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SPREAD OF ANTIBIOTIC RESISTANT *Escherichia coli* FROM BROILER TO HUMAN POPULATIONS

Mamoona Amir[1,2], Muhammad Riaz[1,*], Yung Fu Chang[2], Saeed Akhtar[1], Habibullah Nadeem[3], Zulfiqar Ahmad[4] and Muhammad Nadeem[5]

[1]Institute of Food Science & Nutrition, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan 60800, Pakistan; [2]Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853 (USA); [3]Department of Bioinformatics and Biotechnology, Government College University, Faisalabad; [4]Department of Food Science and Technology, University College of Agriculture and Environmental Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan; [5]Department of Environmental Sciences, COMSATS University Islamabad, Vehari-Pakistan
[*]Corresponding author's e-mail address: riaz@bzu.edu.pk

Escherichia coli is a commensal microorganism while spread of its resistant pathotypes may cause life threatening issues. In this study, fecal samples (n =150 each) of broiler, slaughterers and non-slaughterers were observed for resistance rate of their *E. coli* isolates against 16 antibiotics. Significantly ($P \leq 0.05$) higher *E. coli* counts (3.4×10^5 CFU/g) were observed in fecal samples of broiler than slaughterers (3.1×10^4 CFU/g) and non-slaughterers (2.8×10^4 CFU/g). Higher antibiotic resistance rates were observed in broiler isolates than slaughterer against all the tested antibiotics except Oxytetracycline, Cephalexin and Ceftriaxone; for which the differences were non-significant. As compared to isolates of non-slaughterers, isolates of slaughterers had significantly ($P \leq 0.05$) higher antibiotic resistance rates against 8 of the tested drugs. The results revealed that 100% broiler, 78% slaughterer and 17% non-slaughterer isolates were simultaneously resistant to more than 4 antibiotics. Both *Tet-A* and *Tet-B* genes (tetracycline-resistance genes) were concurrently detected in 21% of broiler, 15% of slaughterer and 5% of non-slaughterer isolates. Differences in antibiotic resistance rates between the isolates of slaughterers and non-slaughterers clearly depicts one of the possible spreading route of resistant *E. coli* from broiler to human population. Hence, it is recommended that slaughterers must adopt hygienic protocols to avoid the spread of resistant bacteria.
Keywords: Antibiotic resistance, poultry disease, pathogenesis, *E. coli*, tetracycline-resistance genes.

INTRODUCTION

Escherichia coli is a commensal organism; but its few strains may cause diseases in humans and animals. Pathogenic strains of the microbes differ from normal microbiota due to presence of virulence factors— genes that are directly involved in pathogenesis. Expression of virulence factors of *E. coli* disrupts host physiology and consequently causes diseases. Pathogenic strains of *E. coli* are involved in almost 90% of all community acquired and 50% of nosocomial infections like urinary tract infections (Kazemnia *et al.*, 2014). Large numbers of antibiotics are often used (in sub-therapeutic doses) to reduce *E. coli* infections in humans and food animals. In addition to therapeutic use at poultry farms, antibiotics are also used for growth promotion, and prophylactic and meta-phylactic purposes (WHO, 2004). For example, tetracycline is the first line drug used in large quantities for medicinal purposes in domestic animals and for growth promotion at animal farms (Chopra and Roberts, 2001). Beta-lactams agents like cephalosporin are used in veterinary and human populations. Excessive use perhaps is the cause of reduction in effectiveness of cephalosporin (Webster, 2009). Indiscriminate use of antibiotics leads to antibiotic selective pressure and thus resistance in commensal microbiota. Resistance against several common antibiotics has already been documented in commensal microorganisms isolated from chickens and buffaloes (Soomro *et al.*, 2010). Use of cephalosporins at chicken hatcheries resulted in development and spread of resistant microbes (Webster, 2009). Resistant bacteria may gain entry into the human intestinal tract through contamination of food chain and also by continuous occupational exposure to resistant microbes (Witte *et al.*, 1999). Spread of resistant plasmids of *E. coli* from chicken to human's intestinal tract has already been documented (Van den Bogaard *et al.*, 2001). Extensive use of one or more drugs has resulted in multidrug resistance in *E. coli* (Cao *et al.*, 2011). The global problem of antibiotic resistance is overwhelming in developing countries where disease burden is high and cost constraints restrict the application of new drugs. Gastrointestinal, respiratory and nosocomial infections are common in developing countries due to the development and spread of resistant microbes (Okeke *et al.*, 2005). Therefore, the domestic distribution

and application of antibiotics need to be monitored. This information will be crucial for epidemiologists and clinicians in determining which antibiotics have been rendered ineffective through inadvertent resistance marker selection via industrial-scale applications (WHO, 2004).

About 1000 million kg poultry meat is annually produced in Pakistan that is about 27% of the total meat production and about 1% of GDP of the country. Poultry consumption in Pakistan is around 5 kg capita^{-1} y^{-1} and it is increasing each year (FAO, 2013). In Pakistan, antibiotics are easily accessible without any prescription (Mund *et al.*, 2017). This allows poultry growers to use large quantities of antibiotics without any monitoring. Such large-scale use of antibiotics might have implicated in the spread of resistant *E. coli*. However, there is no report from Pakistan on the possible spread of antibiotic resistant bacteria from broiler to humans. Present research activity was conducted to achieve following three objectives: (i) to observe the antibiotic resistance rates in *E. coli* isolates from fecal samples of broilers, slaughterers and non-slaughterers, (ii) to evaluate the prevalence of multidrug resistance in the isolates of broilers and humans, (iii) to compare the prevalence of tetracycline-resistance genes in the isolates of broilers, slaughterers and non-slaughterers.

MATERIALS AND METHODS

***Collection of samples*:** Fecal samples of broilers, volunteer slaughterers (25 to 35 years of age) of broiler and non-slaughterers (25 to 35 years of age) were randomly selected (n=150 each) from different cities (Khanewal, Multan, Dera Ghazi Khan and Bahawalpur) of southern Punjab, Pakistan (Amir *et al.*, 2017). All the methods of the study were preapproved by Ethical Committee of Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan. Not any single bird was specifically slaughtered for the study. The fresh samples of broiler feces, about 15–30 g, were collected from butcher's shops in sterile zip-locked plastic bags. For the collection of human stool samples, the participating subjects were guided in detail about the sampling protocol (Abrahamson *et al.*, 2017). The stool samples were collected in sterile plastic jars. During sample collection, history about health status and use of antibiotics was also noted. All collected samples were placed in an ice box and transported to the institutional laboratory within 4 h.

***Isolation of E. coli*:** Isolation of *E. coli* and biochemical confirmation were performed as described in a previous report (Amir *et al.*, 2017; Byomi *et al.*, 2017). Briefly, fecal samples (25 g each) were separately homogenized in a stomacher using 225 mL Butterfield's phosphate-buffered water. The diluted blend of about 10 µL was first streaked on MacConkey agar and incubated at 37°C for 24 h. Lactose fermented colonies were then streaked on Levine's Eosin Methylene Blue (L-EMB) agar plates. The suspected colonies with metallic sheen were further confirmed as *E. coli* by IMViC and sugar biochemical reactions. Confirmed colonies of *E. coli* were transferred into 2 mL Luria Burtani broth (Thermo Fisher Scientific) and incubated at 37°C for 24 h. *E. coli* strain ATCC 25922 was used as a reference strain.

***Antibiotics resistance*:** The *E. coli* cultures were spread on Muller Hinton Agar (Oxoid) plates (CLSI, 2014). Antibiotic discs were obtained from Oxoid (UK), stored at 4°C and placed at room temperature before application. A number of drugs were evaluated for the prevalence of antibiotic resistance in the *E. coli* isolates: Penicillin (5 units), Amoxicillin (30 µg), Cephradine (30 µg), Azithromycin (15 µg), Ciprofloxacin (10 µg), Gentamycin (30 µg), Nalidixic acid (30 µg), Chloramphenicol (30 µg), Oxytetracycline (30 µg), Cephalothin (30 µg), Cephalexin (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Cefoperazone (75 µg), Cefotaxime (30 µg) and Cefepime (30 µg). Isolates resistant to more than two drugs were considered as multidrug resistant (Van den Bogaard *et al.*, 2001).

***Tetracycline-resistance genes*:** For identification of tetracycline-resistance genes, DNA extraction was performed by following the method of Kagambèga *et al.* (2012). PCR (Ingenius 3, UK) amplification for identification of tetracycline-resistance was carried out by using primer pairs 5′-GTAATTCTGAGCACTGTCGC-3′ and 5′-CTGCCTGGACAACATTGCTT-3′ *Tet-A*, 5′-CTCAGTATTCCAAGCCTTTG-3′ and 5′-ACTCCCCTGAGCTTGAGGGG-3′ *Tet-B* (Thermo Fisher Scientific) (Sengeløv *et al.*, 2003). The amplicon sizes of *Tet-A* and *Tet-B* were 956 and 414 base pairs, respectively. PCR reactions were performed in following conditions: (i) for *Tet-A*; 3 min at 94°C followed by 25 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C followed by 10 min at 72°C, (ii) for *Tet-B*; 3 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 52˚C and 1 min at 72°C followed by 10 min at 72°C. PCR products were analyzed by electrophoresis in 1.5% agarose gel and stained by ethidium bromide.

***Statistical analysis*:** Data of colony counts were analyzed by employing one-way analysis of variance followed by least significant difference (LSD) test at $P \leq 0.05$. To analyze the association among antibiotic resistance rates of the isolates, Chi-square test was applied by using *SPSS* (IBM version 21 Armonk, NY, released 2012).

RESULTS

***Prevalence of E. coli*:** *Escherichia coli* was present in all the fecal samples of broilers, slaughterers and non-slaughterers (data not shown). Colony counts *E. coli* in fecal samples of broiler (3.3×10^5 CFU/g) was significantly ($P \leq 0.05$) greater

than the colony counts in slaughterers (3.1×10^4 CFU/g) and non-slaughterers (2.8×10^4 CFU/g) (Fig. 1).

***Antibiotic resistance rates*:** Of the three population groups, the highest antibiotic resistance rates were observed in fecal isolates of broiler while closely followed by those from slaughterers and in the non-slaughterers (Table 1). Fecal isolates of broiler and slaughterer were simultaneously and highly resistant to Oxytetracycline than any other tested drug. A high resistance rate in *E. coli* isolates from fecal samples of broiler was observed against Penicillin (95%), Oxytetracycline (95%), Nalidixic acid (94%), Ciprofloxacin (83%), Amoxicillin (73%), Gentamycin (70%) and Cephradine (69%). Antibiotic resistance rates in the isolates of broiler were significantly higher than the isolates of slaughterers against 11 out of 16 antibiotics (excepting only Amoxicillin, Oxytetracycline, Cephalexin, Ceftriaxone and Cefepime).

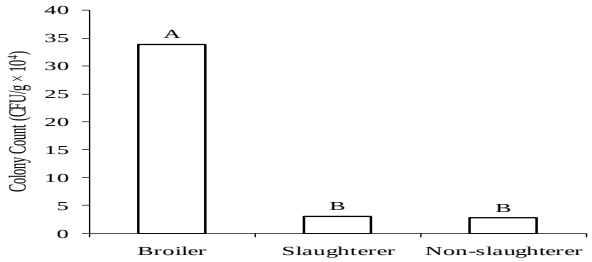

Figure 1. Colony count of (CFU/g) of fecal samples of broiler, slaughterers and non-slaughterer.

Table 1. Prevalence of antibiotic resistance in *Escherichia coli* isolated from 150 (each) fecal samples of broilers, slaughterers and non-slaughterers.

Antibiotics	Broilers	Slaughterers	Non-slaughterers
	Observed counts		
Penicillin	142a	52b	45b
Amoxicillin	109a	94a	49b
Gentamycin	105a	66b	26c
Oxytetracycline	142a	138a	50b
Azithromycin	68a	39b	39b
Chloramphenicol	90a	30b	17b
Nalidixic acid	141a	99b	50c
Ciprofloxacin	125a	61b	33c
Cephradine	104a	73b	56b
Cephalothin	47a	19b	16b
Cephalexin	70a	61a	26b
Ceftazidime	72a	39b	24b
Ceftriaxone	29a	18ab	7b
Cefoperazone	77a	23b	2c
Cefotaxime	65a	34b	16c
Cefepime	0a	0a	0a

[a,b,c]For each disc, each superscript letter denotes a subset of sample categories whose column proportions do not differ significantly from each other at $\alpha=0.05$. For all discs, zero cells (0%) had expected count less than 5.

In slaughterers and non-slaughterers, *E. coli* isolates showed statistically similar tendency of antibiotic resistance rates against half of the tested antibiotics (8 antibiotics). For remaining 8 antibiotics (Amoxicillin, Gentamycin, Oxytetracycline, Nalidixic acid, Ciprofloxacin, Cephalexin, Cefoperazone and Cefotaxime), the isolates of slaughterers had significantly ($P \leq 0.05$) higher antibiotic resistance rates than those of non-slaughterers. All *E. coli* isolates from broilers, slaughterers and non-slaughters were sensitive to Cefepime.

***Multidrug resistance*:** It was observed that 100% isolates of broilers, 78% isolates of slaughterers and 17% isolates of non-slaughterers were simultaneously resistant against more than 4 drugs (Table 2). Against more than 7 drugs, 87% isolates of broilers, 12% isolates of slaughterers and 2% isolates of non-slaughterers were simultaneously resistant. Alarmingly, 21% of broiler origin isolates were simultaneously resistant against at least 10 out of 16 tested antibiotics.

Table 2. Multidrug resistance (%) in *Escherichia coli* isolates from fecal samples of broilers, slaughterers and non-slaughterers against sixteen antibiotics.

Number of antibiotics resistant	Broiler (n=150)	Slaughterer (n=150)	Non-slaughterer (n=150)
0	0 (0)	1 (1)	2 (3)
1	0 (0)	0 (0)	17 (11)
2	0 (0)	2 (1)	39 (26)
3	0 (0)	9 (6)	34 (23)
4	0 (0)	21 (14)	30 (20)
5	5 (3)	36 (24)	15 (10)
6	7 (5)	38 (25)	7 (5)
7	9 (6)	26 (17)	2 (1)
8	22 (15)	13 (9)	2 (1)
9	41 (27)	3 (2)	0 (0)
10	36 (24)	1 (1)	0 (0)
11	15 (10)	0 (0)	0 (0)
12	10 (7)	0 (0)	0 (0)
13	4 (3)	0 (0)	0 (0)
≥14	1 (1)	0 (0)	0 (0)

***Tetracycline-resistance genes*:** A total of 23, 18 and 9% isolates, respectively, of broilers, slaughterers and non-slaughterers had one or both of the Tetracycline-resistance genes (Table 3). Majority of the isolates from each sample category possessed both *Tet-A* and *Tet-B* genes.

Table 3. Tetracycline-resistance genes detected in fecal isolates of broiler, slaughterer and non-slaughterers.

Sample Categories (n = isolates resistant against Oxytetracycline)	*Tet – A* (%)	*Tet – B* (%)	*Tet – A + Tet – B* (%)
Broiler (n= 142)	23	21	21
Slaughterers (n = 138)	18	15	15
Non-Slaughterers (n = 50)	7	7	5

DISCUSSION

Colony counts of *E. coli* observed in fecal samples of broilers were significantly higher *($P \leq 0.05$)* as compared to slaughterers and non-slaughterers (Figure 1). The results were in line to the findings of Van den Boggard *et al.* (2001) who also observed higher load of *E. coli* in poultry feces as compared to poultry slaughterers and farmers. Jiménez *et al.* (2003) observed 3.54 log CFU/ml of *E. coli* isolates in poultry carcass having 11% fecal contamination. Trawinska *et al.* (2016) observed higher colony counts (6.37 log CFU/g) of *E. coli* isolates in poultry feces than the present study (Fig. 1). The difference between the colony counts in the studies might relate with differential microbial load in the regions and also varying sanitary conditions at poultry farms. *Escherichia coli* can readily colonize the ceca of poultry and it is excreted in feces, suggesting poultry as the reservoir of the organism. Massive number of bacteria in poultry feces may also be due to the routine feeding of broilers with contaminated feed (da Costa *et al.*, 2007).

Owing to high bacterial count at poultry farms, a number of antibiotics are used to control infections. Surprisingly, about two thirds of global antibiotics sales occur without prescription. In Pakistan, there is unmonitored use of antibiotics at poultry farms. A major factor for selecting the antimicrobial resistance in *E. coli* (Table 1) may be antibiotic usage at large scale in poultry farms (Sayah *et al.*, 2005). Along with use of antibiotics at poultry farms, the crowding and poor sanitation at poultry farms and butcher shops may also contribute in development and spread of resistant *E. coli* (Van den Bogaard *et al.*, 2001).

Similar to fecal isolates of broiler, high antibiotic resistance rates in fecal isolates of slaughterers (Table 1) reflect the dissemination of resistant bacteria/plasmid to their relevant slaughterers (Van den Bogaard *et al.*, 2001) through various ways. Resistant bacteria are transferred to humans from occupational exposure and improper disposal facilities (Van den Bogaard and Stobberingh, 2000). Direct contact of slaughterers with carcass, continuous exposure to poultry feces and contaminated slaughtering environment (Amir *et al.*, 2017) might be the major factors for dissemination of resistant bacteria from poultry to slaughterers.

We observed a noticeable prevalence of resistance (0 to 37%) in isolates of non-slaughterers against several drugs (Table 1). Antibiotics are widely prescribed to treat human infections. Among the prescribed medicines in Punjab (Pakistan), 70% were antibiotics (Aslam *et al.*, 2016). Moreover, antibiotics are largely used in food producing animals. As a result, meat, pasteurized milk, eggs other food items may contain antibiotic residues. Microbiota of human's large intestine might become resistant after continuous exposure to antibiotic residues.

The resistance rate in fecal *E. coli* isolates of broiler was highest (95%) against Oxytetracycline (Table 1). Others observed 78 to 82% resistance rates in fecal isolates of *E. coli* against tetracycline (Van den Bogaard *et al.*, 2001; Miles *et al.*, 2006). Higher percentage of Oxytetracycline resistant *E. coli* in present study (Table 1) reflect the development of antibiotic resistance in broiler due to greater application of antibiotics at farm level (Van den Bogaard *et al.*, 2001). Tetracycline has been commonly used at animal farms and its member Oxytetracycline is approved as feed ingredient for the purpose of growth promotion (Chopra and Roberts, 2001). Surprisingly, a similar Oxytetracycline resistance rate was observed in broiler (95%) and slaughterers (92%); while significantly lower in non-slaughterers (33%) (Table 1). Tetracycline resistance rates in human isolates are generally lower than 40% (Melo *et al.*, 2015; Miles *et al.*, 2006). Therefore, the higher Oxytetracycline resistance rates in isolates of slaughterers (Table 1) may be due to exposure to contaminated environment of poultry shops (Amir *et al.*, 2017).

Similar to our results of resistance (22%) in isolates of non-slaughterers against Ciprofloxacin (a fluoroquinolone) (Table 1), 24% fluoroquinolone resistant *E. coli* isolates were observed in healthy adults and 26% in healthy children (Garau *et al.*, 1999). Resistance against fluoroquinolones occurs by chromosomal mutation in a small number of resistant mutants. These bacteria may increase by clonal expansion due to continuous exposure to fluoroquinolone. This clonal expansion may switch over to other population as it was observed in Dutch poultry and poultry farmer population (Oteo *et al.*, 1999; Garau *et al.*, 1999).

Resistance against cephalosporin has been dramatically increased since 2000 (Webster, 2009). Resistance of *E. coli* isolates of broiler against Cephalexin in this study (46%; Table 1) was lower as compared with a study of Iran (86%) but higher than a study of USA (22%) (Kazemnia *et al.*, 2014; Braykov *et al.*, 2016). Moreover, resistance of *E. coli* isolates from slaughterers and non-slaughterers against Cefoperazone and Ceftriaxone (about 15%, Table 1) was much lower than that of uropathogenic *E. coli* isolates (≤70%) (Bashir *et al.*, 2011). However, not any single isolate of *E. coli* showed resistance against Cefepime (Table 1), very similar findings were observed by Cho *et al.* (2012) in poultry workers.

Differences in resistance rates of *E. coli* might be related to different use of antibiotics in various countries at farm level

depending upon availability, cost and enforcing laws. For example, in Netherland, where antibiotic use is monitored, only about 10% of fecal *E. coli* isolates of poultry were resistant against Ciprofloxacin (London *et al.*, 1993). In countries where use of antibiotics is not monitored at poultry farms, the antibiotic resistance rates are often higher (Nhung *et al.*, 2016). For example, against some antibiotics, the antibiotic resistance rate in fecal isolates of broiler was over 80% (Table 1).

Escherichia coli is one of the beta-lactamase producing organisms and may contribute in the emergence of multidrug resistance. Similar to our study (Table 2), high multidrug resistance in *E. coli* isolated from poultry feces as compared to poultry slaughterers is already reported (Van den Bogaard *et al.*, 2001). The horizontal transfer or sharing of plasmids, which develops a mosaic structure responsible for capturing resistant genes, consequently give rise to the multidrug-resistant phenotype (Leplae *et al.*, 2006). It has already been reported that resistant strains from poultry gut readily approach carcass and often contaminate the meat with multidrug-resistant strains (Nazer, 1980). High multidrug resistance in isolates of broiler feces in present and previous studies reflected the recent antibiotic usage (Van den Bogaard *et al.*, 2001; Miles *et al.*, 2006). However, application of antibiotics during rearing of broiler select intestinal *E. coli* for resistance that remain in the animal even if no further antibiotics are used (Chaslus-Dancla *et al.*, 1987).

In this study, relatively higher prevalence of *Tet-A* and *Tet-B* genes in fecal isolates of slaughterers as compared to fecal isolates of non-slaughters may relate with the spread of these genes from fecal isolates of broiler (Table 3). Our results are in line to Miles *et al.* (2006) who observed similar tetracycline-resistant determinants in avian and human populations. It has already been documented that many avian and human isolates carry the same Tetracycline-resistance genes (Chopra and Roberts, 2001). These resistant agents could be transferred to disease causing organisms and might be challenging in disease control (Miles *et al.*, 2006). Our results suggest a possibility of spreading antibiotic resistant *E. coli* and Tetracycline-resistance genes from broilers to slaughterers.

***Conclusions*:** To our knowledge, this study represents the first large scale survey in Pakistan on antibiotic resistance rates in *E. coli* isolated from poultry and humans against various groups of antibiotics. Alarming levels of antibiotic resistance rates were observed in fecal isolates of broilers and slaughterers. Tetracycline-resistant determinants (*Tet-A* and *Tet-B* genes) were detected almost at similar levels in fecal isolates of broilers and slaughterers, while a lower level was observed in non-slaughterers. This study identified a possibility of the spread of antibiotic-resistant *E. coli*, multidrug-resistant *E. coli* and tetracycline-resistance genes from broilers to slaughterers. High antibiotic resistance rates among *E. coli* is potentially a life-threatening issue and could only be mitigated by strict regulation of law on usage of antibiotics at the farm level. To avoid any possible morbidity and mortality of slaughterers and consumers due to antibiotic resistance, there is an immediate need of implementing the guidelines for hygienic slaughtering to mitigate or stop the spread of resistant *E. coli*. For this purpose, the developing countries should prioritize and implement a modified guidelines and approach in every slaughter house.

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