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Short Communication

Determination of Antibiotic Resistance Prototype and *in vitro* R Plasmid Conjugation of *Escherichia coli* Isolates from Broiler Feces

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Abstract

The objective of the present investigation was to ascertain the prevalence of antibiotic resistance amongst *E. coli* strains obtained from broiler chicken in two different poultry farms against antibiotics that have a limited or no use in poultry and to advocate a probable mechanism of antibiotic resistance transfer between strains. A high resistance rate was seen against azteronam (100%), streptomycin (88-92%) followed by amoxicillin (68-76%), ceftrioxone (60-68%) and cefepime (56-68%). However reasonably lower levels of resistance were seen towards co-amoxiclave (40%), ciprofloxacin (24-28%), and ofloxacin (36-40%). Meropenem emerged as least resistant (16-28%) antimicrobial agent. Likewise, virtually entire sum of isolates were reported as resistant to not less than three antibiotics (multidrug resistant). It was noticed that above half of the isolates tested were capable of conjugative R plasmid transfer between strains. We finally concluded that there existed a significant evidence of multidrug resistance in *E. coli* isolates obtained from two poultry farms, capable of conjugative transfer of resistance between isolates and probably to animals and human.

Keywords: antimicrobial agents, broiler chicken, conjugative R plasmid multidrug resistance

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บทคัดย่อ

รูปแบบการดื้อยาปฏิชีวนะในหลอดทดลองและการถ่ายโอนพลาสมิด R แบบคอนจูเกชั่นของเชื้อ *Escherichia coli* ที่แยกได้จากอุจจาระไก่กระทง

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วัตถุประสงค์ของการศึกษาครั้งนี้ เพื่อหาความชุกของการดื้อยาปฏิชีวนะของ เชื้อ *E. coli* สายพันธุ์ที่เพาะแยกจากไก่กระทง ใน 2 ฟาร์มที่ไม่มีประวัติการใช้ยาปฏิชีวนะ และเพื่ออธิบายกลไกของการถ่ายโอนพลาสมิด R แบบคอนจูเกชั่น โดยพบว่ายาที่มีอัตราการดื้อยาสูงสุดได้แก่ azteronam (100%), streptomycin (88-92%), amoxicillin (68-76%), ceftrioxone (60-68%) และ cefepime (56-68%) ส่วนยาที่มีอัตราการดื้อยาต่ำสุด คือ Meropenem (16-28%), ciprofloxacin (24-28%), Ofloxacin (36-40%) และ amoxiclav (40%) นอกจากนั้น ตัวอย่างเชื้อที่แยกได้มีการดื้อยาร่วมกัน อย่างน้อย 3 ชนิด เชื้อที่แยกได้มีความสามารถในการถ่ายโอนพลาสมิดและคอนจูเกชั่นระหว่างสายพันธุ์ ซึ่งอาจมีผลต่อการถ่ายโอนการดื้อยามาสู่คน

คำสำคัญ: ยาปฏิชีวนะ ไก่กระทง การถ่ายโอนพลาสมิด R แบบคอนจูเกชั่น

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Introduction

The utilization of antibiotic drugs towards infection control and mainly as growth promoters in feed aimed to increase poultry production. A good number of such antibiotics is not entirely taken in the fowl intestine, and as a result above 90% of the executed quantity is emitted in the chicken feces (Kumar et al., 2005[b]). For the reason that farms debris frequently have a use as manure, the charisma of antimicrobials on environment and human health is an issue of mounting concern (Boxal et al., 2003; Rooklidge, 2004). Antibiotic usage is presumed as an imperative factor that endorsed the appearance, assortment, as well as propagation of resistant microorganisms both in human and veterinary practice (Neu, 1992). Profound usage of antimicrobials for confrontation in bacterial population including natural flora of disenchant entity (animals and humans) or inhabitants (Piddock, 1996). The widespread use of antimicrobial drugs exerts a positive stress on pathogen bacteria as well as commensals and thus implicating the development of multidrug resistant bacteria (Baquero et al., 1997). The epiphany of multidrug resistant (MDR) food propping pathogen like *E. coli* and *Salmonella spp.* have developed into a community health apprehension worldwide (Baquero et al., 1997; Rooklidge, 2004). Seeing that bacteria procure a good number of resistant genes via parallel gene transfer, the transferred genetic materials like transposons and plasmids are frequent axis for the spread of resistant genes to assorted microbes (Lee et al., 2007). *E. coli* is common inhabitant of human and animal normal flora, conversely a few of these *spp.* are notorious for pathogenicity. Such microorganism are notorious for the spread of colibacillosis in fowl, which is considered as an imperative impulse of monetary failure for the poultry industry (Amara et al., 1995). Likewise, the *E. coli* preserves copious infections in human resembling septicemia, neonatal meningitis and urinary tract infections (Ewers et al., 2004; Johnson et al., 2008). The avian intestines are envisaged as a reservoir of impending *E. coli* with veterinary prospective that may be transferred straight from birds to human beings (Ewers et al., 2009; Furtula et al., 2010). The acquittance of antibiotic drug usage in feed of poultry modifies the gut flora by means of discerning stress in resistant microbial population (such as resistant *E. coli*) with the aim to get into atmosphere and food procession (Diarra et al., 2007).

After successful spread of drug resistant *E. coli* isolates from one host species to other, it is probable to propagate the antibiotic resistant genes by horizontal transfer which may head for the swift surfacing of antibiotic resistance among clinical isolates of bacteria (Johnson et al., 2008). The spread of

resistant genes is significantly ameliorated on being an element of a mobile gene cassette, since this provides a prospect for horizontal transfer by numerous mechanisms assimilating integrons. The integron mediating antimicrobial resistant is the chief mechanism for spread of resistance in both Gram-positive and negative bacteria (Hall et al., 2002). Integrons are known to harbor prevalence of resistance genes within mobile resistance elements (transposons and plasmids) which allocate transmit of resistance between bacteria which may include transmission of resistance from swine to human microbiota (Hall et al., 2002; Guardabassi et al., 2004). The poultry business in Pakistan has presented a tremendous growth with a worth of more than 90 billion Pak rupees. More than 15000/- poultry farms in this country are producing above 370000/- metric tones poultry meat and 48.60 million poultry eggs. It has been estimated that about 70% of the total antibiotics produced today are fed to poultry birds and livestock (Amjad et al., 2006). A previous report provides a general preview of the antibiotics in poultry and prevalence of resistance (Eissa, 1981; Ansari and Khatoon, 1999; Khalid et al., 2002) including antibiotics that are used in poultry. The present study aimed to evaluate the prevalence of resistance in broiler chicken feces against antimicrobial agents that have a limited or no use in poultry and to establish the likelihood of conjugative R plasmid transfer between the isolates.

Materials and Methods

Sampling and Processing: Fresh fecal droppings (approximately 100 g) were obtained from flock waste in two viable broiler chicken farms in District DI Khan, KPK Pakistan. The fecal samples were collected from 3 to 7 week mature chicken and immediately transferred to lab for bacteriological assay within 4 hours. The samples were subsequently attenuated (10^{+1}) in 0.9% NaCl with 20% (v/v), glycerol and stored at -20^{0}C in anticipation of assay.

Identification: The bacterial isolates were confined by biochemical and cytomorphological mean (MacFaddin, 2000) using MacConkey agar (Oxoid, UK), Motility-indole-lysine media (Oxoid, UK) triple-iron agar (Oxoid, UK), Simmon's citrate agar (Oxoid, UK) and Urea agar (Oxoid, UK). The morphological and biochemical idiosyncrasy of the isolated bacterial strain was harmonized by way of statistics Bergey's Manual of Systematic Bacteriology (Krier and Holt, 1984). The *E. coli* strain ATCC 29050 was used as reference. The acknowledged *E. coli* isolates were relocated to 2 ml Luria broth (Oxoid, UK) and incubated for 18–24 hours at 37^{0}C. One milliliter of currently developed bacterial culture was shifted to 0.8 ml of sterile 80% glycerol in a sterile tube, vortexed and kept at -80^{0}C until further use.

Antimicrobial susceptibility testing: The antimicrobial agents used during present investigation primarily meant to evaluate efficacy of those antibiotics that have very limited or no use in poultry to ascertain the R factors transfer from environment to chicken and animals. Antimicrobial susceptibility tests were set forth via disc diffusion assay in accordance with National Committee for Clinical Laboratory Standards (NCCLS, 2002). Subsequent antibiotic agents were employed during the study (Oxoid, UK); Meropenem 5 µg, Amoxicillin 30 µg, Amoxicillin/Clavulanic acid 30 µg, Ceftrioxone 10 µg, Cefepime 30 µg, Ciprofloxacin 5 µg, Ofloxacin 5 µg, Azteronam 30 µg and Streptomycin 30 µg. The isolates were streaked from the depository on Miller's LB agar (Oxoid, UK) and incubated at 37^{0}C for 20–24 hours prior to being transferred to 5 ml sterile 0.9% normal saline to contest the '0.5' MacFarland standard. By using a sterilized cotton scrub, cultures were applied on Mueller-Hinton (Oxoid, UK) plates. Consequently, the antimicrobial disc was supplemented and plates were incubated for 20-24 hours at 37^{0}C. The thickness of the region of inhibition adjoining each antimicrobial discs was deliberated in millimeters. Isolates were deduced resistant in accordance with breakpoint recommended by the NCCLS procedure (NCCLS, 2002).

Transfer of resistance: The likelihood of resistance transfer via conjugation between isolates was settled by broth mating. Twenty four hours bacterial cultures of donor (selected on basis of resistance pattern) and of *E. coli* recipient (soil isolates) were developed in BHI (Brain Heart Infusion Broth) (Oxoid, UK). Identical 2 ml recipient and donor strains were mixed up in 2 ml BHI with soft trembling at 37^{0}C for 2 hours to accord conjugation. Cardinal dilutions of 10^2–10^5 of the conjugation mix was shifted to iso-sensitest agar plates (Oxoid, UK) containing 30 mg/l of loxacin acid and one of the antibiotics toward which the donor was non responsive. If no conjugation occurred subsequently to 2 hours, the mating was recurred for 24 hours at 37^{0}C exclusive of agitation. The strains failing conjugation were ruled out from further processing. The occurrence of transfer was deliberated as the quotient of the integer of transconjugants to the integer of colony forming units of the donor strain (Nijisten et al., 1996).

Results and Discussion

A sum of 25 *E. coli* strains were secluded from 2 commercial poultry farms in District DI Khan, KPK Pakistan and processed for the determination of antimicrobial resistance pattern and *in vitro* conjugative R plasmid transfer. Approximately all strains that are representatives of both commercial poultry farms elucidated resistance to antimicrobial agents. The outcome of zones of inhibition (mm) were articulated as proportion of isolates resistant, susceptible and intermediate to each antibiotic. Moreover, a dynamic trend towards elevated resistance was seen in isolates of both poultry farms whereas meropenem was noticed as the most susceptible antimicrobial agent (Table 1) as has previously been reported (Byarugaba et al., 2011). However, the resistance pattern of the isolates from the 2 farms was upsetting as outlined in Table 2. During the present investigation, an analogous trend

Table 1 Percentages of *E. coli* isolates from broiler chicken, susceptible (S), intermediate (I) and resistant (R) to antimicrobial agents by NCCLS disc diffusion methods.

Antimicrobial agent	Code	Concentration (µg/ml)	NCCLS Break points (zone mm)	Farm A (n=25)			Farm B (n=25)		
				S (%)	I (%)	R (%)	S (%)	I (%)	R(%)
Meropenem	MEM	5	≤2	80	4	16	82	0	18
Amoxicillin	AML	30	≤13	12	12	76	16	16	68
Amoxicillin/Clavulanic acid	AMC	30	≤13	56	4	40	52	8	40
Ceftrixone	CRO	10	≤15	32	8	60	28	4	68
Cefepime	FEP	30	≤14	40	4	56	32	0	68
Ciprofloxacin	CIP	5	≤15	68	8	24	72	0	28
Ofloxacin	OFX	5	≤12	60	4	36	60	0	40
Azteronam	ATM	30	≤4	0	0	100	0	0	100
Streptomycin	SPT	30	≤11	8	4	88	8	0	92

Table 2 Resistance pattern of *E. coli* isolates obtained from 2 different poultry farms

Farm A		Farm B	
Isolate Code	Resistance pattern	Isolate Code	Resistance pattern
X1	AMC, CRO, FEP, ATM, SPT	Z1	MEM, AMC, FEP, OFX, ATM, SPT
X2	MEM, CRO, CIP, ATM, SPT	Z2	MEM, AMC, CRO, FEP, OFX, ATM, SPT
X3	AML, FEP, CIP, ATM, SPT	Z3	MEM, CRO, FEP, OFX, ATM, SPT
X4	AMC, CRO, OFX, ATM, SPT	Z4	MEM, AMC, CRO, OFX, ATM, SPT
X5	MEM, AML, CRO, OFX, ATM	Z5	MEM, AMC, CRO, FEP, ATM, SPT
X6	AMC, OFX, ATM, SPT	Z6	MEM, AMC, ATM, SPT
X7	MEM, AMC, CRO, FEP, OFX, ATM, SPT	Z7	MEM, AMC ,FEP,ATM, SPT
X8	AMC, CRO, FEP, ATM, SPT	Z8	CRO, FEP, ATM, SPT
X9	AMC, FEP, ATM, SPT	Z9	CRO, FEP, ATM, SPT
X10	AMC, CRO, ATM, SPT	Z10	AML, AMC, CRO, FEP, ATM, SPT
X11	AMC, CRO, FEP, ATM, SPT	Z11	CRO, FEP, CIP, OFX, ATM, SPT
X12	MEM, AMC, ATM, SPT	Z12	AMC, FEP, CIP, OFX, ATM, SPT
X13	AMC, FEP, ATM, SPT	Z13	AMC, FEP, ATM, SPT
X14	AMC, FEP, ATM, SPT	Z14	CRO, FEP, CIP, OFX, ATM, SPT
X15	AMC, FEP, ATM, SPT	Z15	AMC, CIP, OFX, ATM, SPT
X16	AMC, CRO, FEP, ATM	Z16	CRO, FEP, ATM
X17	AMC, FEP, ATM, SPT	Z17	AMC, CIP, OFX, ATM, SPT
X18	AMC, CIP, ATM, SPT	Z18	AMC, CRO, ATM, SPT
X19	CRO, FEP, CIP, ATM, SPT	Z19	AMC, ATM, SPTC
X20	CRO, FEP, CIP, ATM, SPT	Z20	AMC,CRO, ATM, SPT
X21	AMC, CRO, FEP, OFX, ATM	Z21	AMC, CRO, ATM, SPT
X22	CRO, CIP, OFX, ATM, SPT	Z22	CRO, FEP, ATM, SPT
X23	AMC, OFX, ATM, SPT	Z23	CRO, FEP, CIP, OFX, ATM, SPT
X24	AMC, CRO, OFX, ATM, SPT	Z24	CRO, FEP, ATM, SPT
X25	AMC, CRO, CIP, OFX, ATM, SPT	Z25	CRO, FEP, ATM

towards cephalosporin resistance was determined, as reported in previous reports (Dhanji et al., 2010; Idress et al., 2011). More importantly, although meropenem was not included in chicken feed, numerous resistances was noticed against this antimicrobial agent. The meropenem, like other metalo β-Lactam antibiotics, possesses a zwitteronic structure that makes a protective shield just about β-lactam ring, and hence overrules the effects of lactamases (Shah et al., 2004). Synonymously, we noticed high resistance level against amoxicillin that was associated with a profound record of use in the poultry feed used in both broiler farms. The existence and high incidence of amoxicillin resistance within *E. coli* from broiler chickens concur with former findings (Van den et al., 2001; Furtula et al., 2010; Tabatsbaei et al., 2010). However much striking results were noticed when the antibiograms of co amoxiclave (amoxicillin/ clavulanic acid) were observed, that on the whole were much less compared to amoxicillin alone which is a clear indication of synergism between the two antibiotics (Miles et al., 2006; Elviss et al., 2009). Markedly low levels of resistance

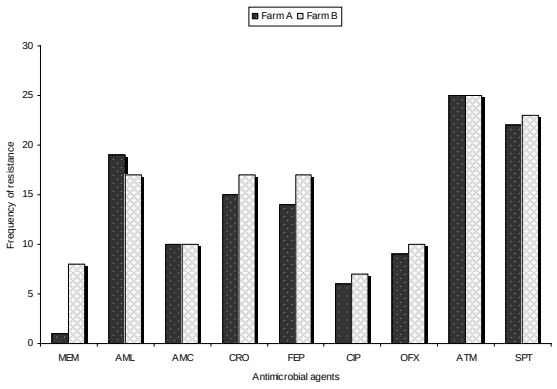

Figure 1 Frequency of multiresistance in E.coli isolates against antimicrobials.

compared to other antibiotics were observed for floroquinolones despite a minute difference in the susceptibilities of *E. coli* isolates obtained from both

Table 3 Most prevalent Resistance pattern observed in *E.coli* isolates

Antimicrobials	Resistance pattern	
	Farm A (n=25)	Farm B (n=25)
ATM,SPT	22	24
AMC,CRO	9	8
AMC,FEP,ATM,SPT	4	8
MEM,CRO	3	4
AMC,CRO,OFX,ATM,SPT	3	3
MEM,AMC,CRO,FEP,OFX,ATM,SPT	1	1
AMC,OFX	8	6
AMC,CIP	2	4

Table 5 Resistance patterns of donor and transconjugants

Isolate	Resistance Pattern	Transconjugants Profile
Farm A		
X1	AMC,CRO,FEP,ATM,SPT	AMC,FEP
X2	MEM,CRO,CIP,ATM,SPT	MEM,CIP
X12	MEM,AMC,ATM,SPT	-
X22	CRO,CIP,OFX,ATM	-
Farm B		
Z1	MEM,AMC,FEP,OFX,ATM,SPT	AMC,FEP
Z9	AML,AMC,CRO,FEP,ATM,SPT	-
Z25	CRO,FEP,ATM	CRO,FEP
Z18	AMC,CRO,ATM,SPT	-

farms as reported previously (Hofacre et al., 2000). These findings imply the floroquinolones for use in the poultry as a result of appreciable efficacy and lower levels of resistance in human subjects (Bonten et al., 1992; London et al., 1993).

During the present investigation a significant majority of isolates were resistant to more than three antibiotic drugs and consequently considered as multidrug resistant (MDR). It was surprisingly observed that resistance to only one or 2 antimicrobials was not evident in the 2 farm isolates. Moreover, the resistance pattern of all isolates from both poultry farms was nearly alike and we did not observed any significant difference in resistance pattern. These finding are supported by many reports (Vergidis et al., 2008; Okeke et al., 2007). Moreover, the frequency of antimicrobial resistance in our study was significantly eminent (Fig 1) and a considerable majority of the antimicrobial agents possessed frequency of resistance prevalence more than 5 except meropenem (less than 5). The azteronam, streptomycin, amoxicillin, ceftrioxone and cefepime were reported as highly resistant (Fig 1). Moreover, the amoxicillin + streptomycin was the most prevalent resistance pattern observed in both (A and B) farms followed by amoxicillin+ceftrioxone (Table 3).

The transferability of antibiotic resistance by multiresistant bacteria has remained the focus of some studies (Nijstein et al., 1996; Gonzalez et al., 2005). Furthermore, during the present investigation, a significant majority of *E. coli* isolates were noticed to be multiresistant, which pretended us to delimitate the R plasmid transfer via conjugation. It was noticed that more than half of the strains were proficient in transferring the R plasmid (Table 5) as reported previously (Nijstein et al., 1996; Yates et al., 2004). Moreover, this phenomenon suggests the development of resistance towards antimicrobial agents that neither remained a part of feed nor provided any evidence of use in infections control in either broiler chicken farm.

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