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ANTIMICROBIAL DRUG RESISTANCE EVALUATION AND MONITORING IN *ESCHERICHIA COLI* ISOLATED FROM POULTRY ENVIRONMENT AT DIFFERENT TIME INTERVALS

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A high prevalence of multiple drug resistance was observed in populations of *E. coli* isolated in 1992-93 from poultry carcasses, fluff, hatchery environment and water at different broiler farms in Karachi, Pakistan. Five hundred isolate of *E. coli* were made of which 375 were tested for their sensitivity to five antimicrobials using the tube dilution method. Similarly, during 1995-96, 430 *E. coli* isolates were made (from the same farms) of which 315 were tested for their sensitivity to the same antimicrobials studied in 1992-93. *E. coli* isolates during 1992-93 and 1995-96 showed increase in resistance from 50 to 56% against amoxycillin, from 62 to 71% against neomycin, from 97 to 100% against oxytetracycline, from 95 to 100% against tetracycline and from 95 to 98% against trimethoprim. It appears that exposure of *E. coli* of poultry origin in Pakistan to the five studied antimicrobials could well be the cause of increasing antimicrobials resistance. This data supports the growing contention that subtherapeutic doses of antimicrobials should be eliminated as a means of promoting rapid growth.

Key words**:** Antibiotics, Antimicrobials, Drug resistance, Sensitivity.

Introduction

Coliform infections in poultry are often caused by the strains of *E. coli*. Disease problems range from diarrhoea, septicemia, hemorrhagic enteritis, chronic respiratory disease, air sacculitis, yolk sac infections, omphalitis, synovitis and osteomyelitis (Gross 1984; Cloud *et al* 1985; Valvano *et al* 1992).

The disease may result from *E. coli* infection alone as the primary agent or in combination with other agents as a complicating or secondary infections, e.g. New Castle disease, bursal infection, coccidiosis and mycoplasmosis (Gordon and Jordan 1982). Poor management and sanitation practices at the hatchery and farm along with environemtal stresses such as high ammonia level (>20 ppm), inadequate ventilation, overcrowding, extremes of temperature, poor quality of feed and water are considered predisposing factors for infection (Goodwin *et al* 1993).

Several antibiotics such as penicillin and tetracyclines have been used as feed additives for farm animals since 1950s. The subtherapeutic doses have been established to promote growth and prevent bacterial disease (Van Houweling *et al* 1978; Visek 1978; Scioli *et al* 1983). This promoting effect is due in part to control intestinal bacteria that can interfere with the animals ability to absorb nutrients or to the control of pathogens. As a result animals become healthier, grown faster and stronger without suffering from bacterial infections. This use of antibiotics creates optimum conditions for the rapid selection of bacteria resistance to antibiotics (Durand *et al* 1987).

In addition to this, the use of subtherapeutic doses of antibiotics to make animals healthier might create a human health risk which have been expressed 20 years long ago and as well as recently (Van Houweling *et al* 1987; Bongers *et al* 1995; Al-Ghamdi *et al* 1999). Antibiotic resistant bacteria from animals can be transferred to humans through contaminated water or food. Resistant bacterial species that are known to be transferred through food chain include *Salmonella*, *Campylobacter*, *Enterococci* and *Escherichia coli* (Van Houweling *et al* 1978). Municipal wastewater is a primary source of bacteria to the aquatic environment (Linton *et al* 1974) and antibiotic resistant bacteria have been widely reported in rivers (Al-Jebouri 1985; Al-Jebouri and Al-Meshhadani 1985) and lakes (Jones *et al* 1986).

Enterobacteriaceae are notorious in that they rapidly transfer antibiotic resistance among each other and also to enteric pathogens like *Salmonella typhi* (Martinez *et al* 1987; Blanco *et al* 1997).

Slaughter house workers, cooks and food handlers have been found to have a higher incidence of resistant *E. coli* in their gut than does the general population (Van Houweling *et al* 1978).

In Pakistan and in many other developing countries quite a number of antiobiotics are being imprudently used to control *E. coli* infections in poultry. The reason for using these antibiotics (amoxycillin, neomycin, tetracycline, oxytetracycline

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and trimethoprim) is that they are being used widely in veterinary as well as in human medicine in Pakistan and in several other countries.

This study has been undertaken with a view to monitor trend of antibacterial drug resistance especially against *E. coli* so that measures could be developed and implemented for controlling this growing problem.

Materials and Methods

Isolation of E. coli. Isolation of *E. coli* was made by inoculating the clinical specimen (egg yolk, piece of trachea, lungs or liver of diseased birds), fluff, swab of various surfaces of hatchery and water (Table 1) into MacConkey's broth. The inoculated samples were incubated at 37˚C for 18-24 h . Broths exhibiting a change in color from red to yellow were subcultured onto Eosin Methylene Blue (EMB, Oxoid) agar plates to grow putative enteric bacteria.

Identification and preservation of E. coli. Isolates were identified by routine biochemical and serological (using type specific antisera, Difco) characters. After identification and final confirmation. *E. coli* isolates were stabbed into presterilized semi solid Tryptic Soya Agar (TSA, Oxoid) in screw capped vials and incubated at 37˚C for 24 h. These vials were then kept at 4˚C in a refrigerator until use.

MIC Determinations

Culture media. Muller Hinton's Broth (MHB, Oxoid) was prepared as directed by the manufacturer and used for the determination of minimum inhibitory concentration (MIC) (NCCLS 1990).

Antimicrobials used. (a).Amoxycillin (Smoth Klein and French), (b). Neomycin (Squibb), (c). Oxytetracycline (Lisko Pharma), (d). Tetracycline (Epla Lab. Lted.), (e). Trimethoprim (Epla Lab. Ltd.) These antimicrobials are widely used in Pakistan in poultry industry.

Preparation of stock solutions of antimicrobials. Stock solutions containing 1000 µg/ml of antimicrobials were prepared, filter sterilized using presterilized 0.22 µm Millipore membrane filters, dispensed in 10 ml quantities in sterile vials and stored at - 15˚C.

Inoculum preparation. For this purpose *E. coli* isolates were inoculated into 5 ml nutrient broth and incubated at 37˚C for 18-24 h. Broth cultures were homogenized using a vortex mixer and then turbidity adjusted to match that of the 0.5 McFarland standard (approxi 10^8 CFU/ml). A portion of cell suspension was 100 times diluted (10^6 CFU/ml) and used to inoculate experimental tubes (Baker *et al* 1983).

Experimental protocol. Two-fold dilution technique was used for the determination of Minimum Inhibitory Concentration (MIC) of single antimicrobial.

One ml of the diluted culture (ca $1x10^6$ CFU/ml) inoculated into each tube containing 1 ml of antimicrobial diluted in MHB to get a recommended final inoculum concentration of $5x10^5$ CFU/ml (Baker *et al* 1983). Experimental tubes were incubated at 37˚C for two days and the growth was observed each day. A control tube containing 4 ml of MHB with no antimicrobial was also inoculated and incubated in the same way. In the series the last tube not showing any visible growth was considered as the MIC.

Table 1
Sources of isolation of *E. coli* for the study during 1992-93 and 1995-96

Sources of isolates	No. of isolates tested	
	1992-93	1995-96
Infected poultry birds (lung, trachea & liver)	125	110
Hatchery	95	65
Fluff	55	55
Water	100	85
Total	375	315

Isolations were made in both phases of study (1992-93 and 1995-96) from the same farms.

Results and Discussion

For this study, work was done in two phases. In first phase (1992-93) 500 and in the second phase (1995-96) 430 *E. coli* isolates were made. Of which 375 (1992-93) and 315 (1995-96) were randomly selected and subjected to antimicrobial sensitivity testing. The MICs of different antimicrobials was determined against various isolates of *E. coli* from poultry environment.

Using MIC interpretive standards for the resistant and susceptible categories as recommended by the National Committee for Clinical Laboratory Standards (NCCLS 1990), *E. coli* isolates exhibiting their MIC at or below breakpoint concentrations were classified as "susceptible" and those ones showing MIC greater than breakpoint concentrations were designated as "resistant".

Antimicrobial sensitivity pattern study during 1992-93 and 1995-96. Table 2 shows the MIC distribution pattern of five antimicrobials against *E. coli* isolates of poultry origin during 1992-93 and 1995-96.

The relative resistance of 375 and 315 *E. coli* isolates against different concentrations of 5 antimicrobials studied during 1992-93and 1995-96 is shown in Table 3. Table 3 also expresses percent increase in antimicrobials resistance among *E. coli* isolates of poultry origin from 1992 to 1996 at various MIC levels. It is observed from the results that a fairly large number of *E. coli* isloates (>90%) were found resistant to 3 antimicrobials (oxytetracycline, tetracycline and trimethoprim) during both phases of the study as shown in Table 4 and in Fig 1.

As per NCCLS (1990) interpretive standards for resistance and susceptibility it is observed that in 1992-93 50% isolates were resistant to amoxycillin, which increased to 56% in 1995-96. The effectiveness of neomycin decreased from 38 to 29% from 1992 to 1996. Oxytetracycline was effective only against 3% isolates in 1992-93 that decreases to 0% in 1995-96 i.e all the 315 isolates were found resistant to oxytetracycline. Similar susceptibility/resistance pattern was also observed with tetracycline. In case of trimethoprim 20 (5%) isolates were found susceptible in 1992-93, which decreased to 7(2%) in 1995-96.

The increase in resistance of *E. coli* against amoxycillin, neomycin and trimethoprim from 1992 to 1996 was statistically significant at $p < 0.05$, $p < 0.001$ and $p < 0.01$, respectively and insignificant with oxytetracycline and tetracycline as determined by calculating standard deviation.

According to the present study, resistance in poultry isolates of *E. coli* against antimicrobials seems to be increasing and this continues to be a cause of concern to clinicians, veterinarians, poultry farmers, scientists and researchers. In a press release, WHO criticized the excessive use of antimicrobials especially as growth promoters in animals destined for human consumption to avoid growing risk to human health (WHO Press Release 1997).

The antibacterial resistance pattern shown by *E. coli* isolates of poultry origin indicates that either they have been frequently exposed to these antimicrobials in the past or that the resistance may be inborn (intrinsic). It is difficult to collect the data regarding the actual use pattern of these antimicrobials in these farms because of the hesitation of farmers. However, the experience indicates that these strains of *E. coli* must have developed resistance due to indiscriminate and inadequate use of antimicrobials both in veterinary and human medicine. Therefore, the use of these antimicrobials in controlling *E.coli* infections in poultry becomes meaningless.

Table 2

Distribution MICs* of antimicrobials against *E. coli* isolates of poultry origin studied during 1992-93 and 1995-95

Antimicrobials used	MIC range (µg/ml)									
	0-5		5-10		10-20		20-40		40-80	
	1992-93	1995-96	1992-93	1995-96	1992-93	1995-96	1992-93	1995-96	1992-93	1995-96
Amoxycillin	143(38)	94(30)	23(6)	23(7)	22(6)	22(7)	19(5)	19(6)	168(45)	157(50)
Neomycin	82(22)	51(16)	41(11)	30(10)	19(5)	10(3)	12(3)	7(2)	221(59)	217(69)
Oxytetracycline	4(1)	0	8(2)	0	0	0	0	0	363(97)	315(100)
Tetracycline	19(5)	0	0	0	11(3)	0	8(2)	0	337(90)	315(100)
Trimethoprim	20(5)	7(2)	0	0	4(1)	0	8(2)	4(1)	343(92)	303(97)

Figures in parentheses are percentages; MIC of each isolate for each antimicrobial is determined in triplicate.

Table -3

Relative resistance of *E. coli* isolates of poultry origin during 1992-93 and 1995-96

Antimicrobials used	Relative resistance of *E. coli* to varying concentrations of antimicrobials (%)														
	5			10			20			40			80		
	1992-93 %	1995-96 %	%increase in Anti-microbial resistance	1992-93 %	1995-96 %	% increase in Anti-microbial resistance	1992-93 %	1995-96 %	% increase in Anti-microbial resistance	1992-93 %	1995-96 %	% increase in Anti-microbial resistance	1992-93 %	1995-96 %	% increase in Anti-microbial resistance
Amoxycillin	62	70	8	56	63	7	50	56	6	45	50	5	45	50	5
Neomycin	77	84	7	67	74	7	63	71	8	59	69	10	59	69	10
Oxytetracycline	98	100	2	97	100	3	97	100	3	97	100	3	97	100	3
Tetracycline	95	100	5	95	100	5	92	100	8	90	100	10	90	100	10
Trimethoprim	95	98	3	95	98	3	94	98	4	94	97	3	92	97	5

Average of triplicate analysis

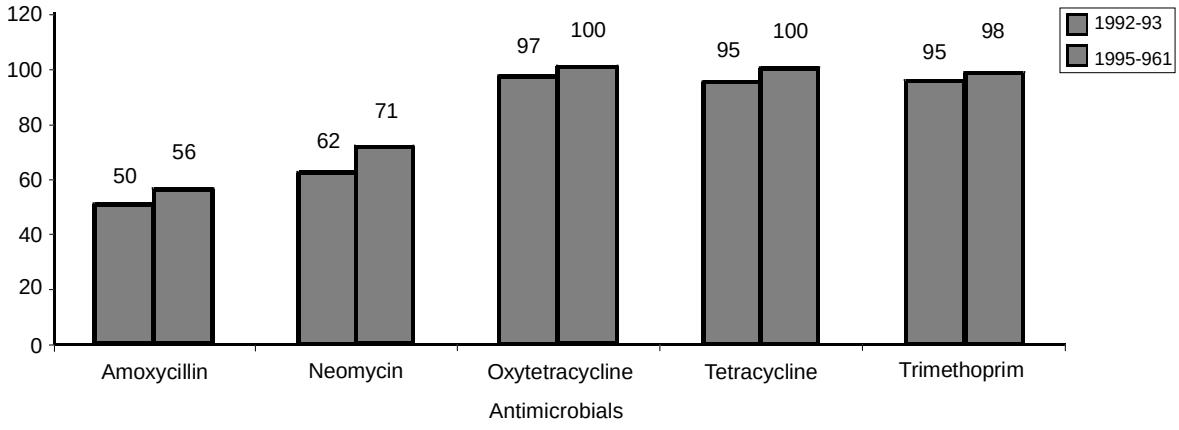

Fig 1. Changing resistance pattern of *E. coli* isolates of poultry origin during 1992-93 and 1995-96 against 5 different antibiotics.

Table 4

Changing resistance pattern of *E. coli* isolates of poultry origin during 1992-93 and 1995-96

Test antimicrobials	Break point* Concentration µg/ml	1992-93		1995-96	
		Susceptible†	Resistant‡	Susceptible	Resistant‡
Amoxyclillin	16	185 (50)	187 (50)	139 (44)	176 (56)
Neomycin	16	142 (38)	233 (62)	91 (29)	224 (71)
Oxytetracycline	8	12 (3)	363 (97)	0	315 (100)
Tetracycline	8	19 (5)	356 (95)	0	315 (100)
Trimethoprim	8	20 (5)	355 (95)	7 (2)	308 (98)

Figures in parentheses are percentages; *Break point of an antimicrobial agent is the concentration that can be achieved in the serum with maximal therapy. Levels are expressed as µg/ml. These values are adapted from NCCLS (19); ‡ MIC (µg/ml) > break point concentration † MIC (µg/ml) < break point concentration

Resistance to tetracycline, streptomycin, ampicillin and sulphonamide has been reported in poultry isolates in Iran; likewise resistance was found to be associated with the use of antibiotic/drug for prophylaxis or growth promotion (Scioli *et al* 1983; Nazer 1980). Studies conducted by Lopes *et al* (1979) have demonstrated selection tetracycline, chloramphenicol and nitrofurazone resistant *E. coli* isolates from poultry fed on rations containing these antimicrobials. Al-Sam *et al* (1993) reported the selection of ampicillin resistant *E. coli* in chicken fed on feed containing 1.7 and 5 g t^{-1} of ampicillin.

Bebora *et al* (1994) studied MIC levels of 6 antibiotics against 37 *E. coli* isolates from septicaemic cases of chickens. All these isolates were found resistant to septran and 51.4% to tetracycline. In our study all bacterial isolates were resistant to tetracycline.

Al-Ghamdi *et al* (1999) also reported a variable degree of antibiotic resistance in *E. coli* poultry isolates. They recommended that the use of antibiotics as growth promoters be banned and that their use be restricted to treating infections. Some of the antimicrobials tested in the present study (e.g tetracycline and oxytetracycline) are being widely used as growth promoters in poultry nutrition in Pakistan. All our *E. coli* isolates have shown resistance against oxytetracycline and tetracycline, which may be due to variety of reasons including their use in poultry ration as growth promoters (Fekety and Pratt 1986).

In the present investigation bacterial isolates were totally resistant to oxytetracycline, tetracycline and highly resistant to trimethoprim; this data is similar to that reported earlier (Bongers *et al* 1995; Amara *et al* 1995).

Although tremendous technological development in poultry industry has taken place and many infections have been successfully controlled by modern drugs and vaccination programme. However, inappropriate, indiscriminate, uncontrolled and inadequate use of antimicrobials has led to the problem of drug resistance in poultry pathogens by disturbing the natural ecological balance which serve to control the growth of potential/opportunistic pathogens. If ecological balance of natural microbial population is disturbed, as it is when an antiobiotic acts upon important resident bacteria,

other organisms that are normally kept in check in ecosystem could multiply and cause a number of complications.

The use of antimicrobials as a means of prophylaxis and as growth promoting substances is on increase in Pakistan. Farmers tend to use antimicrobials as prophylactic agents in an attempt to get quick profits completely forgetting the long-term risks involved in this approach. Besides, it is easier and perhaps temporarily cheaper for the farmers to use antimicrobials for prophylaxis in place of good environmental hygiene practice. This is currently being practiced widely in Pakistan for the last 30 years since the introduction of PIA Shaver in 1960.

The need for continuous epidemiological monitoring of development of antibiotic resistance by human and animal pathogens and indicator bacteria has been emphasized (Durand *et al* 1987; Blanco *et al* 1997; Kelley *et al* 1998).

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