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ANTIBIOTIC RESISTANCE OF *ESCHERICHIA COLI* ISOLATED FROM BROILER CHICKENS

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ABSTRACT

The purpose of this study was to detect the antibiotic resistance of forty-one *Escherichia coli* isolates from the intestinal contents of slaughtered broiler chickens using the disk diffusion method according to Kirby-Bauer. Mueller-Hinton agar plates were inoculated with 0.1 ml overnight broth cultures of individual *E. coli* isolates and the disks with the following concentrations of antibiotics were applied onto them: ampicillin (10 µg), cefotaxime (30 µg), gentamicin (10 µg), streptomycin (10 µg), azithromycin (15 µg), tetracycline (30 µg), ciprofloxacin (30 µg) and levofloxacin (3 µg). After the incubation at 37 °C for 16—18 hours, the inhibition zones were measured and interpreted in accordance with the Clinical and Laboratory Standard Institute (CLSI) zone diameter breakpoints. Almost all *E. coli* isolates showed resistance to tetracycline (92.68 %), most of them were resistant to gentamicin (75.61 %) and levofloxacine (70.73 %). Phenotypic resistance to tetracycline was further confirmed with the help of the Polymerase Chain Reaction (PCR) procedure focused on the presence of specific tet(A) and tet(B) genes. These genes were detected in all 41 *E. coli* isolates. On the contrary, *E. coli* isolates were highly susceptible to both azithromycin and streptomycin. In conclusion, the study highlighted the role of commensal *E. coli* bacteria isolated from the intestines of broiler chickens as an important reservoir of tetracycline resistance genes.

Key words: antibiotics; broilers; disk diffusion method; *Escherichia coli*; PCR

INTRODUCTION

Escherichia coli comprises a significant part of the normal microflora of all warm-blooded animals. This bacterium constitutes a major concern to the public health and food safety issues as it is more than just a harmless intestinal inhabitant; it can also be a highly versatile, and frequently also deadly pathogen.

The gastrointestinal tract of broiler chickens is inhabited by more than 900 bacterial species and this microbial

community consists of both commensal and pathogenic bacteria [1, 22]. Several different *E. coli* strains cause diverse intestinal and extra-intestinal diseases by means of virulence factors that affect a wide range of cellular processes [20]. However, in immunocompromised humans and other animals, even the normal non-pathogenic *E. coli* strains are capable of causing infection [6].

Antibiotics are used worldwide in food-producing animals for many reasons, including prevention of diseases, treatment of infections, growth promotion and increased production. In past decades, the inappropriate use of antibiotics in human and veterinary medicine has led to an increasing rate of antimicrobial resistance [13] and a rapid spread of drug-resistance among both pathogenic and commensal bacteria [14]. Therefore, the European authorities laid down general and specific principles of official controls on the products of animal origin intended for human consumption in order to ensure the compliance with feed and food laws, including animal health and animal welfare rules. At all stages of the food production chain, the food business operators must ensure that food products meet the requirements of food law and that those requirements are being adhered to in an effective way [17, 18].

Tetracyclines which are commonly used in poultry farming have been reported as one of the drugs against which bacteria are most resistant. Tetracycline resistance has been reported in poultry even without any previous administration of this antibiotic [2]. Due to frequent occurrence, *E. coli* strains resistant to tetracycline can be used as an indicator of antibiotic-resistant bacteria in poultry farming [8].

Therefore, our study was focused on the detection of tetracycline resistance genes in *E. coli* bacteria inhabiting the intestines of commercial broiler chickens.

MATERIALS AND METHODS

E. coli isolates were obtained from the intestinal contents of 18 broiler chickens (cross COBB 500) slaughtered at the age of 42 days in a private poultry slaughterhouse Hydina Slovensko s. r. o. in Košice (Slovakia). Immediately after evisceration, the intestines were transported to the Department of Food Hygiene and Technology of the University of Veterinary Medicine and Pharmacy in Košice, while being kept permanently at a refrigeration temperature. Microbiological testing had commenced as soon as the samples arrived at the laboratory. The appropriate decimal dilutions of the intestinal content in a sterile 0.85 % saline were spread in a volume of 0.1 ml on the surface of Endo Agar plates (HiMedia, India) and incubated at 37 °C for 24 hours. Colonies with typical appearance were further identified and confirmed using the biochemical test kit ENT 16 fp (Diagnostics Inc., Slovakia) where the reference strain *Escherichia coli* CCM 4225 (Czech Collection of Microorganisms, Czech Republic) was used as a positive control.

Table 1. Antibiotic susceptibility limits for Enterobacteriaceae [4]

Test/Report group	Antimicrobial agent	Disk content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)		
			S	I	R
Penicillin	Ampicillin	10 µg	≥ 17	14—16	≤ 13
Cephems	Cefotaxime	30 µg	≥ 26	23—25	≤ 22
Aminoglycosides	Gentamicin	10 µg	≥ 15	13—14	≤ 12
	Streptomycin	10 µg	≥ 15	12—14	≤ 11
Macrolides	Azithromycin	15 µg	≥ 13	–	≤ 12
Tetracycline	Tetracycline	30 µg	≥ 15	12—14	≤ 11
Fluoroquinolones	Ciprofloxacin	5 µg	≥ 21	16—20	≤ 15
	Levofloxacin	5 µg	≥ 17	14—16	≤ 13

S—susceptible; I—intermediately resistant; R—resistant

The susceptibility of *E. coli* isolates to eight antibiotics was tested by the Disk diffusion method according to Kirby-Bauer [4]. Bacterial suspensions adjusted to a 0.5 McFarland standard were spread in a volume of 0.1 ml on the surface of Mueller-Hinton agar plates (HiMedia, India) and the disks with the following concentrations of particular antibiotics (Oxoid, United Kingdom) were applied: ampicillin (10 µg/disk), cefotaxime (30 µg/disk), gentamicin (10 µg/disk), streptomycin (10 µg/disk), azithromycin (15 µg/disk), tetracycline (30 µg/disk), ciprofloxacin (30 µg/disk) and levofloxacin (3 µg/disk). The diameters of inhibition zones were measured after incubation at 3 °C for 16—18 hours. In accordance with CLSI zone diameter breakpoints (Table 1) the individual *E. coli* isolates were reported as susceptible (S), intermediate resistant (I), or resistant (R) to a particular antimicrobial agent [5].

The presence of tet(A) and tet(B) genes (577 bp and 634 bp) associated with resistance to tetracycline was determined by the Polymerase chain reaction and the set of primers shown in Table 2. The PCR mixture in a volume of 20 µl contained 2.5 µl of template DNA, 0.3 µl of each primer (ProScience Tech s. r. o., Slovakia), and 4.0 µl of HOT Firepol® Blend Master Mix (Solis BioDyne, Estonia). Amplification in the DNA thermo-cycler (Techne, United Kingdom) has started with an initial denaturation at 95 °C for 12 minutes and was followed by 25 cycles of denaturation at 95 °C for 20 seconds, annealing at 56 °C for one minute and elongation at 72 °C for 2 minutes. The process was completed with a final extension at 72 °C for 10 min. After electrophoresis in 1.5 % agarose gel stained with the GelRed™Nucleic Acid gel stain (Biotium Inc., USA), the amplicons were visualised by the UV transilluminator Mini Bis Pro® (DNR Bio-Imaging Systems Ltd., Israel). The 100 bp DNA ladder (Solis BioDyne, Estonia) was used as a size standard.

Table 2. Primers used for the detection of tetracycline resistance [16]

Resistance genes	Sequences	Size [bp]
***tet*(A)**	(F) GGT TCA CTC GAA CGA CGT CA (R) CTG TCC GAC AAG TTG CAT GA	577
***tet*(B)**	(F) CCT CAG CTT CTC AAC GCG TG (R) GCA CCT TGC TGA CTC TT	634

RESULTS

Forty-one *E. coli* isolates were obtained by inoculation of the chicken intestinal contents on the surface of Endo Agar selective-differential medium. These formed typical pink/red colonies with a metallic sheen. The identity of suspect isolates was further confirmed biochemically using the commercially distributed biochemical test kit ENT 16 fp.

Antimicrobial susceptibility testing

The results of the disk diffusion method in forty-one *E. coli* isolates are shown in Table 3. In this study, a high degree of resistance to almost all antibacterial agents tested was detected among *E. coli* isolates (Fig. 1). Resistance to tetracycline was observed most frequently (38 isolates), followed by that to gentamicin (31 isolates), levofloxacin (29 isolates) and ciprofloxacin (28 isolates). In opposite, resistances to azithromycin (3 isolates) and streptomycin (5 isolates) were the least frequent.

All of the *E. coli* isolates showed resistance to at least one of the eight antibiotics tested. Only one isolate was resistant to a single antibacterial substance (ampicillin). Resistance to two antibiotics was confirmed in 5 *E. coli* isolates. Among the remaining 38 isolates of *E. coli*, 9 were simultaneously resistant to three antibiotics, 12 isolates

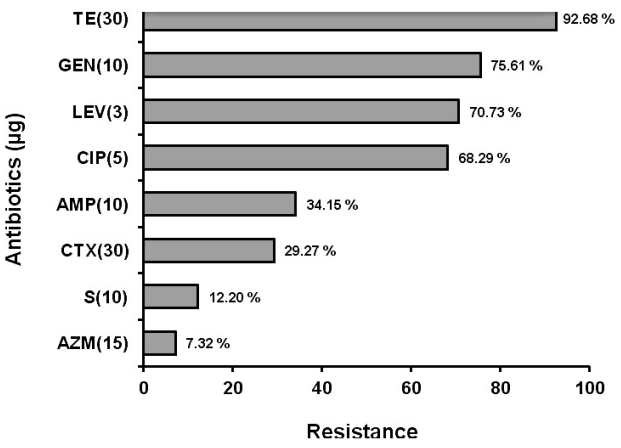

Fig. 1. Resistance of *E. coli* isolates to selected antibiotics

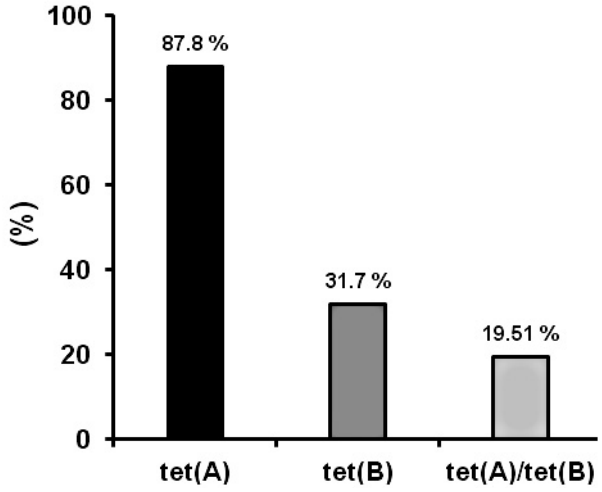

Fig. 2. Occurrence of tetracycline resistance genes in *E. coli* isolates

showed resistance to four antibiotics, 11 isolates were resistant to five antimicrobial agents and another 3 isolates even to 6 out of 8 antibiotics tested.

PCR detection of tetracycline resistance genes

As seen in Table 3, the resistance to tetracycline was phenotypically manifested in almost 93 % of the *E. coli* isolates. However, the PCR method has detected the presence of tetracycline resistance genes in all 41 isolates tested. Resistance to tetracycline was encoded by the tet(A) gene in 36 *E. coli* isolates, with the presence of tet(B) gene confirmed in 13 *E. coli* isolates. Eight isolates possessed both tetracycline resistances genes tested (Figs. 2, 3).

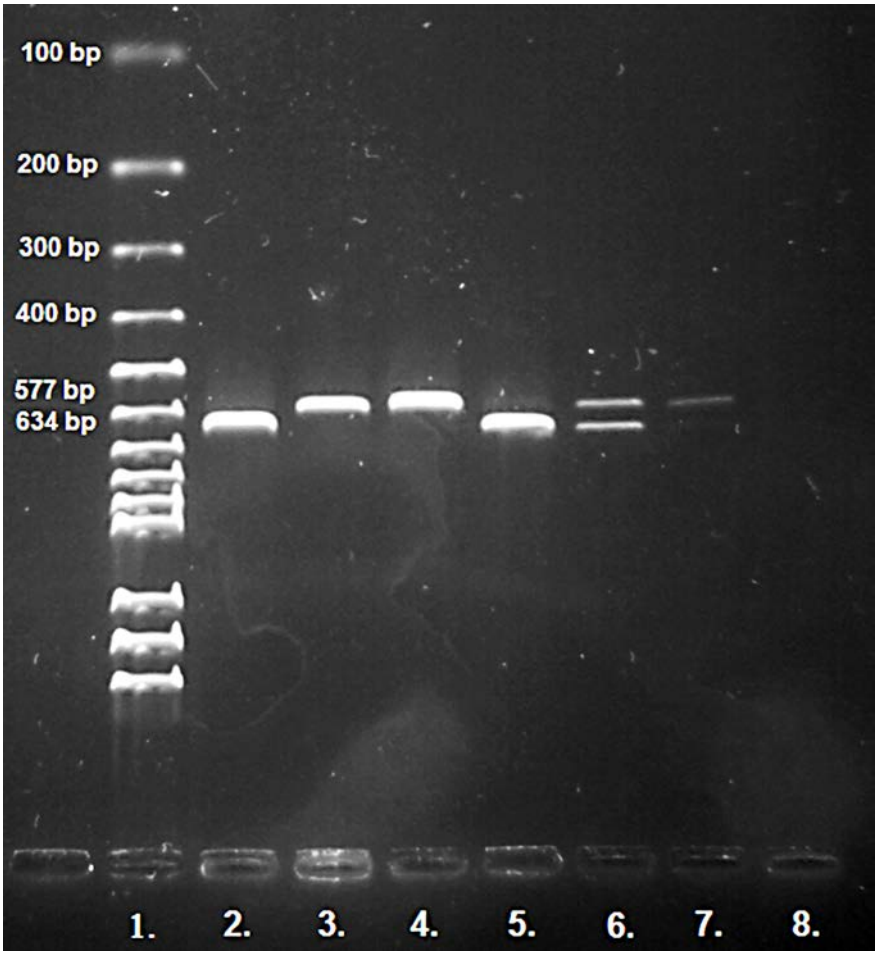

Fig. 3. Detection of tet(A) and tet(B) genes in *E. coli* isolates

Line 1: ladder (100 bp); Lines 2—7: *E. coli* isolates T9, T10, T11, T14, T15, T26;
Line 8: negative control; 577 bp: tet(A); 634 bp: tet(B)

Table 3. Evaluation of inhibition zone diameters (mm) for selected antibiotics in *E. coli* isolates

Isolate	AMP_{10}	CTX_{30}	AZM_{15}	TE_{30}	LEV_{3}	CIP_{5}	GEN_{10}	S_{10}
T1	6 (R)	29 (S)	6 (R)	8 (R)	9 (R)	10 (R)	20 (S)	6 (R)
T2	6 (R)	30 (S)	28 (S)	6 (R)	9 (R)	8 (R)	20 (S)	18 (S)
T3	6 (R)	30 (S)	25 (S)	9 (R)	13 (R)	12 (R)	19 (S)	15 (S)
T4	6 (R)	28 (S)	13 (S)	20 (S)	25 (S)	25 (S)	19 (S)	17 (S)
T7	6 (R)	28 (S)	20 (S)	12 (I)	12 (R)	12 (R)	20 (S)	14 (I)
T8	6 (R)	31 (S)	22 (S)	19 (S)	6 (R)	11 (R)	25 (S)	20 (S)
T9	6 (R)	27 (S)	29 (S)	9 (R)	6 (R)	6 (R)	20 (S)	7 (R)
T10	6 (R)	27 (S)	20 (S)	7 (R)	22 (S)	23 (S)	20 (S)	17 (S)
T11	6 (R)	29 (S)	28 (S)	8 (R)	9 (R)	11 (R)	22 (S)	6 (R)
T12	6 (R)	26 (S)	27 (S)	8 (R)	20 (S)	17 (I)	16 (S)	15 (S)
T13	15 (S)	13 (I)	22 (S)	10 (R)	11 (R)	15 (R)	6 (R)	30 (S)
T14	14 (R)	6 (R)	17 (S)	6 (R)	9 (R)	6 (R)	7 (R)	28 (S)
T15	15 (R)	13 (I)	24 (S)	9 (R)	10 (R)	7 (R)	7 (R)	25 (I)
T16	19 (S)	16 (S)	9 (R)	6 (R)	12 (R)	22 (S)	7 (R)	33 (S)
T17	9 (R)	6 (R)	25 (S)	7 (R)	8 (R)	7 (R)	9 (R)	30 (S)
T18	19 (S)	6 (R)	27 (S)	7 (R)	12 (R)	5 (R)	6 (R)	27 (S)
T26	20 (S)	10 (R)	25 (S)	10 (R)	10 (R)	7 (R)	6 (R)	30 (S)
T27	20 (S)	11 (R)	23 (S)	9 (R)	20 (S)	25 (S)	6 (R)	30 (S)
T28	20 (S)	11 (S)	23 (S)	10 (R)	12 (R)	10 (R)	9 (R)	30 (S)
T29	15 (S)	9 (R)	27 (S)	7 (R)	9 (R)	6 (R)	7 (R)	25 (I)
T30	12 (R)	8 (R)	25 (S)	7 (R)	14 (I)	14 (R)	6 (R)	27 (S)
T31	15 (S)	14 (I)	26 (S)	9 (R)	8 (R)	7 (R)	7 (R)	26 (S)
T32	16 (S)	15 (S)	24 (S)	9 (R)	12 (R)	14 (R)	8 (R)	28 (S)
T33	20 (S)	15 (S)	24 (S)	11 (R)	14 (R)	11 (R)	7 (R)	25 (I)
T34	19 (S)	13 (I)	7 (R)	9 (R)	13 (R)	13 (R)	7 (R)	26 (S)
T35	14 (I)	6 (R)	23 (S)	7 (R)	10 (R)	9 (R)	7 (R)	26 (S)
T36	16 (S)	15 (S)	21 (S)	8 (R)	13 (R)	13 (R)	7 (R)	26 (S)
T49	15 (S)	7 (R)	24 (S)	8 (R)	18 (S)	20 (I)	6 (R)	28 (S)
T52	16 (S)	15 (S)	22 (S)	7 (R)	20 (S)	22 (S)	6 (R)	26 (S)
T53	17 (S)	19 (S)	27 (S)	7 (R)	12 (R)	17 (I)	6 (R)	28 (S)
T61	17 (S)	6 (R)	29 (S)	8 (R)	10 (R)	6 (R)	6 (R)	27 (S)
T62	15 (S)	14 (I)	25 (S)	10 (R)	15 (I)	13 (R)	6 (R)	27 (S)
T63	15 (S)	11 (R)	25 (S)	6 (R)	20 (S)	22 (S)	6 (R)	25 (I)
T64	16 (S)	11 (R)	23 (S)	7 (R)	10 (R)	7 (R)	7 (R)	27 (S)
T65	17 (S)	15 (S)	23 (S)	6 (R)	24 (S)	25 (S)	6 (R)	27 (S)
T66	15 (S)	15 (S)	18 (S)	11 (R)	12 (R)	14 (I)	7 (R)	30 (S)
T67	14 (S)	14 (I)	27 (S)	7 (R)	14 (I)	14 (I)	7 (R)	19 (R)
T68	15 (S)	15 (S)	23 (S)	7 (R)	6 (R)	10 (R)	6 (R)	25 (I)
T69	14 (I)	13 (I)	24 (S)	7 (R)	8 (R)	7 (R)	10 (R)	26 (S)
T70	17 (S)	17 (S)	24 (S)	6 (R)	21 (S)	19 (I)	7 (R)	25 (I)
T72	17 (S)	15 (S)	20 (S)	9 (R)	12 (R)	7 (R)	6 (R)	10 (R)

S—susceptible; I—intermediately resistant; R—resistant

DISCUSSION

Bacteria *E. coli* frequently contaminate food-producing animals as well as foods of animal origin. Some strains supple a part of the common human and other animal intestinal microflora, while others may cause diseases. Avian *E. coli* strains are opportunistic pathogens that are capable of extra-intestinal infection after the exposure of birds to various stressors [21].

Nowadays, antimicrobial resistance is a problem of global public health and food security. This phenomenon can spread globally among microbial species and genera and the mechanisms are not fully understood. Currently, the food chain is seen as one of the most important pathways of spreading antimicrobial resistance [15]. The major factor selecting for antimicrobial resistance in bacteria is antibiotic use, followed by crowding and poor sanitation [7]. These three factors are typical of intensive poultry farming and explain the high prevalence and degree of resistance in faecal *E. coli* of poultry in this particular study and others.

In this study, most bacteria isolated from the intestinal contents of broiler chickens showed a multi-resistant phenotype. The prevalence of tetracycline, gentamicin and ciproflaxin resistance was noticeably high, indicating that intestinal *E. coli* could serve as a reservoir of antimicrobial resistance genes. Almost 93 % of *E. coli* isolates showed a phenotype of tetracycline resistance. However, the presence of tet(A) and/or tet (B) genes were detected in all 41 isolates. Tetracycline resistance genes tet(A) and tet(B) detected in this study were also found in resistant *E. coli* isolates of human and other animal origin in other studies. Soufi et al. [20] reported high rates of antimicrobial resistance among *E. coli* isolates, and the values were similar to the ones in our study. These findings might be linked to the excessive use of sulphonamides, tetracyclines and penicillins in food-producing animals that can result in the selection and transmission of antimicrobial resistance [6]. A high prevalence of tetracycline resistant *E. coli* isolated from broiler chickens was also reported by Sengelov et al. [19]. The authors detected tet(A) in 41.2 % and tet(B) in 52.9 % of the isolates from healthy broilers. In isolates originated from diseased broilers, tet(A) was present in 72.2 % and tet(B) in 27.8 % of the strains. The results of Momtaz [12] confirmed that among 57 *E. coli* isolates from chicken meat samples, resistance to tetracycline had occurred most frequently (91.2 %) and was followed by the resistance to sulfamethoxazol (45.6 %), chloramphenicol and trimethoprim (29.8 %). The antimicrobial resistance profile of *E. coli* strains from broilers of West Azerbaijan province confirmed the presence of tetracycline resistance genes in 54.5 % of the isolates. Among them, 47.7 % were positive for tet(A), 9 % for tet(B) and 2.3 % for both of the above mentioned tetracycline resistance genes [11]. On the contrary, Zibandeh et al. [24] reported the tet(A) to be the only tetracycline resistance gene detected in 72.5 % of *E. coli* isolates taken from the chickens on the day before slaughter.

Tetracyclines are broad spectrum antibiotics used in all food-producing animals (including poultry) because they are cheap and easily available. Therefore, the widespread use of tetracyclines often leads to resistance [3] not only among pathogenic, but also commensal intestinal bacteria, such as *E. coli* [8], resulting in the transmission of resistant bacterial strains from poultry to humans via the food chain [9]. In *E. coli*, the genes tet(A), tet(B), tet(C), tet(D), and tet(E) are associated with an efflux mechanism and make an important part of the tetracycline resistance [3].

It is a well-known fact that integrons play an important role in the dissemination of antimicrobial resistance among Gram-negative bacteria. These genetic structures are able to capture, excise and express genes, frequently included in mobile elements such as plasmids [6]. Therefore, molecular methods, and especially polymerase chain reaction, have been widely used to study antimicrobial resistance genes. As reported by Soufi et al. [20], most of the integrons were detected in food isolates. Marchant et al. [10] noted the correlation between the presence of integrons and resistance to tetracycline in *E. coli* isolates from healthy broiler chickens. The authors found integrons in 49 % of the chicken isolates belonging to the oldest (1999) and the latest (2006) available Spanish surveillance programs, while resistance to tetracycline was determined in 94 % of integron-positive isolates.

However, it should be taken into account that the co-selection of multi-resistant bacteria by the use of different antimicrobial agents, for which resistance genes are associated in the same microorganism, could also occur [23]. Further studies should be done to recognize the main reasons of how commensal non-pathogenic *E. coli* ends up in the wrong place and start acting as pathogenic, and causing harm to the organism.

This study highlighted the role of commensal *E. coli* bacteria isolated from broiler chickens as an important reservoir of antimicrobial resistance genes. During the processing, these bacteria can easily be ingested, enter the human intestines, proliferate and render the person a carrier of these resistant microorganisms. Ultimately, the occurrence of resistant bacteria results in poor human medicine practice, causing a big impact on human health.

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