The Journal of Animal & Plant Sciences, 29(6): 2019, Page: 1580-1586
ISSN: 1018-7081

STUDY OF THE CURRENT RESISTANCE PATTERN TO QUINOLONES AND OTHER ANTIMICROBIALS BY MULTIDRUG RESISTANT *ESCHERICHIA COLI* RECOVERED FROM RETAIL CHICKEN MEAT

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ABSTRACT

Microbial food safety has become a worldwide public health concern. The indiscriminate use of antimicrobials in poultry had rendered bacteria resistant to multiple drugs. The dissemination of resistant genes through the food chain has probably lead to multidrug resistant strains of *Escherichia coli* in chicken meat. With this background in mind, the current study was conducted to ascertain the current antimicrobial susceptibility and multiple drug resistance patterns in *E. coli* recovered from retailed chicken meat. About 100 chicken meat samples were collected from different retail meat shops located across Lahore city. About 85 samples (85%) were found positive for *E. coli*. Further confirmation was done through PCR by amplifying their universal stress protein (*usp*A) gene. As per Kirby Bauer disk diffusion method, *E. coli* displayed the susceptibility towards gentamicin (86%) and ciprofloxacin (67%). The 100% resistance was observed towards chloramphenicol, colistin, tylosin each, 93% against doxycycline and 92% against ampicillin, and oxytetracycline, each. Significant resistance was observed against the quinolone group (enrofloxacine, 81.1%). All the isolates were resistant to more than 8 drugs belonging to 3 or more than 3 different classes of drugs. About 23.5% of the isolates were resistant against nine different antimicrobials and 15.3% showed resistance against eleven different drugs. The results of the *in vitro* susceptibility testing and multidrug resistance pattern observed in the study may provide valuable guidance to adopt good hygienic practices and curb unnecessary antimicrobial usage.

Keywords: Antibiotics; Chicken; *Escherichia coli*; Resistance; Retail meat.

Abbreviations: CLSI= Clinical Laboratory Standard Institute; EMB= eosin methylene blue agar; ExPEC= extraintestinal pathogenic *E. coli*; MDR= multidrug resistant; MR= methyl red; TSB= tryptic soy broth; *usp*A= universal stress protein gene; UTI= urinary tract infections; VP= voges proskauer.

INTRODUCTION

Chicken meat is a rich source of protein and low in carbohydrate. However, its high water contents support the growth of pathogenic bacteria (Ahmad *et al*., 2013). Poultry meat is considered to carry more food-borne pathogens than red meat. As a result of which more food-borne illnesses are associated with poultry meat which in the modern world is considered a great public health concern. It is considered important because it affects the health and well-being of the people as well as has a major economic influence on the country by imposing a substantial burden and reducing economic productivity. It is important to note that in majority of the infections it is not possible to identify the source of food, carrying pathogens, especially in food-borne illnesses (Akbar and Anal, 2013), however, chicken products are suspected to be the source of drug resistant bacteria that cause food-borne diseases in humans. In poultry, the multidrug resistant bacteria are routinely found (Koga *et al.*, 2015), the major reason being that poultry producers have been utilizing antibiotics for more than 50 years (Faridah *et al*., 2004). They are used as growth enhancers, as a prophylaxis measure and for the treatment of diseases (Shahbazi *et al.*, 2015). Despite the fact that this use of antibiotics has improved the health and welfare of poultry by decreasing the occurrence of diseases however, it has also resulted in harmful concentration of drug residues in the meat (Karmi, 2014). Because of these residues that persist in the edible tissue, they may get introduced in the human diet (Offiah and Adesiyun, 2015). This may be one of the reasons for producing multidrug resistance in the bacterial populations leading to therapeutic failures (Karmi, 2014).

Because of increasing trend of antimicrobial resistance, most of the countries including Sweden, United Kingdom, Denmark, The Netherland and other EU countries have banned the use of all essential antimicrobials as prophylaxis in animal production. US has banned the use of fluoroquinolones in poultry sector (Roth et al., 2019) while China has banned the use of Colistin sulphate as poultry growth promoter (Wongsuvan, 2018). However, banning of these antimicrobials has not eliminated the occurrence of resistance in pathogens. This is because of indiscriminate

use of the antimicrobials either by farmers or field veterinarians especially in case of viral infections.

Normally, meat products are the food category that are considered to be the chief reservoir of multi drug resistant *E. coli* as these products are observed to be the most commonly contaminated with this organism. Despite the fact that most *E. coli* strains comprises of a part of the normal flora of the intestine but some can occur as a contamination of the food. Such contamination may occur during production, processing, distribution and through retail (Zou *et al.,* 2014). Therefore, *E. coli* can be used as the bio-indicator of antibiotic resistance. Among it the extra intestinal pathogenic *E. coli* (ExPEC) can be categorized by having many virulence factors. These virulence factors can be transferred through poultry meat to humans via food chain (Koga *et al.*, 2015).

Lahore is located in the east of Pakistan and is the provincial capital of Punjab. Since chicken meat is the most commonly consumed food in Pakistan (Shaheen *et al.,* 2015) therefore in Lahore city it is no exception. In Pakistan, people prefer to buy chicken from live chicken market because customer prefers onsite slaughtering as compared to commercially available packed, frozen chicken (Khan *et al.*, 2015). Therefore, our study was focused on examining the occurrence of antimicrobial resistance amongst the multidrug resistant (MDR) isolates of *E. coli.* They were isolated from the chicken meat sold on retail shops that used onsite slaughtering method. The purpose of the study was also to get an idea about the current scenario of the resistance pattern against normally prescribed antimicrobials for MDR *E. coli* infections particularly quinolones. This may lead to a possible remedial action towards good hygienic practices and may curb the unnecessary antimicrobial use.

MATERIALS AND METHODS

Acquisition of chicken meat samples: A total of 100 (n= 100) raw chicken meat samples were obtained from retail chicken meat shops located in 10 towns (9 administrative towns and 1 cantonment area) of Lahore. From each location, randomly 10 shops were selected and from each shop one sample of either chicken leg, chicken wing, chicken breast were collected in sterile plastic bags and the samples were instantly processed for the isolation of *E. coli*. Further testing was done on 85 strains of *E. coli* isolated from these meat samples and processed for antimicrobial susceptibility testing.

Isolation and biochemical characterization of the *E. coli* isolates. For the isolation, 25gm of the meat sample was added in 20 ml tryptic soy broth (TSB) (Bacto, BD U.S) for enrichment and incubated at 37^{o}C for 24 hours. From it 10µl of the enriched sample was streaked on MacConkey agar (Oxoid, England) and incubated at 37^{o}C for 24 hours. From these plates only lactose fermenting colonies indicated by pink colour were selected and streaked on eosin methylene blue agar (EMB) (Oxoid, England) plates. They were incubated at 37^{o}C for 24 hours and later only the colonies displaying green metallic sheen were taken as positive for *E. coli*. Each isolate was further confirmed by biochemical testing using indole test, methyl red (MR), voges proskauer (VP) test and citrate utilization test (Cappuccino and Sherman, 2008).

Genetic characterization of the *E. coli* isolates. The *E. coli* isolates were confirmed on a genetic basis and for that purpose the DNA was isolated by boiling method as described by Levisohn and Kleven, (2000). Briefly a single colony of *E. coli* was carefully taken from the culture on MacConkey agar, using a sterile tooth pick, and was suspended into 200µl sterile water in a microfuge tube. The tube was put into a boiling water-bath for 15 minute and later centrifuged at 4000 rpm for 2 min. The resulting supernatant containing the isolated DNA was separated and stored at -20^{o}C. The isolated DNA was used for the amplification of universal stress protein (*usp*A) gene through PCR. The PCR was executed using Dream Taq PCR master mix (Thermo Fisher Scientific, U.S) with 5µl of isolated DNA and 0.5µM of forward and reverse primers (Shaheen *et al.,* 2015). The PCR conditions applied were as: initial denaturation at 94^{o}C for 5 min, denaturation at 94^{o}C for 2 min, annealing at 70^{o}C for 1 min, extension at 72^{o}C for 1 min (30 cycles), final extension at 72^{o}C for 10 min. The primers used for the amplification are described in Table 1.

Antimicrobial susceptibility testing: Antimicrobial susceptibility pattern of the *E. coli* isolates was checked using Kirby-Bauer disk diffusion method (Bauer *et al*., 1966). The 85 isolates obtained were tested for their susceptibility against 15 different classes of antibiotics (Oxoid, England) that were regularly prescribed in case of infection viz. gentamicin (10µg), ampicillin (10µg), ceftriaxone (30µg), amoxicillin/clavulanic acid (30µg), levofloxacin (5µg), streptomycin (10µg), sulphamethoxazole/trimethoprim (25µg), ciprofloxacin (5µg), enrofloxacine (10µg), neomycin (30µg), doxycycline (30µg), oxytetracycline (30µg), chloramphenicol (30µg), colistin (10µg) and tylosin (30µg). The sensitivity testing was carried out using Mueller Hinton agar (Oxoid, England) and the plates were incubated at 37°C for 24 hours. The zones of inhibition observed were recorded and compared according to Clinical Laboratory Standard Institute (CLSI) guidelines (Wayne, 2011). The interpretive standards of breakpoints, recommended by CLSI method, are given in Table 2. Multidrug Resistance (MDR) was considered as resistance to more than 8 antimicrobials (belonging to 3 or < 3 different classes) (Faridah *et al*., 2004) among those tested in the study.

Statistical analysis: The results were written in percentages. Statistical analysis was done using Chi-square test and CI of 95% with a value of $P < 0.05$ was considered significant. The test was applied using statistical package SPSS version 22.0 (SPSS, USA).

RESULTS

Prevalence of *E. coli* and antimicrobial susceptibility pattern: From the 100 chicken meat samples, 85 samples (n= 85, 85%) were observed to be positive for the *E. coli*. The antibiotic susceptibility of the *E. coli* isolates was checked against 15 different routinely prescribed antibiotics. *E. coli* was observed to be most susceptible towards aminoglycoside antibiotic gentamicin (73, 86%), quinolone antibiotics ciprofloxacin (57, 67%) and levofloxacin (51, 60%), and cephalosporin antibiotic ceftriaxone (32, 37%). Detailed analysis of the resistance pattern of the *E. coli* isolates displayed 100% (85) resistance towards the amphenicol antibiotic chloramphenicol, polymixin antibiotic colistin as well as the macrolide antibiotic tylosin. More than 92% resistance was observed against ampicillin (β-lactam antibiotic), neomycin (aminoglycoside), doxycycline and oxytetracycline (tetracycline). Significant resistance (88%) was observed towards the fluoroquinolone antibiotic enrofloxacin and sulfonamide sulphamethoxazole /trimethoprim (85%).

Multiple drug resistance patterns in *E. coli:* In this study those *E. coli* isolates that were resistant to more than 8 antimicrobials (belonging to 3 or < 3 different classes) (Faridah *et al*., 2004) were considered as MDR strains. The 85 resistant isolates tested showed 34 different MDR patterns (Table 3). The 23.5% of the isolates were resistant against nine different antimicrobials and 15.3% showed resistance against eleven different drugs. About 14.1% of the isolates were observed to be resistant against ten antimicrobials and 10.6% resistance was found to be against seven and thirteen antimicrobials. Only 9.4% and 3.5% resistance was observed against twelve and fourteen antimicrobials, respectively. None of the isolates were found to be resistant to a single or double antimicrobial.

Table 1. Primers used for the amplification of universal stress protein (*usp*A) in the isolated *E .coli* strains.

Primers	Primer sequence (5'- 3')	Position on *usp*A gene	Product size	Reference
Forward	CCGATACGCTGCCAATCAGT	4-23	884 bp	(9)
Reverse	ACGCAGACCGTAGGCCAGAT	868-887		

Table 2. Interpretive standards for disk diffusion breakpoints for the selected antimicrobials against *E. coli* isolates (N=85).

Antibiotic name	Antibiotic Class	Code Assigned	Zone Diameter Breakpoints, Nearest whole mm		
			S	I	R
Gentamicin	Aminoglycoside	GEN	≥ 15	13-14	≤ 12
Ampicillin	β-lactam	AMP	≥ 17	14-16	≤ 13
Ceftriaxone	Cephalosporin	CRO	≥ 23	20-22	≤ 19
Amoxicillin/ Clavulanic acid	Beta-lactamase inhibitor	AMC	≥ 18	14-17	≤ 13
Levofloxacin	Quinolone	LEV	≥ 17	14-16	≤ 13
Streptomycin	Aminoglycoside	STR	≥ 15	12-14	≤ 11
Sulphamethoxazole/ Trimethoprim	Sulfonamide	SXT	≥ 16	11-15	≤ 10
Ciprofloxacin	Fluoroquinolone	CIP	≥ 21	16-20	≤ 15
Enrofloxacine	Fluoroquinolone	ENR	≥23	17-22	≤16
Neomycin	Aminoglycoside	NEO	≥ 17	13-16	≤ 12
Doxycycline	Tetracycline	DOX	≥ 14	11-13	≤ 10
Oxytetracycline	Tetracycline	OCT	≥ 15	12-14	≤ 11
Chloramphenicol	Amphenicol	CHL	≥ 18	13-17	≤ 12
Colistin	Polymixin	COL	≥15	-	≤14
Tylosin	Macrolide	TYL	-	-	-

Key: S, sensitive; I, Intermediate; R, Resistant; N, Number of total positive isolates

Table 3. Antimicrobial resistance patterns observed in MDR *E. coli* isolates from retail chicken meat in Lahore (N=85).

Antimicrobial Resistance Patterns	No. of [A]MDR *E. coli* isolates	% of Resistance
AMP, CHL, COL, CRO, ENR, NEO, STR	2	2.4
AMC, AMP, CHL, COL, DOX, OTC, STR	2	2.4
AMC, CHL, COL, DOX, NEO, OTC, STR	3	3.5
CHL, CIP, COL, ENR, LEV, NEO, OTC	2	2.4
CHL, CIP, COL, ENR, LEV, NEO, STR	1	1.2
AMC, AMP, CHL, COL, DOX, ENR, NEO, STR	1	1.2
AMP, CHL, COL, CRO, DOX, ENR, OTC, STR	1	1.2
AMC, AMP, CHL, COL, DOX, NEO, OTC, STR	2	2.4
AMP, CHL, COL, DOX, NEO, OTC, STR, SXT	2	2.4
AMP, CHL, COL, DOX, ENR, NEO, OTC, SXT	3	3.5
CHL, COL, DOX, ENR, NEO, OTC, STR, SXT	1	1.2
AMP, CHL, CIP, COL, CRO, ENR, LEV, NEO, STR	1	1.2
AMC, AMP, CHL, COL, CRO, DOX, ENR, NEO, STR	2	2.4
AMC, AMP, CHL, COL, CRO, DOX, ENR, OTC, STR	1	1.2
AMP, CHL, COL, CRO, DOX, NEO, OTC, STR, SXT	1	1.2
AMC, AMP, CHL, COL, CRO, DOX, ENR, NEO, OTC	6	7.1
AMC, AMP, CHL, COL, DOX, ENR, NEO, OTC, STR	2	2.4
AMC, CHL, COL, CRO, DOX, ENR, NEO, OTC, STR	1	1.2
AMP, CHL, COL, DOX, ENR, NEO, OTC, STR, SXT	2	2.4
AMP, CHL, COL, DOX, ENR, LEV, NEO, OTC, STR	4	4.7
AMC, AMP, CHL, COL, CRO, DOX, ENR, OTC, STR, SXT	3	3.5
AMC, AMP, CHL, COL, DOX, ENR, NEO, OTC, STR, SXT	1	1.2
AMP, CHL, COL, CRO, DOX, ENR, GEN, NEO, OTC, STR	4	4.7
AMC, AMP, CHL, COL, CRO, DOX, ENR, NEO, OTC, STR	1	1.2
AMP, CHL, COL, CRO, DOX, ENR, NEO, OTC, STR, SXT	3	3.5
AMP, CHL, CIP, COL, CRO, DOX, ENR, LEV, NEO, OTC, SXT	2	2.4
AMP, CHL, CIP, COL, DOX, ENR, GEN, NEO, OTC, STR, SXT	1	1.2
AMC, AMP, CHL, COL, CRO, DOX, ENR, NEO, OTC, STR, SXT	5	5.9
AMC, AMP, CHL, COL, DOX, ENR, GEN, NEO, OTC, STR, SXT	1	1.2
AMC, AMP, CHL, COL, DOX, ENR, LEV, NEO, OTC, STR, SXT	4	4.7
AMC, AMP, CHL, CIP, COL, DOX, ENR, LEV, NEO, OTC, STR, SXT	5	5.9
AMP, CHL, CIP, COL, DOX, ENR, GEN, LEV, NEO, OTC, STR, SXT	3	3.5
AMC, AMP, CHL, CIP, COL, CRO, DOX, ENR, LEV, NEO, OTC, STR, SXT	9	10.6
AMC, AMP, CHL, CIP, COL, CRO, DOX, ENR, GEN, LEV, NEO, OTC, STR, SXT	3	3.5

Key: GEN, Gentamicin; AMP, Ampicillin; CRO, Ceftriaxone; AMC, Amoxicillin/ Clavulanic; acid; LEV, Levofloxacin; STR, Streptomycin; SXT; Sulphamethoxazole/Trimethoprim; CIP, Ciprofloxacin; ENR, Enrofloxacine; NEO, Neomycin; DOX, Doxycycline; OCT, Oxytetracycline; CHL, Chloramphenicol; COL, Colistin; TYL, Tylosin

N, Number of total positive isolates

[A]Multidrug Resistance (MDR) was considered for those isolates that were resistant to 8 or more than 8 antimicrobials (belonging to 3 or < 3 different classes) (15)

DISCUSSION

The spread of diseases due to contaminated food are considered the most prevalent health problem (Ahmad *et al*., 2013). The alarming rate at which the antimicrobial resistance is spreading among meat borne pathogens also poses a grave public health risk (Shaheen *et al.,* 2015). Although the meat from a healthy animal is free of microorganisms however it may get contaminated during various stages of processing. Therefore, raw meat may contain many pathogenic microbes, particularly *E. coli*, making it a risk for human health (Ahmad *et al*., 2013). The prevention of contamination of chicken meat during the processing and transportation is of great significance for public health because it is directly associated with food borne diseases. The most important

sources of pathogens in this case may be the alimentary tract, skin and feathers particularly during slaughtering, plucking of feathers, cutting and disembowelment (Ahmed *et al*., 2014).

In the study the recovery rate of *E. coli* in the raw chicken meat was very high (85%) in the city of Lahore. This high occurrence of *E. coli* is not unexpected since *E. coli* is a part of the normal microbial flora of the gastrointestinal tract of poultry birds. However, during processing of the meat it might get contaminated with pathogenic MDR *E. coli* (Akond *et al*., 2009). A study by Shaheen *et al.* (2015) coincided with our results as the author also reported 88% *E. coli* isolates from chicken meat samples from retail shops in Lahore. Another study done in the Lahore described the prevalence of *E. coli* to be 78% that is also similar to the results of our study (Razzaq *et al.*, 2016). The high incidence rate of *E. coli* in our study as well as those related indicated that the butchers did not consider proper hygienic and sanitary conditions during handling and processing of meat at retail shops. However, in other studies there was some variation in the occurrence of *E. coli* in the retailed meat. A previous study conducted by Ahmad *et al*. (2013) also described the scenario of contamination of *E. coli* in chicken meat from retail shops in Lahore. Yet, in the study, out of 140 meat samples the prevalence of *E. coli* was observed to be 45% among them. The prevalence of *E. coli* in raw chicken meat is not limited to Pakistan but is spread in different countries all over the world. In India, a study by Pavithra and Ghosh (2013) showed 16.7% *E. coli* isolated from meat obtained from retail meat shops. A study carried out in Japan by Minh *et al.* (2016) described the prevalence of *E. coli* to be 92.3% from raw chicken meat samples which is surprising as well. In Argentine, this prevalence rate was lower to only about 3% (Alonso *et al*., 2012). In Romania however, this prevalence was around 51% that probably explains the difference in hygienic conditions during meat processing in the country (Colobătiu *et al*., 2014). In Saudi Arabia, the prevalence of *E. coli* in raw meat samples was 2% but they belonged to the serotype *E. coli* O157:H7 (Hessain *et al.*, 2015). It is apparent from the mentioned studies that the problem of pathogenic strains of *E. coli* in raw chicken meat is a constant occurrence and strict hygienic practices are needed during processing of the meat to curb the problem.

The antimicrobial susceptibility pattern for the *E. coli* strains was determined to check the effectiveness of the prescribed drugs routinely administered for treatments. In our study most susceptibility was observed against gentamicin (86%) that coincides with another study done in Lahore describing the susceptibility of the drug against *E. coli* to be 77% (Shaheen *et al.,* 2015). In Brazil, the susceptibility against this antibiotic was also observed to be about 75% (Koga *et al.*, 2015) and in Romania as many as 92% isolates were observed to be susceptible (Colobătiu *et al*., 2014). The scenario was however different in the US as only 24% of the isolates were found to be susceptible towards it (Zou *et al.,* 2014).

Other antibiotics towards which most susceptibility was observed were the quinolone antibiotics ciprofloxacin (67%), and levofloxacin (60%). Our study coincides with the study by Shaheen *et al.* (2015) that reported 69% *E. coli* isolates to be susceptible to ciprofloxacin in Lahore city. Quinolones and fluoroquinolones are some of the most important antibiotics used for treatments in human and animals. Despite being forbidden in countries such as Australia for animals (Ingram *et al.*, 2013) they are still employed in other countries keeping in effect with the withdrawal period (6, 11, 16, 24, 29). In humans, they are frequently used for the treatment of enteric and urinary tract infections (UTI). Nevertheless, the emergence of quinolone-resistant *E. coli* in meat had increased (Koga *et al.*, 2015). Because of it, it has been suggested that the poultry meat carrying these resistant isolates may be the reservoir for the UTI causing *E. coli* in humans (Manges, 2015). The large number of MDR *E. coli* reported in UTI cases in Lahore (Tanvir *et al.*, 2012) may be due to these isolates in meat or the residues of quinolone antibiotics left in them. The 88% resistance to the fluoroquinolone antibiotic enrofloxacin reported in our study may be due to this reason. In India, an even frightening scenario of 100% resistance towards fluoroquinolone*s* by *E. coli* isolates in meat has been reported by Pavithra and Ghosh (2013).

Our study reported maximum resistance (100%) among the *E. coli* isolates towards the antibiotics chloramphenicol, colistin, tylosin and more than 92% resistance against ampicillin, neomycin, doxycycline and oxytetracycline. Similar resistance results were reported in cases of these antibiotics (Shaheen *et al.,* 2015; Zhang *et al.*, 2016). However, such a high resistance against chloramphenicol points to an alarming situation. Since the regulations of Food and Drug Administration (FDA) have forbidden the use of this antibiotic in food animals. It could still be detected in some meat samples in European countries as well as in the third world countries. In Iran it is reported to be widely in use in poultry farms despite being legally prohibited (Attari *et al*., 2014). The scenario may not be much different in Pakistan as well as it is evident by the high resistance percentage obtained in the study.

Our study revealed 34 different MDR patterns among the isolates (Table 3). The majority (14.1%) of the *E. coli* isolates were observed to be resistant against ten antimicrobials that can be compared to the study in our neighbouring country India by Pavithra and Ghosh (2013) describing majority of the isolates being resistant to seven commonly prescribed antibiotics. In our study about 10.6% isolates showed resistance against seven and thirteen antimicrobials. A study by Donado-Godoy *et al.*

(2015) and by Parvez *et al.* (2016) also described the MDR nature of *E. coli* isolates and they were observed to be resistant to three or more than three different classes of antibiotics. In China, majority of the *E. coli* isolates (85.5%) from retail meat samples were resistant to at least one antibiotic (Zhang *et al.*, 2016). Since none of the *E. coli* isolates in our study were resistant to one or two antimicrobials this gives us an idea about the increasing prevalence of MDR in *E. coli* isolates in Pakistan as compared to neighbouring countries. It is because of the excessive use of antimicrobials in food producing animals for prophylaxis purposes and treatment. In such cases nearly 80% of the use of such antimicrobials is unessential.

Conclusions: In conclusion, high recovery rate of MDR *E. coli* in retail chicken meat collected from Lahore indicates the misuse of antimicrobials in poultry sector on a large scale as well as the poor hygienic and sanitary practices of the butchers during evisceration, and processing of chicken meat. Therefore, there is a need to check the extensive use of antimicrobials in poultry industry and education for the butchers to adopt good hygienic practices.

Conflict of interest: The authors declare they have no conflict of interest.

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