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Transferable Plasmid Mediating Multi-Antibiotic Resistance in Non-Pathogenic *Escherichia coli* Isolates from Chicken Flocks

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Abstract: Thirty *Escherichia coli* strains isolated from chicken flocks were analyzed to determine their antibiotic resistance patterns and plasmid profile. Thirty *E. coli* isolates showed 21 patterns of resistance to the antimicrobial agents used in this study. All the *E. coli* isolates showed high resistance to multiple drugs with 100% resistance observed against Tetracycline, Ampicillin, Amoxicillin and Cloxacillin. Three *E. coli* isolates (10%) were resistant to all used antibiotics. After that, the most common antimicrobial resistance pattern of these isolates was contained Tetracycline, Ampicillin, Amoxicillin, Cloxacillin, Enrofloxacin, Trimethoprim, Flumequine and Oxytetracycline. In addition six *E. coli* isolates were sensitive to Trimethoprim.All multidrug resistant strains had different size plasmids of ~3.5-42 kb. After transformation just 42 kb plasmid was recovered. The present study confirmed high incidence of resistance in *E. coli* isolated from poultry, which is probably due to increased use of antibiotics as feed additives for growth promotion and prevention of disease, resistance transfer among different bacteria and possible cross-resistance between antibiotics used in domestic animals and those used in human medicine.

Key words: *Escherichia coli* · Chickens · Multidrug resistant · Plasmid profile

INTRODUCTION

Non-pathogenic, multiple-drug-resistant *E. coli* in the intestine are probably an important reservoir of resistance genes and drug-resistant [1-3]. Resistance genes are often located on extra chromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes. The acquisition of a new gene may occur by genetic transformation, but when resistance genes are located on plasmids, they can be mobilized by conjugative transfer.

Antibiotics are used in animals as in humans for therapy and control of bacterial infections. In intensively reared food animals, antibiotics may be administered to whole flocks rather than individual animals. In addition, antimicrobial agents may be continuously fed to food animals such as broilers as antimicrobial growth promoters (AMGP) [4].

The first objective of this study was to obtain an estimate of frequency of resistance to common antimicrobial agents in non-pathogenic *E. coli* strains isolated from chicken flocks in the west Azerbaijan province of Iran by an internationally standardized method. The second objective was to evaluate diversity and distribution of plasmid mediated transferable antibiotic resistance to a sensitive bacterial strain.

MATERIALS AND METHODS

Thirteen commercial farms keeping broiler or laying hens from various regions of west Azerbaijan province randomly were selected for the study. Fresh fecal material was collected via cloacae swab and kept cold while being transported to the laboratory. The samples were processed on the same day as collection.

After overnight incubation, growths on MacConkey agar (MCA) plates suggestive of *E. coli* colonies were further streaked onto EMB agar and subjected to IMViC tests, as described by Quinn *et al*. [5]. *E. coli* isolates selected for resistance testing were re-streaked on Luria Bertani (LB) agar.

Antimicrobial susceptibility tests were performed by the standard disc diffusion [6]. Touch at least four morphologically similar colonies with a sterile loop.

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Transfer the growth into 5 ml of sterile LB broth in universal tubes. Incubate the broth with shaking at 35-37°C, until the visible turbidity is equal to or greater than the 0.5 McFarland standards [7]. Adjusted the density of the organism suspension prepared to equal that of the 0.5 McFarland standards by adding sterile distilled water. Used the adjusted suspension within 15 min to inoculated Mueller-Hinton agar plates by dipping a sterile cotton-wool swab into the suspension and remove the excess liquid by turning the swab against the side of the container. Spreaded the inoculum evenly over the entire surface of the plate by swabbing in three directions [7]. Allowed the plate to dry before applying different antibiotic discs (Padtan Teb, Iran).

Following incubation, clear zones surrounding antibiotic disks were measured and compared to reported zones established by manufacturer of the antibiotic disks. Isolates were determined to be sensitive or resistant based on their respective zone sizes.

Plasmid DNA extracted according to the protocol of Birnboim and Doly [8]. Purified plasmids from multidrug resistant isolates were used to transform chemically-competent *E. coli* DH5α by heat shock [9]. Transformants were analyzed on selective media containing four different antibiotics (25µg/ml AMX, 20µg/ml AM, 50µg/ml CX and 25µg/ml TE) after incubation at 37°C for 18-24 h. After 5 subculture of transformed colonies, their antibiotic resistance and plasmid pattern analyzed.

RESULTS

Pure colonies of bacteria were isolated onto MCA plates from all the samples. All isolates were identified as *E. coli* based on morphological and biochemical characteristics.

All isolated *E. coli* showed resistance to at least six of the following antimicrobials, CX, AM, AMX, TE, FM, NFX, N, TMP and T, thus a pattern of multiple drug resistance was observed in all of them. The highest rates of resistance were against TE, AM, AMX and CX (100%), followed by FM (73.3%), T (66.6%), NFX (60.6%), N (53.3%) and TMP (50%). Thirty *E. coli* isolates elicited 21 different patterns of antibiotic resistance to the agents used in this study (Table 1). The most common resistance pattern was TE, N, AM, CX, AMX, T, TMP, NFX, FM (10%) and the least common resistance pattern was TE, N, AM, CX, AMX (3.3%; Table 1).

E. coli strains isolated from the various poultry types, demonstrated 21 resistance patterns with TE, N, AM, CX, AMX, T, TMP, NFX and FM being the most predominant (Table 1). This pattern occurred 3 times (10%) and was followed by the TE, N, AM, CX, AMX, T, TMP, NFX and FM pattern and TE, N, AM, CX, AMX, TMP, NFX and FM pattern that occurred 2 times. Two other patterns, TE, N, AM, CX, AMX, T, TMP, FM and TE, N, AM, CX, AMX, T, TMP, FM appeared one times. It was also noted that most of the resistance patterns contained from 6 to 9 antibiotics indicating multidrug resistance among the organisms.

Table 1: Antimicrobial resistance patterns of *E. coli* isolates from poultry flocks

	Resistance patterns*	Frequency of Dissemination
1	TE, N, AM, NFX, CX, AMX, T, TMP, FM	3
2	TE, AM, NFX, CX, AMX, T,	2
3	TE, N, AM, CX, AMX,	1
4	TE, AM, CX, AMX, NFX,	1
5	TE, N, AM, CX, AMX, T, TMP	1
6	TE, AM, CX, AMX, T, TMP, NFX, FM	1
7	TE, N, AM, CX, AMX, T, TMP, NFX, FM	2
8	TE, AM, CX, AMX, T, NFX, FM	1
9	TE, AM, CX, AMX, FM	1
10	TE, AM, CX, AMX, T, NFX, FM	1
11	TE, N, Ap, CX, AMX, T, FM	1
12	TE, AM, CX, AMX, T, Tp, NFX, FM	1
13	TE, N, Ap, CX, AMX, T, TMP, FM	1
14	TE, N, Ap, CX, AMX, FM	1
15	TE, N, Ap, CX, AMX, TMP	1
16	TE, N, Ap, CX, AMX, TMP, FM	2
17	TE, N, Ap, CX, AMX, TMP, NFX, FM	2
18	TE, AM, CX, AMX, T, TMP, NFX,	1
19	TE, AM, CX, AMX, T, NFX, FM	4
20	TE, N, Ap, CX, AMX, T, NFX, FM	1
21	TE, AM, CX, AMX, NFX, FM	1

*CX, Cloxaciline; AM, Ampicillin; AMX, Amoxicillin, TE, Tetracycline; FM, Flumequine; NFX, Enrofloxacin; N, Neomycin; TMP, Trimethoprim and T, Oxytetracycline.

All strains contained between one and three plasmids, with sizes ranging from 3.5 to 42 kb. After transformation of *E. coli* DH5α competent cells with extracted plasmid DNA, only the 42 kb plasmid was recovered from transformants and resistance to all antibiotics were transferred except resistance against N, TMP and TE.

DISCUSSION

The amount of antimicrobial agents used for therapeutic and non-therapeutic purposes in agriculture far exceeds what is used for humans in many parts of

the world [10] Since exposure to antimicrobial agents is the most important factor with regard to development of antimicrobial resistance, animals and animal products could thus be significant sources of resistant bacteria for the human population [11]. However, the ease with which bacteria acquire new resistance genes by self-transmissible and mobilizable plasmids and conjugative transposons may represent a more significant contribution to the increasing incidence of resistant strains [12].

The results from this study showed alarming resistance frequencies in non-pathogenic *E. coli* from poultry in North-west of Iran. This was particularly the case for TE, AM, CX and AMX. All *E. coli* isolates were shown 100% resistant to TE, AM, CX and AMX and three of thirty were resistant to all 9 used antibiotics.

In earlier studies, 94-96% resistance to TE and 100% resistance to TE-AM and AM-CX were reported in *E. coli* isolates of chicken and quile origin [13-17]. High resistance to tetracycline seen in *E. coli* isolates is in accordance with earlier reports, in which high resistance to these antibiotics (57.0-100%) was reported in chicken isolates [18, 17]. As reported by von den Bogaard *et al.* [19] a high prevalence of resistance in the samples from all slaughterhouses was observed for Amoxicillin (70-94%), Oxytetracycline (78-98%), Trimethoprim (62-96%) and neomycin (38-67%). Our results on moderate resistance to TMP observed in the present study is in contrast to the reports by Al-Ghamdi *et al*. [18] and Over *et al*. [20] respectively, in which high resistance to these antibiotics were reported in chicken and turkey isolates. On the other hand 100% resistance to Cotrimoxasol reported by Roy *et al*. [17] in isolated *E. coli* from Japanese quail and their environment. Results on resistance of *E. coli* isolates to AM (100%) and NFX (60.6%) are in contrast with a report by Amara *et al.* [21] in which low to medium resistance (AM 15-40% and NFX 4.0-9.7%) was reported in *E. coli* isolates of chickens.

Other workers reported that 90% of *E. coli* strains isolated from poultry, were resistant to the two Tetracyclines (Chlortetracycline and Oxytetracycline) and 20% of studied strains were resistant to Neomycine [22]. Resistance against NFX and FM was 60% and 73.3% respectively, which was almost similar to those previously reported from Iran [13, 23]. Nazer [14] reported multiple drug resistance in *E. coli* strains isolated from poultry in Iran and it was revealed that all of the cultures were resistant to TE, AM, Streptomycin and Sulfoamide. In a previous study, it was shown that a high percentage of *E. coli* (86.5%) isolated from avian faeces were resistant to one or more antibiotics [24]. To control and prevent poultry diseases, breeders administer subtherapeutic and therapeutic levels of antimicrobial agents to chickens via food and water. This practice also improves feed efficiency and accelerates weight gain [25]. Administration of antimicrobial agents to poultry however has provided a selective pressure which explains the detection of resistant bacteria and as a result, many bacteria associated with poultry products are commonly resistant to antimicrobial agents [16, 24, 26-28]. According to different reports, transmission of resistance plasmids of *E. coli* from poultry to human commonly occurs [19]. Previous studies have revealed that high level of resistance to Chlortetracycline and Oxytetracycline is of concern due to possible cross resistance with antibiotics used in human medicine and there is a link between the use of antimicrobial agents in poultry and other food producing animals and the emergence of human pathogens with decreased susceptibilities or complete resistance to antibiotics used for treatment of human infections [29-32].

These data confirmed that significant increase in appearance of drug resistant strains in poultry is due to uncontrolled use of antimicrobial agents as food additives, for therapy and control of bacterial infections. *E. coli* strains are routinely exposed to a wide range of antimicrobial agents and has a very wide natural distribution and a propensity for plasmid carriage [33, 34]. Resistance to tetracycline, chloramphenicol or trimethoprim is relatively common in clinical pathogens in Australia, including *E. coli* and is frequently plasmid-mediated [35]. We isolated a collection of multi-resistance plasmids from non pathogenic isolates of *E. coli*. Plasmids harbouring multiple antimicrobial resistance determinants, transfered resistance against 7 of 10 tested antibiotics (except TE, N and TMP) when transferred from non pathogenic *E. coli* to susceptible *E. coli* DH5α strain. As reported by Oppegaard *et al.* [36] the penicillin and tetracycline-resistant *S. aureus* causing mastitis in the herd shared a single plasmid of approximately 20 kb harbouring the *tetA*(*K*) determinant, while the *blaZ* gene is chromosomally located. But *S. aureus* and *E. coli* differ with regard to resistance genes and genetic exchange between *Staphylococci* and coliform bacteria is unlikely to occur [37].

Rosner [38] reported that in the gram-negative bacterium *E. coli*, exposure to the weak acid salicylate induces a condition of phenotypic resistance to a number of chemically unrelated antibiotics, including tetracycline, chloramphenicol, â-lactams and quinolones.

These observations imply that plasmid mediated antimicrobial resistance is a global problem that does not respect any boundaries, either between animals and humans, or bacterial species and genera, demonstrating the strong capacity of plasmids to be horizontally transmitted.

Many questions remained unanswered about mechanisms driving the dissemination of plasmids along the different bacteria isolated from human and animals. The exact contribution of antimicrobials use for animal and human therapy, on the one hand and animal growth promotion and prevention of infection in humans, on the other, to the positive selection of specific resistance genes also remains uncertain. However, further research extending the knowledge of antimicrobial resistance mechanisms and evaluation of role of different plasmids in transferring antimicrobial resistance will facilitate the development of effective preventive and control strategies against this phenomenon.

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