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Full Length Article

Patterns of Antimicrobial and Multi Drug Resistance in *E. coli* and *Salmonella* Isolates of Commercial and Non-Commercial Poultry

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Abstract

The extensive and abuse of antibiotics have contributed to the universal spread of antibiotic resistance (AR). Commercial poultry birds harbor more antibiotic-resistant microorganisms than the backyard chicken, but the status is not published in quails. This study was designed to investigate the status of AR microbiota in **C0:** backyard chickens, **C1:** commercial broiler, **Q0:** backyard/wild quails and **Q1:** commercial quails (n=20). *Escherichia coli* (*E. coli*) and *Salmonella* isolates from carcass and ceca of these chickens and quails were investigated for incidence and extent of AR using disk diffusion method. The results of overall microbiota of the experimental birds revealed that C1 showed a greater ($P < 0.01$) AR as compared with C0 for ampicillin, chloramphenicol, ciprofloxacin, gentamicin, neomycin, norfloxacin, oxytetracycline, and sulfamethoxazole, with about 57.39, 57.24, 38.78, 62.92, 36.51, 67.61, 55.83 and 55.68% greater incidence of AR, respectively. Similarly, Q1 also exhibited a greater ($P < 0.01$) AR than Q0 for these antibiotics, with about 65.59, 58.44, 54.38, 54.38, 55.68, 51.62, 54.87, and 64.93%, respectively. Moreover, the results of individual microbial numbers of both the pathogenic bacterial isolates from C1 and Q1 exhibited a higher ($P < 0.01$) AR for all tested antimicrobials than those isolated from C0 and Q0. Additionally, the *E. coli* and *Salmonella* isolates of C1 and Q1 were more ($P < 0.05$) multi drug resistant (MDR) as compared with C0 and Q0. Furthermore, the extent of AR was greater in *E. coli* and *Salmonella* isolates of C1 and Q1 in contrast to those of C0 and Q0. Finally, C1 and Q1 harbored a greater number of MDR bacterial species than those in C0 and Q0 thus may act as risk factors for antimicrobial dissemination.

Keywords: Antimicrobial resistance; Multidrug resistance; Backyard poultry; Quail; Pathogenic bacteria

Introduction

The non-judicious use of antimicrobial agents in poultry feed has been associated with an increased incidence of antibiotic resistance (AR) in the microorganisms of poultry origin. Commercial poultry farming is highly profitable because it produces poultry meat with the least investment within 5–6 weeks. Poultry farmers observe intense care in commercial broiler farming, and particularly in the developing countries, such as Pakistan, in-feed antibiotic drugs are prevalent for preventing a variety of poultry diseases (Kamboh *et al.* 2018a). Although antibiotics are essential for disease prevention and control, these have also found another use as growth promoters in the poultry industry (Obajuluwa *et al.* 2021; Schwarz *et al.* 2001). A number of recently published reports indicated that the meat gained from the broilers reared on conventional antibiotic mixed feeds harbored high counts of AR bacteria as compared to the one obtained from organically reared chickens (Miranda *et al.* 2008a, b; Kamboh *et al.* 2018b). The incidence of AR is a serious concern for human health because the use of antimicrobial agents has been continuously increasing the types, strains, and numbers of antibiotic-resistant bacteria

(ARB). Since humans eat the poultry products, including meat and eggs, the effectiveness of the antimicrobial medicines which are used in poultry feed, has also reduced in human populations (Asai *et al.* 2006; Caniça *et al.* 2019). Since the development of a novel group of antimicrobial agents is really a challenging task (Ikele *et al.* 2020), hence humans cannot afford their existing antimicrobial drugs to be inefficient to control their diseases. The major reason for the increasing spread of AR is unawareness of the masses about this problem. Hence research needs to be done to provide conclusive evidence about the prevalence of AR in the sources of human foods, particularly poultry meat.

Microbiota lives in the intestines of poultry birds serve as their essential survivors against invading pathogens. Pathogenic microorganisms, such as *Escherichia coli*, and *Salmonella* compete with microbiota and cause diseases to poultry birds. *E. coli*, a commensal bacterium, is globally considered a major reason for the morbidity and mortality of humans and animals by causing food-borne infections (Miskinyte *et al.* 2013; Khan *et al.* 2015). It can survive in several hosts. Its pathogenicity and increase in AR have raised concerns regarding community health and socioeconomic values (Pegues 2005). Moreover, *Salmonella* is considered a growing threat to human health, as it exhibits highly deadly pathogenic behavior in poultry birds. Moreover, *Salmonella* contaminates poultry meat and spreads infection in humans through the consumption of infected poultry products (Nair and Johny 2019; Han *et al.* 2020). Moreover, *Salmonella* is also a human pathogen that also enjoys poultry as an alternate host. Two major species of *Salmonella*, *viz.*, *Salmonella enterica* and *S. bangori* have been reported as sources of foodborne diseases in the US (CDCP 2013). There is a remarkably higher incidence of antibiotic-resistance in the microbiota of chickens grown on diets containing antibiotic drugs as compared to the ones reared without antimicrobial drugs (Zhang *et al.* 2011). Similarly, lower AR bacteria are generally found in the organically grown poultry birds in contrast to traditionally reared ones (Smith-Spangler *et al.* 2012). The existing published reports used chicken as the model bird for the prevalence and dissemination of AR in commercial and non-conventional poultry farming systems but investigations in commercial and backyard/wild quails are yet to be published.

The present study, therefore, used two poultry models, *i.e.*, commercial and backyard/wild chickens and quails to elucidate the potential risks of AR. In this study, the incidence of AR was explored in *E. coli* and *Salmonella* isolated from intestines and meats of commercial and backyard/wild chickens and quails. The results of this study will be beneficial for the poultry industry to understand the perils of AR in commercial poultry, and provoke new research for the production of antibiotic-free poultry.

Materials and Methods

Sample collection

Commercial and backyard/wild chickens and quails were procured from a local live bird market of Hyderabad, Sindh, Pakistan. The birds were assigned to four treatment groups, *i.e.*, **C0:** backyard chickens, **C1:** commercial broiler, **Q0:** backyard/wild quails and **Q1:** commercial quails. Each treatment group contained twenty birds (n=20 each). The birds of each treatment group were exsanguinated using a sharp sterilized knife on different days according to the requirements of the Directorate of Advanced Studies, Sindh Agriculture University, Tandojam. The carcasses were eviscerated and portioned using sterilized equipment. The cecal contents of each bird were collected under aseptic conditions and stored in a laboratory freezer at −24°C until required for analysis. The whole carcasses were subject to chilling (4°C) after slaughter and analyzed for the isolation of pathogenic microbes within 2 h.

Isolation of *E. coli* and *Salmonella*

The isolation of two pathogenic microorganisms, including *E. coli* and *Salmonella*, from the ceca, was performed by taking 1 g samples, placing them in 9 mL of 0.9% sterile saline solution in a sterilized beaker, and vortexed. The samples were subsequently diluted to prepare ten-fold dilution using the saline solution. About 1 mL of each 10-fold diluted sample was cultured on the media, as described by Habib *et al.* (2015).

On the other hand, the whole carcass was hand rinsed under aseptic conditions in 100 mL of 0.85% sterile saline solution, according to the method of Kilonzo-Nthenge *et al.* (2008). About 25 mL of this solution was taken and mixed with 225 mL of 0.1% peptone water. The samples were then incubated at 37°C for 24 h (pre-enrichment culturing).

The sub-culturing of *E. coli* and *Salmonella* was done by selecting the suspected bacteria and individually inoculating them onto differential bacteriological agar media (Oxoid, Co., UK) under aseptic conditions. The samples were incubated at 37°C for 12 h and pure cultures were obtained. The conformation of bacterial species was performed according to the method of Monica (1985).

Assessment of antibiotic resistance

The AR patterns of both the pathogenic isolates were verified according to the protocol described by CLSI Guidelines (2012). Briefly, the AR of the pathogenic bacterial (*E. coli* and *Salmonella*) isolates was assessed by the disk dispersion method. The colonies of the pathogenic bacteria were added into nutrient broth. The turbidity (0.5 McFarland standard) of the broth was adjusted. These were spread on sensitivity agar plates using sterile swabs. Subsequently, these were dried. In the next step, the selected

antibiotic discs of the antibiotic drugs were put on the aforementioned sensitivity plates by maintaining an even distance. The amounts of the antibiotic drugs were about 5 μg for ciprofloxacin; 10 μg each for ampicillin, gentamicin, and norfloxacin; 25 μg for sulfamethoxazole; and 30 μg each for chloramphenicol and oxytetracycline. Subsequently, these plates were incubated at 37°C. The inhibition zones were measured after 24 h of the incubation from the centre of the disc. The classification of AR breakpoints was done using the protocol published by Lalitha (2004), according to which the bacteria which had an inhabitation zone ≥ 21 mm were termed as 'Susceptible'; those between 17–20 mm were considered 'Intermediate'; and the ones having ≤ 16 mm were classified 'Resistants'. Multidrug-resistant (MDR) strains were those whose isolates displayed resistance against 3 or more antibiotics (Kamboh *et al.* 2018b). The data for these parameters were collected in triplicate.

Statistical analysis

The general calculations and data analysis were performed using Microsoft Excel (v. 2010). The comparison of the levels of AR between the four treatment groups ($P < 0.05$) was performed by Fisher's exact test using JMP software (5.0.1.a, SAS, USA).

Results

The incidence of antimicrobial resistance

The data regarding AR of bacterial isolates obtained from the backyard and commercial chickens and quails against 5 μg ciprofloxacin, 10 μg each of ampicillin, gentamicin, neomycin, norfloxacin, 25 μg of sulfamethoxazole, and 30 μg each of chloramphenicol and oxytetracycline, and indicated significant differences ($P < 0.01$) with only one exception (Table 1). In this study, the comparison between the treatment groups revealed that the bacterial isolates obtained from both of the backyard chickens and quails exhibited significantly lower levels of AR than those obtained from commercial birds against the eight antimicrobial drugs tested in this study. The bacterial isolates obtained from the C1 group exhibited the highest AR against each of ampicillin, ciprofloxacin, neomycin, norfloxacin, sulfamethoxazole, and oxytetracycline. In contrast, the bacterial isolates obtained from the Q1 group exhibited the highest AR against each of the chloramphenicol, gentamicin in this study. On the other hand, the comparison between the antibiotic drugs revealed that the highest levels of AR in the bacterial isolates were observed against ampicillin for all the four bird groups. Moreover, the second-worst results were observed against gentamicin in C0, neomycin in each of C1 and Q0, and ciprofloxacin in Q1. Finally, the lowest levels of AR of the bacterial isolates were observed against norfloxacin in each of C0 and Q0, sulfamethoxazole in each of Q0 and Q1, and chloramphenicol in C1.

The resistance of *E. coli* against antimicrobial drugs

The percentages of *E. coli* isolated from the intestines of commercial and non-commercial chickens and quails selected in this study were shown in Fig. 1. The criteria described by Lalitha (2004) was used to classify the *E. coli* resistance against the eight antimicrobial drugs, according to which the pathogen was susceptible to the antibiotic drug if the inhibition zone on the disk was ≥ 21 mm, whereas the pathogen was resistant to the drug if the inhibition zone was: ≤ 16mm, while the zone between 17–20 mm was considered intermediate. In this study, the comparison between the treatment groups revealed that the *E. coli* isolates of the commercial birds showed significantly higher resistance than the backyard ones against all the tested antimicrobial drugs. Moreover, the *E. coli* isolates of C1 exhibited a significantly ($P < 0.01$) higher levels of AR than those of C0, whereas those of Q1 higher than those of Q0. Furthermore, the *E. coli* isolates of C1 exhibited higher levels of AR than those of Q1 against five drugs (ampicillin, neomycin, norfloxacin, sulfamethoxazole, and oxytetracycline), but lower against two drugs (gentamicin and chloramphenicol). On the other hand, the comparison between the antibiotic drugs revealed that the *E. coli* isolates exhibited the highest levels of AR against ampicillin in all the four treatment groups. Finally, the *E. coli* isolates exhibited the lowest levels of AR against gentamicin in C1, and against sulfamethoxazole in Q1. However, the *E. coli* isolates of backyard chickens showed the least resistance against four drugs (ciprofloxacin, neomycin, norfloxacin, and sulfamethoxazole). Finally, the *E. coli* isolates of backyard quails exhibited no resistance against four drugs (ciprofloxacin, norfloxacin, chloramphenicol, and oxytetracycline) in this study.

The resistance of *Salmonella* against antimicrobial drugs

The data regarding the percentages of *Salmonella* isolates obtained from the intestines of commercial and non-commercial chickens and quails selected in this study were shown in Fig. 2. The isolates obtained from commercial birds exhibited significantly ($P < 0.01$) higher AR than those isolated from non-commercial ones against all the tested antimicrobial drugs. Moreover, the *Salmonella* isolates of C1 exhibited significantly higher levels of AR than those obtained from C0, whereas those of Q1 higher than those of Q0. Furthermore, the *Salmonella* isolates of C1 exhibited higher levels of AR than those of Q1 against all the tested antimicrobial drugs in this study. On the other hand, the comparison between the antibiotic drugs revealed that the *Salmonella* isolates exhibited the highest levels of AR against ampicillin in all the four bird groups. Moreover, the *Salmonella* isolates exhibited the least of AR against two

Table 1: Overall percentages of bacterial isolates of commercial and non-commercial poultry resistant to antimicrobial agents

Antimicrobials	μg	C0 n=44		C1 n=64		Q0 n=44		Q1 n=56	
		#	%	#	%	#	%	#	%
Ciprofloxacin	5	5	11.36	44	68.75	1	2.27	38	67.86
Ampicillin	10	14	31.82	56	87.50	6	13.64	44	78.57
Gentamicin	10	8	18.18	35	54.69	3	6.82	35	62.50
Neomycin	10	6	13.64	52	81.25	4	9.09	34	60.71
Norfloxacin	10	3	6.82	41	64.06	1	2.27	34	60.71
Sulfamethoxazole	25	5	11.36	43	67.19	1	2.27	32	57.14
Chloramphenicol	30	7	15.91	35	54.69	2	4.55	33	58.93
Oxytetracycline	30	6	13.64	49	76.56	2	4.55	33	58.93

C0: backyard chickens; C1: commercial broiler; Q0: backyard/wild quails; Q1: commercial quails (*P* Value < 0.001 for all antimicrobials)

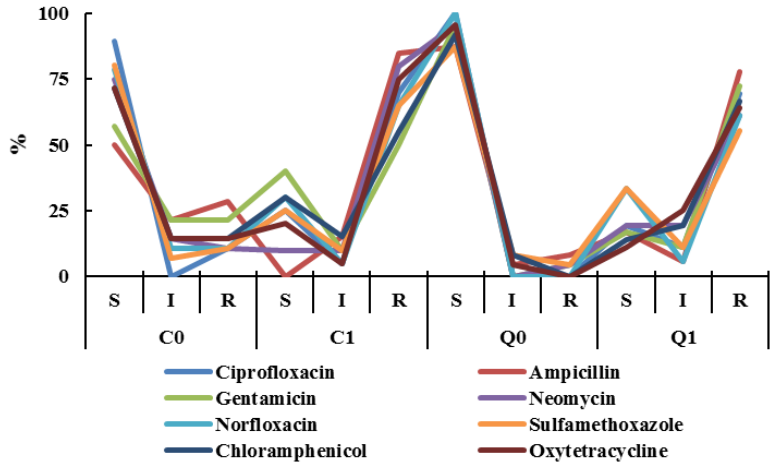

Fig. 1: Percentages of *Escherichia coli* isolated from commercial and non-commercial poultry susceptible (S), intermediate (I) and resistant (R) to antimicrobial agents by disk diffusion method. C0: backyard chickens (n=28); C1: commercial broiler (n=40); Q0: backyard/wild quails (n=24); Q1: commercial quails (n=36)

antimicrobial drugs including gentamicin and chloramphenicol in Q1. Furthermore, the isolates of both the commercial bird types (C1 and Q1) showed the lowest resistance against chloramphenicol. Finally, the *Salmonella* isolates obtained from backyard chickens and quails exhibited no resistance against norfloxacin and sulfamethoxazole, respectively in this study.

Multidrug resistance patterns

The data regarding the prevalence of MDR in the two bacterial isolates obtained from commercial and backyard poultry were summarized in Fig. 3. The percentages of MDR *E. coli* and *Salmonella* isolates of commercial birds (95 and 77% for C1 and Q1) were significantly higher (*P* = 0.0082 and 0.0076, respectively) than those obtained from non-commercial ones (67.8 and 54.2% for C0 and Q0). Similarly, the levels of MDR *Salmonella* isolates of commercial birds (92.8 and 85% for C1 and Q1) were higher (*P* = 0.0266 and 0.0060, respectively) than their counterparts obtained from the backyard birds (81.2 and 60% for C0 and Q0). The best results were shown by Q0, for which 45.8 and 40% *E. coli* and *Salmonella* isolates showed the resistance against 0–1 antimicrobial drugs, whereas the worst results were shown by C1, for which 42.8 and 28.6% *E. coli* and *Salmonella* isolates showed the resistance against > 6 antimicrobial drugs.

Discussion

Food-borne pathogens have become a leading threat to public health throughout the world. These include *E. coli*, *Salmonella*, *Shigella*, and *Campylobacter*, while raw and partially-cooked poultry meat products harbor them the most. Particularly, *Salmonella* and *E. coli* proliferate to human populations during the handling of untreated poultry carcasses, as well as through consumption of partially or improperly cooked poultry products (Chen *et al.* 2020). Another rising threat to human beings is the incidence of AR microorganisms, for which leading cause has been thought to be the non-judicious use of antibiotic drugs in the poultry feed. A recently published report suggested that in the European countries the *E. coli* isolates obtained from poultry and cattle exhibited greater resistance to antimicrobial drugs, and there were very strong associations between the dosages of antibacterial drugs and their patterns of antibiotic resistance (Chantziaras *et al.* 2013; Ariffin *et al.* 2019). Moreover, FDA has highlighted that the resistance against antimicrobial drugs in the Enterobacteriaceae family can be transferred from poultry

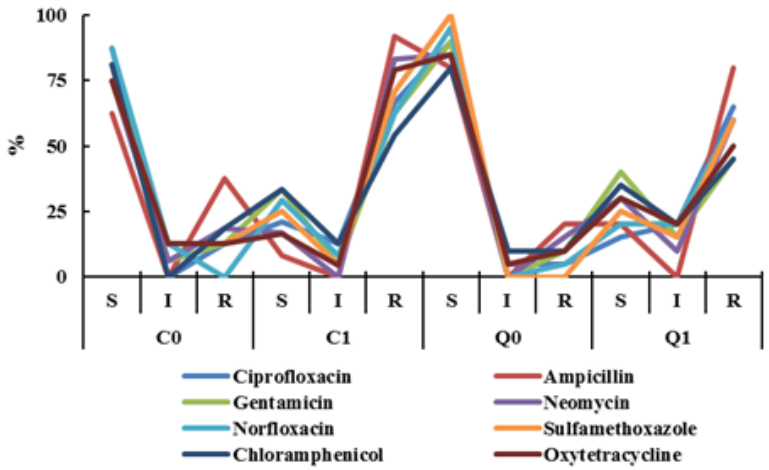

Fig. 2: Percentages of *Salmonella* isolated from commercial and non-commercial poultry susceptible (S), intermediate (I) and resistant (R) to antimicrobial agents by disk diffusion method. C0: backyard chickens (n=16); C1: commercial broiler (n=24); Q0: backyard/wild quails (n=20); Q1: commercial quails (n=20)

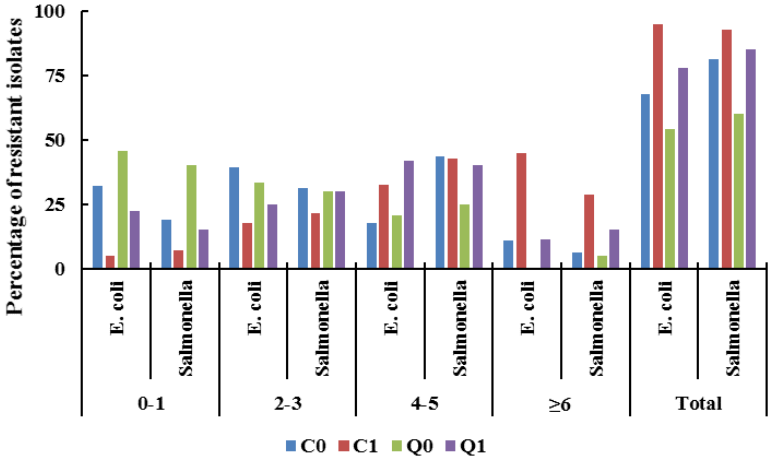

Fig. 3: Percentage of *Escherichia coli* and *Salmonella* isolates of commercial and non-commercial poultry resistant to multiple antimicrobials. C0: backyard chickens; C1: commercial broiler; Q0: backyard/wild quails; Q1: commercial quails

reared on those antimicrobial drugs to human populations (Rafiei and Nasirian 2003), particularly through the consumption of eggs, milk, and meat obtained from such animals and poultry birds (Reig and Toldra 2008).

The primary purpose of using antibiotic drugs in poultry feed and water is to prevent diseases in the commercial flocks. However, owing to the enhanced feed efficiency and weight gain with these drugs, their non-judicious use up to unacceptable levels has increased to achieve maximum monetary benefits from the commercial poultry flocks. Recently published reports have suggested that the non-medical use of these drugs results in rendering these drugs ineffective against the control of few strains of gut microbes, which upon poultry harvest can be transferred to the human food chain (Koga *et al.* 2015). Our previously results (Kamboh *et al.* 2018b) and findings of some other authors (Miranda and Zemelman 2002; Nisar *et al.* 2017; Davis *et al.* 2018; Gad *et al.* 2018) suggest that the incidence of AR microbes was higher in commercially reared chickens as compared with those in domestically reared ones. In this study, the *E. coli* and *Salmonella* isolates obtained from the commercial broiler chickens and quails exhibited alarmingly higher resistance patterns against the eight antibiotic drugs tested in this experiment, as compared with the isolates obtained from backyard chickens and wild quails. In previously published studies, poultry reared without antibiotics reported less antibiotic-resistant *E. coli* as compared with the conventionally reared ones (Zhang *et al.* 2011). Similarly, the chicks reared on an organic diet have also been found to have lower numbers of antibiotic-resistant bacteria than those reared on a commercial diet (Smith-Spangler *et al.* 2012).

Unfortunately, during the last two decades, the nonjudicial use of antibiotics for seeking higher profitability in the poultry sector has tremendously increased in most of the countries having poor economic conditions. Particularly

in Pakistan, the increase in the use of antibiotics has been about 65% in the last 16 years in the poultry sector (Klein *et al.* 2018). The major reason associated with nonjudicial infeed consumption of antibiotics for poultry production includes poor rearing conditions, high microbial contamination in feed and drinking water, imbalanced poultry nutrition, increasing occurrences of urbanization-related nonbacterial infections, which have caused to increased prevalence of AR of pathogenic bacteria (Weaver 2013; Collineau *et al.* 2017; Nandi *et al.* 2017; Klein *et al.* 2018). Particularly, in this report, all the bacterial isolates showed high resistance (about 54%) against chloramphenicol. It is an antibiotic drug that has been banned for use in the poultry diet in several countries such as Europe and the US; however, it is still excessively being used in several developing countries.

Moreover, the findings of this study revealed that the commercial broiler chickens and quails exhibited a higher incidence of MDR strains of both *E. coli* and *Salmonella* than those of non-commercial chickens and quails. These findings agreed to the results of Miranda *et al.* (2008a), who found a higher incidence of MDR enteric microbes in the meat obtained from commercially reared chickens (63.3%) as compared with those obtained from turkeys (56.7%) and organic chickens (41.7%) after the application of aminoglycosides, penicillins, and quinolones in the diets of commercial chicks. Extensive and non-judicial use of antibacterial agents in the poultry feeds makes *Salmonella* and *E. coli* become resistant against those drugs (Fielding *et al.* 2012). The phenomenon of MDR against the antimicrobial drugs investigated in this study was in agreement with the earlier published report (Brown *et al.* 2019).

Several studies have investigated the role of antimicrobial drugs in the poultry production systems in the induction of AR in poultry birds (Chantziaras *et al.* 2013; Kamboh *et al.* 2018b). Moreover, several reports have published the resistance patterns of pathogenic microbial isolates obtained from poultry being reared in the organic and conventional production systems (Miranda *et al.* 2008b; Miranda *et al.* 2009). It has been well documented that the zoonotic microorganisms isolated from conventional poultry production systems exhibit a remarkably higher prevalence of AR in contrast to the ones isolated from organic production systems (Young *et al.* 2009). Particularly, the isolates of *E. coli* obtained from conventional and organic production systems exhibited similar AR patterns in a European study (Österberg *et al.* 2016). Similarly, some other reports also found that the isolates of *E. coli* and *Staphylococcus aureus* from beef (Miranda *et al.* 2009) and intestines (Sato *et al.* 2004, 2005) obtained from conventional rearing system exhibited higher antimicrobial resistance in contrast to the ones obtained from an organic production system. Furthermore, the phenomenon of multidrug resistance was also higher in the bacterial isolates of the liver obtained from commercial broilers as compared to those of backyard chickens (Kamboh *et al.* 2018b).

Conclusion

The AR of both the bacterial isolates (*E. coli* and *Salmonella*) obtained from commercial broiler chickens and quails were higher in contrast to those of their non-commercial counterparts against the eight antimicrobial drugs tested in this study. Moreover, the total bacterial (*E. coli* and *Salmonella*) isolates obtained from all the four bird groups were the most resistant against ampicillin, followed by neomycin in this study. Finally, the incidence of MDR in both bacteria was also higher in the commercial chickens and quails isolates as compared with their non-commercial backyard counterparts.

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Authors Contributions

Asghar Ali Kamboh conceptualized the study, Asghar Ali Kamboh and Tarique Ali Rind performed the research. Muhammad Amar Khan wrote the manuscript, and Kanwar Kumar Malhi contributed to analysis and interpretation of the study data. Rehana Burriro and Riaz Ahmed Leghari helped in analysis and proof reading.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

All data reported in this article are available and will be produced on demand.

Ethics Approval

All study protocols were approved by the Directorate of Advanced Studies, Sindh Agriculture University, Tandojam.

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