Impact of unhygienic conditions during slaughtering and processing on spread of antibiotic resistant *Escherichia coli* from poultry

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Abstract

Antibiotic resistance in *Escherichia coli* is a global health concern. We studied all possible routes of cross contamination of broiler meat with resistant *E. coli* from broiler feces at poultry shops. Various sample categories namely poultry feces, meat (n=225 for each), slaughterer hands, consumer hands, slaughterer knife, canister, tap water, carcass, feed and drinking water (n=50 for each) were collected from local poultry processing market. Samples were screened for prevalence of *E. coli*, resistance of isolates against ten antibiotics and presence of tetracycline-resistance genes in the isolates. Fecal samples had greatest colony count (4.1×10^4 CFU/g) as compared to meat (1.9×10^4 CFU/g) samples. Samples of consumer hands (6%) and tap water (12%) had less prevalence percentages of *E. coli* as compared to slaughterer hands (92%) and drinking water of broiler (86%). Isolates of eight sample categories had high resistant rate (≥90%) against oxytetracycline. On average, about 94% of the isolates from various sample categories possessed multidrug-resistance (MDR). Tetracycline-resistance genes (*tetA* and *tetB*) were identified in all sample categories except isolates of consumer hands and tap water. The distribution of tetracycline-resistance genes was significantly greater in fecal isolates (42%) than meat isolates (25%). The study depicted the spread of resistant *E. coli* in broiler meat through all studied routes of contamination of slaughtering periphery. This problem can be mitigated by strict monitoring of antibiotics use at poultry farms, prevention of cross contamination by adopting hygienic slaughter and vigorously screening the market meat for resistant *E. coli*.

Introduction

Escherichia coli commonly colonize the gastrointestinal tract of animals and some of its strains instigate gastroenteritis, urinary tract infections and meningitis.[1] According to WHO, diarrheal diseases associated with *E. coli* account for over 4% of the total daily global disease burden and about 1.8 million deaths each year, of which 90% are children.[2] Avian Pathogenic *Escherichia coli* (APEC) in poultry causes colibacillosis, septicemia and cellulitis and it may also link to the extra-intestinal pathogenic *E. coli* strains in humans. These extra-intestinal pathogenic *E. coli* strains cause diseases in outside the intestinal tract of humans.[3] To control bacterial infections in humans and animals, huge amounts of antibiotics are used. A number of antibiotics like β-lactams and quinolones are commonly recommended for poultry diseases.[4,5] In many Asian and African countries, large amounts of antibiotics are frequently added in broiler feed as antimicrobial growth promoters and results in antibiotic resistance.[6] Enzymatic degradation of antimicrobial drugs, alteration in bacterial proteins and changes in membrane permeability to antibiotics are mechanisms of development of antibiotic resistance in bacteria. In intensively reared animals, antibiotics are administered to whole flock rather than the individual animal. Therefore, antibiotic selection pressure against resistance in bacteria in broiler is high and their feces are often loaded with antibiotic resistant microbiota.[7] In Pakistan, for example, fecal *E. coli* of food animals were found resistant against many commonly prescribed veterinary medicines.[8]

At slaughter, resistant strains from gut readily spoil the poultry carcass and consequently, meat is often contaminated with multi-resistant *E. coli*.[9] Antibiotics not only select the pathogenic bacteria for resistance but also induce resistance in microbiota of exposed individuals[10] and thus it becomes difficult to treat human ailments.[11] Resistant APEC may transfer their resistant genes to humans through food chain and cause complications in the treatment of urinary tract infections.[3,12] Resistant fecal *E. coli* may become part of human intestinal microbiota directly by individual exposure or indirectly via food chain.[10] Therefore, resistant *E. coli* from poultry may contaminate the environment and are disseminated throughout the ecosystem.[13] Despite uncontrolled use of antibiotics, particularly in developing countries, at poultry farms and high prevalence rate of antibiotic resistance in *E. coli* isolates of broiler, there is no latest report on spread of resistant *E. coli* in the environment.

Tetracycline-resistance genes were identified in *E. coli* isolates of meat and fecal samples of broiler[14] and such resistant genes may spread from poultry feces to humans.[10,15] Transfer of CMY-2 AmpC β-lactamase plasmids of *E. coli* from food animals to humans has also been reported in Iowa, United States.[16] Increase in resistance of Gram-negative bacteria is mainly due to presence of mobile genes that may spread through bacterial population. Furthermore, unprecedented human air travel and migration allowed resistant-bacterial plasmids and clones to transport between countries. Owing to transfer of resistant bacteria, antimicrobial resistance has become a major threat to global public health against effective prevention and treatment of an ever-increasing range of infections caused by *E. coli*.[17-19]

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In many developing countries, including Pakistan, India, Bangladesh, and Afghanistan, more than 90% of human population consumes broiler meat purchased from local poultry processing markets located in open environments of streets, roads and populated areas of cities. Skinning and evisceration are done without taking care of contamination of meat with feces and blood. Unhygienic practices of using unclean knives, dirty containers, and unwashed cutting boards are the possible routes of contamination from poultry feces to poultry meat and surrounding environment. In Pakistan, these poultry shops are the major source of poultry meat.

Keeping in view the unhygienic conditions at the poultry shops, we speculated that these poultry shops might be spreading resistant *E. coli* originating from broiler feces. The key objective of our study was to evaluate spread of fecal *E. coli* and its resistance determinants through poultry slaughtering and processing practices adopted at local markets.

Materials and Methods

Sampling

Fecal and meat samples (n=225 each) of broilers were randomly collected from different poultry shops located in four cities (Khanewal, Multan, Dera Ghazi Khan and Bahawalpur) of South Punjab, Pakistan. Not any single bird was specifically sacrificed for this study. Swabbing of slaughterers' hands, consumers' hands, slaughtering knives, cutting boards, canisters and broiler carcasses was done (n=50 each) from randomly selected poultry shops. A sterilized swab, premoistened with phosphate buffer saline (PBS), was used for each sample. To cover maximum area, the swab was rolled three times for each sample. Each swab was dipped and vortexed in 5 mL of brain heart fusion broth for 10 s.[20]

Samples of broiler feeds and drinking water were also randomly collected from these poultry processing shops. Swabbing of consumer's hands was done before their entrance to the poultry shops (as mentioned above) and samples of tap water (unfiltered and untreated) were collected after running the tap for five minutes. Collected samples were placed in sterile plastic bags and stored at 4°C until further experimentation. All the chemicals and materials used in this study were purchased from Oxoid (UK) or Sigma-Aldrich (USA).

Isolation and colony counts

For isolation of *E.coli*, fecal, meat and feed samples were homogenized in Butterfield's phosphate-buffered water. The diluted blends and water samples (tap/drinking) were inoculated in Lauryl Tryptose Soya (LTS) broth following incubation.[21] All swabs were separately enriched in Lauryl Tryptose Soya (LTS) broth. Positive samples from LTS broth were identified with gas production and transferred on Levine's Eosin Methylene Blue (L-EMB) agar.[22] Dark centered and flat colonies with or without metallic sheen were isolated and confirmed as *E. coli* by IMViC and sugar biochemical reactions. Colonies of *E. coli* were counted and reported in CFU/ml, CFU/g or CFU/cm^2.

Antibiotic resistance

Antimicrobial resistance pattern of isolated *E. coli* strains were carried out with disk diffusion method on Mueller Hinton agar.[23] Ten drugs belonging to eight antimicrobial categories [1st generation cephalosporins (cephradine), 3rd generation cephalosporins (ceftriaxone), phenicols (chloramaphenicol), aminoglycosides (gentamycin), quinolone/flouroquinolone (nalidixic acid, ciprofloxacin), penicillin (penicillin, amoxicillin), tetracycline (oxytetracycline) and macrolides (azithromycin)] were used in this study. Results on *E. coli* isolates were classified to sensitive and resistant groups. Antibiotic control strain *E. coli* ATCC 25922 was used for standardization.[24]

Resistant isolates were further classified to possible multi-drug resistant (MDR), confirmed MDR, possible extensively-drug resistant (XDR) and possible pandrug resistant (PDR).[25] Possible MDR isolates were resistant to at least one drug in less than three out of the used eight antimicrobial categories. Confirmed MDR isolates were resistant to at least one drug in at least three antimicrobial categories. Any confirmed MDR isolate that was resistant to one drug in all but less than or equal to two antimicrobial categories was named as possible XDR and a possible XDR isolate resistant to all the used ten drugs was named as possible PDR.

Tetracycline-resistance genes

All tetracycline-resistant isolates were further observed for presence of tetracycline-resistance genes through PCR. Bacterial genomic DNA was extracted by method of Seidavi *et al.*[26] and confirmed by 16s rRNA gene using oligonucleotide primers ECO-F GACCTCGGTTTAGTTCACAGA and ECO-R CACACGCTGACGCTGACCA with product size 585bp.[27] Moreover, tetracycline resistant genes (*tetA* and *tetB*) were amplified with primers *tetA*-F GGTTCACTCGAACGACGTCA, *tetA*-R CTGTCCGACAAGTTGCATGA, *tetB*-F CCTCAGCTTCTCAACGCGTG and *tetB*-R GCACCTTGCTGATGACTCTT.[28] PCR reactions were carried out in total volume of 25 µl containing 5 µL DNA, 2.5 µL buffer × 10, 2 µL $MgCl_2$, 5 µL dNTP mix, 0.25 µL of each primer and 0.2 µL Taq polymerase.[29] Denaturation process were carried out at 95°C for 5 min and amplification was performed at 94°C for 60 s, 50°C for 45 s, and extension at 72°C for 90 s, with further extension at 72°C for 300 s. PCR products were analyzed by electrophoresis in 1.5% agarose gel and stained by ethidium bromide.

Statistical analysis

Association of prevalence of *E. coli* and antibiotic resistance with sample categories was estimated by employing Chi-square (χ^2) test on SPSS version 21 (IBM Corporation, USA).

Results

Prevalence percentage and colony counts

Greater than 80% isolates of all sample

Table 1. Prevalence of E. *coli* in different categories of samples taken from poultry shops in Pakistan.

Sample categories	N. samples	Observed counts (%)
Broiler feces	225	225^a (100)
Broiler meat	225	225^a (100)
Slaughterer hands	50	46^b (92)
Consumer hands	50	3^c (6)
Slaughtering knife	50	46^b (92)
Cutting board	50	46^b (92)
Canister	50	50^b (100)
Broiler carcass	50	50^b (100)
Tap water	50	6^c (12)
Broiler feed	50	42^b (84)
Drinking water of broiler	50	43^b (86)

^{a,b,c,d}Each superscript letter denotes a subset of sample categories whose column proportions do not differ significantly from each other at α=0.05. For all categories, zero cells (0%) have expected count less than 5.

categories had *E. coli* except that of consumer hands (6%) and tap water (12%) (Table 1). All collected samples of feces, meat, canister and carcass were 100% positive for *E. coli*. Prevalence percentage of *E. coli* in samples of slaughterer hand, knife and cutting board was statistically similar (about 92%).

Observed load of *E. coli* was more than double in broiler feces (4.1×10^4 CFU/g) than meat (1.9×10^4 CFU/g) samples (Figure 1). Drinking water of broiler had about six times more *E. coli* load than tap water (1.5×10^2 CFU/mL). Similarly, slaughterer hands (1.4×10^4 CFU/cm^2) had much greater load of *E. coli* than on consumer hands (11 CFU/cm^2).

Antibiotic resistance and MDR

In present study, antibiotic resistance of *E. coli* isolates from all sample categories against the 10 antibiotics ranged from 0 to 100% (Table 2). Most *E. coli* isolates from feces, meat, carcass and canister were resistant against eight antibiotics. Surprisingly, level of resistance against oxytetracycline was >80% in all sample categories except samples of consumer hands (0%), tap water (4%) and drinking water (20%). Comparing the results of resistance of feces and meat, significant ($P<0.05$) difference in antibiotic resistance against gentamycin, nalidixic acid, penicillin, cephradine, amoxicillin, azithromycin and ciprofloxacin was found. *E. coli* isolates from consumer hands were completely sensitive to all antibiotics while isolates from tap water showed minimum resistance against the tested drugs. Majority of isolates from all sample categories were sensitive against ceftriaxone except isolates of feces and cutting board.

All isolates from slaughterer hands and knife were susceptible to azithromycin (100%) and no significant difference was observed for ceftriaxone, gentamycin, penicillin, cephradine, oxytetracycline and ciprofloxacin for both sample categories.

On average, about 94% of the isolates from various sample categories were resistant to one or more of the drugs from ≥3 antimicrobial categories tested. Alarmingly, 51 isolates from broiler feces and 24 isolates from broiler meat were resistant to all drugs except two and were classified as possible XDR. Moreover, 4 isolates from broiler feces and 1 isolate from canister were completely resistant to all the drugs tested.

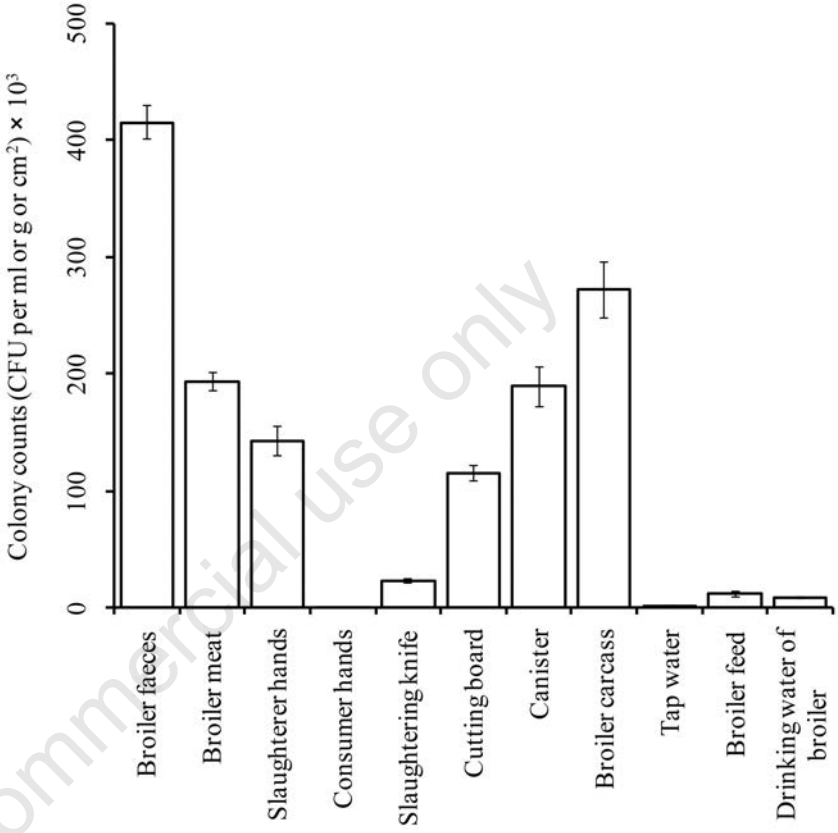

Figure 1. Colony count of *Escherichia coli* in broiler faeces (n=225), meat (n=225), slaughterer hands (n=50), consumer hands (n=50), slaughtering knife (n=50), cutting board (n=50), carcass (n=50), tap water (n=50), canister (n=50), feed (n=50) and drinking water (n=50). Error bars are of standard deviation.

Table 2. Antibiotics resistance in *E. coli* isolated from different categories of samples taken from poultry shops in Pakistan.

Antibiotics	Faeces (n=225)	Meat (n=225)	Slaughterer hands (n=46)	Consumer hands (n=2)	Slaughtering knife (n=46)	Cutting board (n=46)	Container (n=50)	Carcass (n=50)	Tap water (n=6)	Feed water (n=42)	Drinking (n=43)
Ceftriaxone	31^{b}	10^{d}	4^{d}	0^{e}	6^{d}	39^{a}	26^{bc}	20b^{c}	3^{d}	7^{d}	3^{d}
Chloramphenicol	130^{a}	123^{a}	8^{d}	0^{e}	30^{b}	32^{b}	34^{b}	37^{b}	2^{d}	18^{c}	6^{d}
Gentamycin	164^{a}	104^{b}	14^{d}	0^{f}	16^{e}	28^{d}	20^{d}	36^{c}	3^{f}	23^{d}	18de
Nalidixic acid	163^{a}	141^{b}	27^{cd}	0^{e}	31^{c}	32^{c}	36^{c}	36^{c}	5^{e}	22^{cd}	21^{cd}
Penicillin	225^{a}	138^{b}	20^{c}	0^{e}	19^{c}	17^{c}	26^{c}	18^{c}	1^{a}	17^{c}	8^{d}
Cephradine	165^{a}	126^{b}	13^{c}	0^{f}	12^{c}	22^{c}	22^{c}	24^{c}	2^{e}	17^{c}	11^{d}
Amoxycillin	138^{a}	110^{b}	20^{c}	0^{e}	18^{cd}	26^{c}	33^{c}	26^{c}	3^{d}	11^{d}	7^{d}
Azithromycin	106^{a}	73^{b}	0^{e}	0^{e}	0^{e}	12^{d}	21^{c}	24^{c}	0^{e}	8^{d}	4de
Oxytetracycline	225^{a}	222^{a}	44^{b}	0^{d}	43^{d}	42^{b}	45^{b}	47^{b}	2^{cd}	41^{b}	10^{c}
Ciprofloxacin	192^{a}	135^{b}	23^{d}	0^{e}	23^{d}	25^{d}	39^{c}	26^{d}	3^{e}	32^{cd}	17^{d}

^{a,b,c,d,e,f}Each superscript letter denotes a subset of sample categories whose column proportions do not differ significantly from each other at α=0.05. For all categories zero cells (0%) have expected count less than 5.

Tetracycline-resistance genes

PCR products for tetracycline-resistance genes were obtained for 95 faecal, 57 meat, 6 slaughterer hand, 7 slaughterer knife, 6 cutting board, 5 canister, 7 carcass, 8 feed and 2 drinking water isolates (Table 3). For all sample categories, prevalence percentage of genotypic results of tetracycline-resistance was lower than results of phenotypic percentage. The positive rate of *tetA* was greater in feces (44%) while almost same prevalence percentage for *tetA* and *tetB* was observed in all sample categories. Study demonstrated presence of *tetA*, *tetB* and *tetA+tetB* in all sample categories except in isolates of consumer hands and tap water.

Discussion

Escherichia coli is the commensal bacterium and reside in the gut of animals including broiler.[30] Similar to this study (Figure 1, Table 1), greater prevalence of *E. coli* in poultry feces was observed in previous study as compared to other sample categories.[10] Contaminated poultry feed and water are major sources of *E. coli* for broiler.[31,32] Prevalence of *E. coli* in drinking water (86%) and feed (84%) at poultry shops (Figure 1, Table 1) was greater than reported in water (19%) and feed (35%) samples of broiler farms.[31-33] This is probably because of contamination of feed and water pots with broiler feces at poultry shops. Amin *et al.*[34] observed *E. coli* in about 8% samples of tap water of Pakistan that was in line to present study (Table 1). At poultry shops, tap water is used for the washing of utensils, tables, cutting knives, and hands. Relatively lesser *E. coli* load in tap water means that it was not the major source of high *E. coli* prevalence in various samples collected from slaughtering and processing markets.

During slaughtering, carcass of poultry may be contaminated with the gut contents from which *E. coli* may spilled out as a contaminant. Seidavi and collaeuges,[26] found that 88%, 38% and 25% samples respectively of cecum, ileum and duodenum (gut contents) yielded *E. coli* in healthy broiler chickens. Feces of broiler, gut contents and carcass of bird are considerably major sources of contamination of meat. High prevalence percentage of *E. coli* in meat (100%) was in line to study of India (98%) while higher than of Morroco (48%), Washington (39%) and Bangkok (25%).[35-38] Presence of *E. coli* in meat poses serious health issues for consumers at large. Extremely high prevalence rate pinpointed precarious and dangerous unhygienic conditions of slaughtering at retail butcher shops.

We assumed that the screening and then colonization under selection of antibiotics from extraneous sources resulted in resistant *E. coli* colonization of the chicken gut. Work on poultry showed the presence of different antibiotics in reasonable amounts that could select *E. coli* transferred to poultry meat.[39] Resistant bacteria from feces of bird may spread to meat[40] and possibly to surrounding periphery. In most categories of samples, resistant *E. coli* were detected (Table 2). Resistance in broiler *E. coli* might have been developed due to extensive use of antibiotics at poultry farms.[41,42] Majority of isolates from all sample categories were resistant to oxytetracycline (Table 2). Tetracycline resistant *E. coli* isolates from poultry meat, feces, water, carcass and poultry products was also examined in a number of previous studies.[15,30,33,43,44] Elevated level of tetracycline resistance might suggest widespread and extensive use of tetracycline at poultry farms.[10] Tetracycline is the first line drug used for prophylaxis and for growth promotion of livestock.[45] Determined resistance of *E. coli* against oxytetracycline (84%) in poultry feed was much higher than the study of Portugal that showed maximum resistance of 41% against tetracycline.[39] These differences in antibiotic resistance in *E. coli* among previous and the present studies might be due to the differences in purity, dosage, price, laws, access, availability and awareness regarding usage of antibiotics in different regions.[46] In Switzerland, for example, where usage of antibiotic is monitored by the government agencies, low levels of antibiotic resistance against cephalosporins were observed in isolates of poultry faeces.[47]Quinolone is next to tetracycline that is commonly used at clinical sites.[48] Frequency of resistance in *Escherichia coli* isolated from meat and faecal samples (63-72%) to nalidixic acid (Table 2) was lesser than findings of Miles[15] who reported 85% resistance of nalidixic acid against *E. coli*. Greater resistance level of *E. coli* isolates against first generation cephradine (Table 2) suggested that the presence and routine use of antibiotics. This exerts selective pressure in poultry farms, allowing Cephradine-resistant pathogenic *E. coli* strains to dominate the intestinal microbiota of the birds. Compared with other drugs used in this and a previous study,[49] sensitivity of *E. coli* isolates to ceftriaxone in poultry meat perhaps was due to low usage of this antibiotic at broiler farms. Complete resistance (100%) for two drugs importantly in fecal samples indicated routine use of these antibiotics in animal feed again driven up penicillin, tetracycline, quinolones and fluoroquinolone resistance rates. In present study, isolates exhibited alarmingly higher multi-drug resistance (Table 4). Prevalence of such highly resistant isolates poses a challenge not only for poultry industry but also in ailment of humans. Observed MDR, XDR and PDR probably evolved through excessive usage of multiple antibiotics in poultry for growth promoters and therapeutic purposes- a practice that needs to be modified greatly if poultry farmers want to control spread of pathogenic *E. coli*.

Reports on dissemination and amplification of resistant genes including that for tetracycline-resistant genes in the environment are available.[50] Detection of *tetA* and *tetB* genes carried by all studied groups except control groups of consumer hands and tap water (Table 3) demonstrated the distribution of similar resistant determinants in diverse genetic background. This probably indicated the continuous exposure to tetracycline resulted in high percentage of tetracycline-resistant *E. coli* and these

Table 3. Distribution percentages of tetracycline-resistance genes in different categories of samples taken from poultry shops in Pakistan.

Samples categories	Total confirmed *E. coli* isolates (n)	*tetA* (%)	*tetB* (%)	*tetA+tetB* (%)
Broiler feces	225	44	42	42
Broiler meat	225	27	25	25
Slaughterer hands	46	20	13	13
Consumer hands	2	0	0	0
Slaughtering knife	46	15	15	15
Cutting board	46	13	13	13
Canister	50	13	10	10
Broiler carcass	50	14	14	10
Tap water	6	0	0	0
Broiler feed	42	19	19	19
Drinking water of broiler	43	5	5	5

Table 4. Distribution of multidrug resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) *Escherichia coli* in various sample types.

Sample type	N.	Possible MDR	Confirmed MDR	Possible XDR	Possible PDR
Broiler faeces	225	1	224	51	4
Broiler meat	225	10	215	24	0
Slaughterer hands	46	7	39	0	0
Consumer hands	2	2	0	0	0
Slaughtering knife	46	6	40	3	0
Cutting board	46	0	46	11	0
Canister	50	1	49	9	1
Broiler carcass	50	1	49	9	0
Tap water	6	1	5	1	0
Broiler feed	42	7	35	1	0
Drinking water of broiler	43	26	17	0	0
Total	1006	63	943	160	9

isolates may have diversity of resistance genes. Presence of tetracycline-resistance genes in above mentioned sample categories showed significant level of cross-resistance among all routes relevant to broiler meat. This study was in agreement to the results of Miles *et al.*[15] who determined tetracycline-resistance in diverse genetic background. This elevated level of dissemination of *tetA* and *tetB* genes in present study suggested limited therapeutic options for broiler colonized with tetracycline-resistant *E. coli*. It has already been documented that indiscriminate usage of antimicrobial agents in poultry industry had led to dissemination of antibiotic resistant genes from poultry to humans.[40] It is further suggested that colonization of these resistant species will effect human gastrointestinal microbiota by transfer of their plasmids to endogenous microbiota.[10] Therapeutic use of drugs, such as quinolones, in animal feed had resulted in resistance in *Enterobacter* species in humans.[51] Therefore, in-feed antibiotics must be replaced with suitable alternatives.[52]

Conclusions

Prevalence of resistant *E. coli* in most sample categories clearly depicted the level of cross contamination of broiler meat from different routes during slaughtering and processing practices. Greater resistant isolates from all sample categories, specifically in feces and meat, against most antibiotics presented an alarming situation. Spread of tetracycline-resistance genes may precariously depict broiler meat as a danger for human health. These resistant determinants of *E. coli* may not only pose threat for slaughterers but also confront several risks to control most common diarrheal outbreaks in southern Punjab, Pakistan and similar areas. Greater MDR isolates revealed the possibility of indiscriminate use of antibiotics at poultry farms. For the provision of safe and healthy protein source for humans, regulatory authorities must strictly govern the use of antibiotics at poultry farms. There is a dire need to educate poultry farmers for the use of only permitted antibiotics within the recommended dosage. Moreover, slaughterers must be educated how to prevent cross contamination and adopt hygienic practices during slaughtering and processing of chicken meat for sale to prevail the situation. Contaminated raw meat loaded resistant *E. coli* is unfit for human consumption until proper cooking. To control disease burden, therefore, there is dire need to vigorously screen broiler meet for resistant *E. coli*.

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