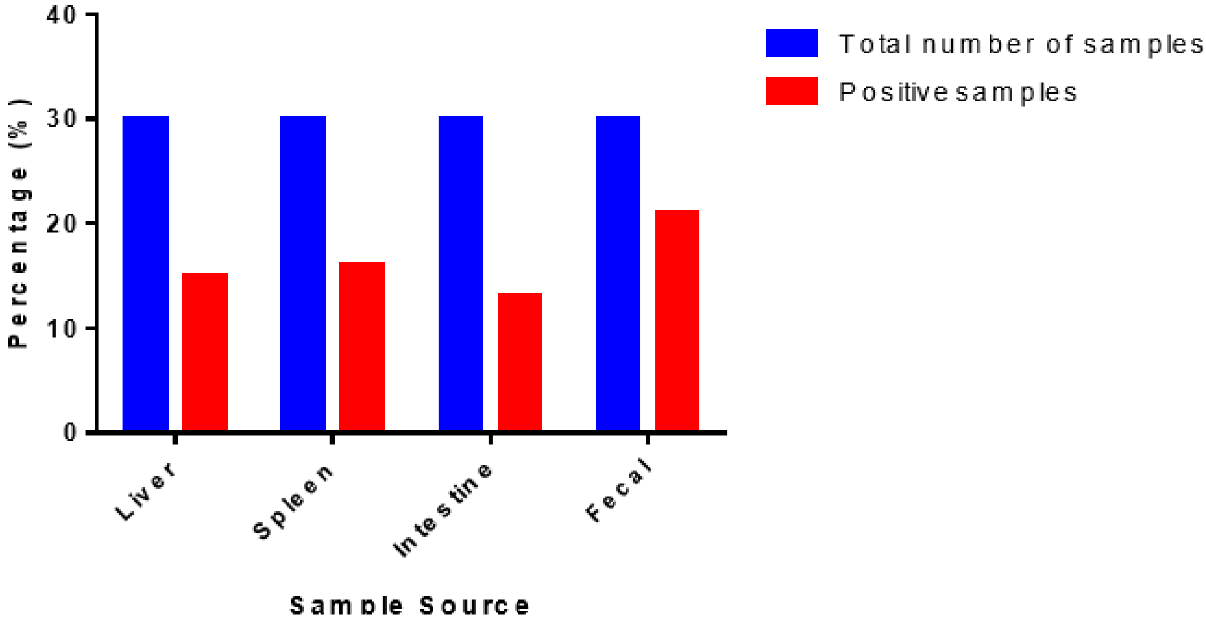

Figure 3.1: Percentage distribution of *E. coli* in different samples

Table 3.2: Antimicrobial resistance pattern of *E.coli* isolated from different chicken samples

Sr. No.	Antibiotic Class	Antibiotic Used	Frequency (n)			Percentage (%)		
			R	I	S	R	I	S
1.	Macrolides	Azithromycin	34	4	27	52.31	6.15	41.54
		Erythromycin	62	0	3	95.38	0	4.61
2.	Quinolones	Ciprofloxacin	51	1	13	78.46	1.54	20
		Nalidixic acid	65	0	0	100	0	0
3.	Aminoglycosides	Gentamicin	33	3	29	50.77	4.61	44.61
		Kanamycin	59	0	6	90.77	0	9.23
		Amikacin	36	2	27	55.38	3.08	41.54

4.	Monobactam	Aztreonam	34	0	31	52.31	0	47.69
5.	Phenicols	Chloramphenicol	43	11	11	66.15	16.92	16.92
6.	Tetracyclines	Tetracycline	62	0	3	95.38	0	4.62
7.	Cephalosporins	Ceftriaxone	33	10	22	50.77	15.38	33.85
		Cefepime	6	0	59	9.23	0	90.77
8.	Penicillins	Amoxycilin	62	0	3	95.38	0	4.61
		Penicillin G	61	0	4	93.84	0	6.15
9.	Polymixin	Colistin sulphate	11	0	54	16.92	0	83.08

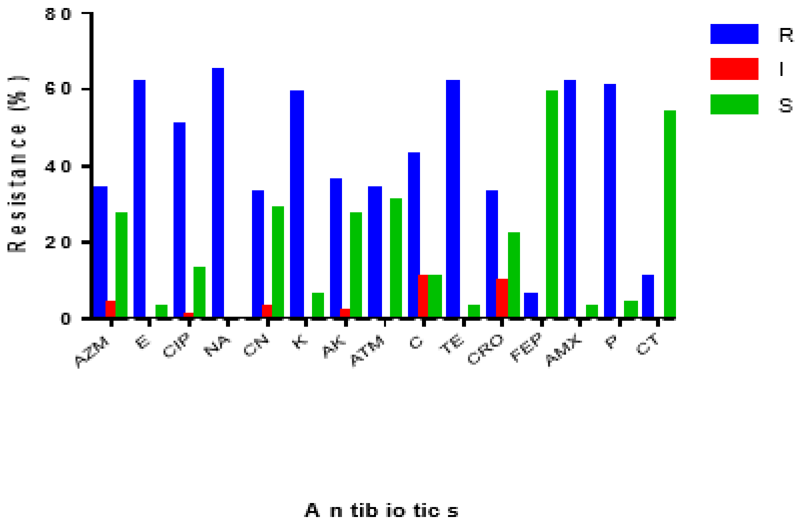

Figure 3.2: Antibiotic susceptibility profiling of *E.coli* isolated from different chicken samples

All the 13 isolates from intestinal source showed resistance against erythromycin, nalidixic acid, tetracycline and amoxycilin. However, only one isolate showed resistance against colistin-sulphate and all the isolates were found susceptible to cefepime. *E.coli* (n=16) isolated from spleen showed complete clinical resistance against nalidixic acid, kanamycin and amoxycilin and zero clinical resistance was observed with cefepime. However, least resistance frequency (n=3) was observed against colistin sulphate. Similarly, all the isolates (n=21) from fecal source showed 100% resistance against erythromycin, nalidixic acid and penicillin G. Unlike, other sample sources, three and four isolates of fecal waste showed resistance against cefepime and colistin sulphate respectively. Similar to fecal sample, 100% resistance was observed against erythromycin and nalidixic acid in *E.coli* (n=15) isolated from liver samples. However, minimum resistance frequency (n=3) was observed against cefepime and colistin sulphate (Table 3.3).

Table 3.3: Antimicrobial resistance frequencies of *E.coli in* different sample sources

Antibiotic used	**Frequency in different sample sources (n)**											
	Intestine			**Spleen**			**Fecal waste**			**Liver**		
	R	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**
Azithromycin	6	5	2	8	7	1	11	9	1	9	6	0
Erythromycin	13	0	0	15	1	0	21	0	0	13	2	0
Ciprofloxacin	9	4	0	13	3	0	18	2	1	11	4	0
Nalidixic acid	13	0	0	16	0	0	21	0	0	15	0	0
Gentamicin	4	8	1	7	7	2	15	6	0	7	8	0
Kanamycin	12	1	0	16	0	0	19	2	0	12	3	0
Amikacin	6	7	0	7	8	1	15	5	1	8	7	0
Aztreonam	8	5	0	9	7	0	9	12	0	8	7	0

Tetracycline	13	0	0	15	1	0	20	1	0	14	1	0
Ceftriaxone	5	5	3	9	5	2	10	7	4	9	5	1
Cefepime	0	13	0	0	16	0	3	18	0	3	12	0
Amoxycilin	13	0	0	16	0	0	20	1	0	13	2	0
Penicillin G	10	3	0	15	1	0	21	0	0	15	0	0
Chloramphenicol	9	2	2	10	4	2	13	1	7	11	4	0
Colistin sulphate	1	12	0	3	13	0	4	17	0	3	12	0

3.3 Multi Drug Resistance Pattern in *E.coli*

An isolate showing resistance against three or more classes of tested antimicrobials was considered as multidrug resistant (MDR) as mentioned by Magiorakos et al, (2012). All the isolated *E.coli* were found to be multidrug resistant. None of the isolate showed resistance against three or less classes of tested antimicrobials. Of these, only one isolate (n=1, 1.53%) showed resistance against four classes of antimicrobial. Rest of isolates (n=64, 98.46%) showed resistance to more than four classes of antibiotics. Maximum number of *E.coli* isolates (n=28, 43.08%) showed resistance against seven different classes of antimicrobial agents followed by six and eight classes (n= 13, 20%) each. Comparably, lower level multi drug resistance was observed against nine classes (n=1, 3.08%) (Table 3.3).

Resistance against No. o Classes	Sample Source				Total Resistant Isolates	
	Liver	Spleen	Intestine	Fecal waste	Frequency (n)	Percentage (%)
3	0	0	0	0	0	0
4	0	0	1	0	1	1.54
5	1	1	2	3	7	10.77
6	2	4	2	5	13	20
7	7	6	6	9	28	43.08
8	3	5	2	3	13	20
9	1	0	0	1	2	3.08

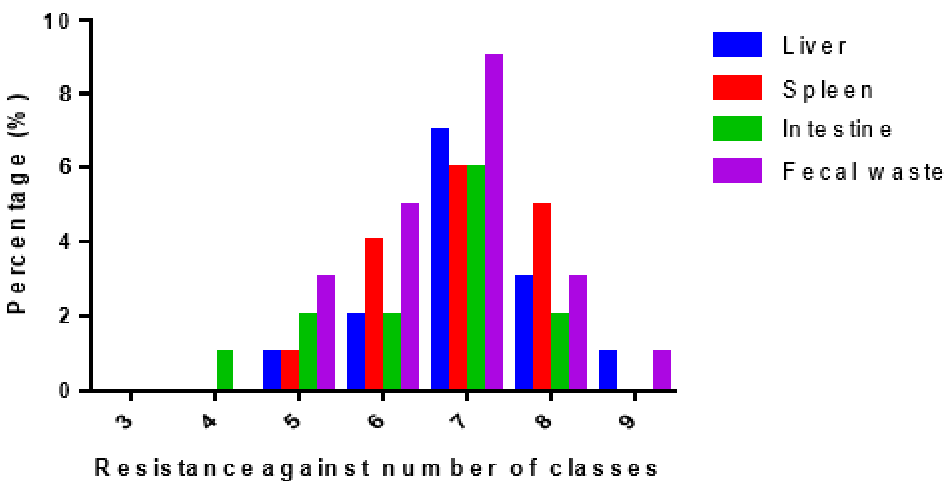

Figure 3.3: Multi drug resistance pattern of *E.coli* isolated from different chicken samples

4. DISCUSSION

Globally, one of the grave challenges of current era is antimicrobial resistance which is a major concern of human, animal and environmental health (Debas et al., 2011). Particularly, in developing countries like Pakistan, antimicrobial resistance (AMR) poses serious risk to public health. In this study, the overall incidence of *E. coli* was 54.17% and in different poultry samples ranged from 43.33 to 70%. However, the frequency of *E.coli* in present study is comparably lower than previous studies from two different provinces of Pakistan which reported 82% *E.coli* in chicken samples (meat, spleen, and liver) from three districts of Khyber Pukhtunkhwa (Mardan, Swabi, and Swat), Pakistan (Liaqat et al., 2022) and 75% from broiler chickens feces and meat samples collected from the retailer shops in Southern Punjab, Pakistan (Amir et al., 2021). Few studies have reported a gradual reducing trend in the prevalence of resistant *E.coli* isolates from chicken samples (Theobald et al., 2019). On the other hand, in literature reports are also available regarding incidence of higher than 50% of *E.coli* in poultry samples (Kim et al., 2020; Tang et al., 2021). From different geographical regions, numerous recent studies have reported the contamination of *E.coli* in poultry samples including Nepal (94%) (Koju et al., 2019), Korea (70.3%) (Kim et al., 2020), Czech Republic (74.8%) (Nedbalcova et al., 2023), Tanzania (100%) (Kiiti et al., 2021). In this study, predominance of *E. coli* load (70%, 21/65) was observed in fecal waste sample. Previous studies has reported the cross contamination of fecal *E. coli* to broiler chicken meat (Amir et al., 2017). Several factor contributed towards the cross contamination and increase in microbial load of meat including improper evisceration procedures, use of contaminated water and mishandling of equipments (Amir et al., 2017; Müller et al., 2001).

The selected *E.coli* isolates were tested against 15 different antibiotics of 9 different classes (Table 2.1). All the isolated *E.coli* were found to be resistant to nalidixic acid (100%) fallowed by erythromycin, tetracycline, amoxycilin (95.38%), penicillin G (93.84%) and kanamycin (90.77%) (Fig. 3.2). The pattern of resistance is similar to previous reports from Pakistan where nalidixic acid was found to be second least effective drug against diarrheagenic *E. coli* (DEC) strains isolated from farmed broiler chickens (Amir et al., 2021) and 100% resistance against nalidixic acid, tetracycline and norfloxacin in *E.coli* from meat, spleen, and liver samples (Liaquat et al., 2022). In addition, another study conducted by Kim et al., (2020) reported 75.7% resistance to nalidixic acid followed by ampicillin (69.1%) and tetracycline (64.0%). Similar findings were also reported from Bangladesh with high resistance of *E. coli* against ampicillin and tetracycline (Sarker et al., 2019). In a survey, tetracycline was reported as a most widely used antibiotic in veterinary sector of Nepal (Global Antibiotic Resistance Partnership, 2015). However, lower level of resistance was observed against colistin-sulphate (16.92%) and cefipeme (9.23%) (Table 3.3). Majewski et al, (2021) reported 100% susceptibility against colistin-sulphate of *E.coli* isolated from organs such as the lungs, liver, heart and spleen of chickens from Western Poland. Dissimilar to present findings, much higher level of resistance (70.1%) was reported against colistin from cloacal swabs from domesticated chicken in district Jhang ,Punjab Pakistan (Saeed et al., 2023). Irrational and excessive usage of antibiotics in livestock as therapeutics and for growth promotion tends to generate a strong selective pressure causing rapid dissemination of antibiotic resistant genes among microorganisms (Chopra and Roberts, 2001). Along with that, excessive usage of antibiotics causes food contamination which has serious implications on human and veterinary health (Zhang et al., 2021).

All the isolated *E.coli* (100%) were found to be multi drug resistant. None of the isolate showed simultaneous resistance against three or less classes of tested antimicrobials. Of the studied *E.coli* strains, 98.46% (n=64/65) showed resistance to more than four classes of antibiotics. Maximum number of *E.coli* isolates (n=28, 43.08%) showed resistance against seven different classes of antimicrobial agents. An alarming high level of multi-drug resistance substantiated the indiscriminate and extensive usage of antibiotics in poultry sector. The high incidence of multidrug resistant *E.coli* in chicken samples can possibly be due to supply of poultry chicken from poultry farms located in federal capital (Islamabad) of Pakistan and adjacent areas due to scarcity of poultry rearing farms in Azad Jammu and Kashmir specifically Bagh. High level of multi drug resistance (89.13%, n=82/92) in poultry associated *E.coli* collected from representative regions of Sindh, Khyber Pakhtunkhwa, Punjab, Balochistan and Islamabad (Capital) of Pakistan has already been documented by Rafique et al, (2020). However, *E.coli* from chicken meat sample collected from retailer shop in Nepal showed 69.7% resistance against three to four or more than four antibiotics (Saud et al., 2019) and 87.9% was reported by Kim et al, (2020) in *E. coli* from chicken meat with susceptibility against only meropenem out of

16 antibiotics tested. However, contrary to these findings, multidrug resistance incidence is far less in *E.coli* recovered from one day chickens having overall multiple drug resistance of 20.5% with resistance of 8.7% against three, 7.3% against four, 3.6% against five and 0.9% against six antibiotics from different classes (Nedbalcova et al., 2023). The present findings of an upsurge in incidence of multi antimicrobial resistance (MAR) index could be due to injudicious and indiscreet usage of clinically important antimicrobials as therapeutics in humans and as prophylaxis or non-therapeutic options for farm animals in Pakistan (Siddique et al., 2021).

5. CONCLUSION

The present findings validated the incidence of *E.coli* in different samples (liver, spleen, intestinal tract and chicken droppings) of chicken collected from Bagh Azad Jammu and Kashmir. The current study delineated 100% multi drug resistant (MDR) isolates which endorsed the requirement of attention towards proper monitoring and regular surveillance of exploitation of antimicrobials as chemotherapeutic agents in human and veterinary health care sector in Pakistan. Along with that, veterinarians are need to be trained for target specific usage of antibiotics as a risk management or containment options for prevention of emergence and dissemination of antibiotic resistance. Present findings provide a baseline for further studies involving the studies of genes harboring resistance and the mechanism of acquisition of resistance in *E.coli* strains.

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