PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *E. COLI* ISOLATED FROM CHICKEN LIVER SOLD IN RETAIL MARKETS

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Summary. The objective of this study was to estimate the prevalence and the antimicrobial resistance of *E. coli* that contaminates raw chicken liver as one of the most popular poultry sub-product sold in retail markets.

Two hundred and forty samples of fresh raw chicken liver were obtained from national poultry producers in different retail marketing sites and tested for the presence of *E. coli*. One hundred *E. coli* strains (41.7%) were isolated and tested for antimicrobial susceptibility. The MICs of 14 antimicrobial agents were determined for each of the isolates using the broth microdilution method with custom-made microtitre plates. EUCAST cut-off values were used for the interpretation of susceptibility of isolated bacteria to antimicrobial agents. The most frequent resistances were demonstrated to streptomycin (100 %), ampicillin (60%), nalidixic acid (50%), ciprofloxacin (47%) and tetracycline (45%). No resistant strains were found to amikacin. Law percentage of resistant strains was recorded to cefoxitin (2%), ceftiofur (7%), chloramphenicol (10%) and amoxicillin/clavulanic acid (15%). MIC's values above dilution ranges were found to all antimicrobials except amikacin. The highest numbers of resistant strains that demonstrated resistance to the highest concentrations of antimicrobial agents were found to ampicillin, nalidixic acid, sulphonamides and tetracycline. The data demonstrate potential risk during food preparation for consumers in the context of resistant *E. coli* as abovementioned antimicrobial agents are used in veterinary and human medicine as well.

Keywords: antimicrobial resistance, *E. coli*, liver, chicken.

E. COLI PAPLITIMAS MAŽMENINĖS PREKYBOS REALIZUOJAMOSE KEPENĖLĖSE, JŲ ATSPARUMAS ANTIMIKROBINĖMS MEDŽIAGOMS

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Santrauka. Tyrimų tikslas – ištirti *E. coli* paplitimą mažmeninės prekybos realizuojamose vištų kepenėlėse, kaip viename iš populiariausių vištienos subproduktų, ir nustatyti šių bakterijų atsparumą antimikrobinėms medžiagoms.

Iš skirtingų mažmeninės prekybos vietų surinkti įvairių Lietuvos gamintojų šviežių žalių vištų kepenėlių mėginiai (240 mėginių) ir atlikti *E. coli* bakteriologiniai tyrimai. Iš mėginių išskirtas vienas šimtas *E. coli* padermių (41,7 proc.) ir nustatytas jų atsparumas antimikrobinėms medžiagioms. Tyrimams taikytas mikroskiedimų metodas plokštelėse, naudojant 14 skirtingų antimikrobinių medžiagų. Rezultatai vertinti pagal ribines jautrumo reikšmes, nurodytas Europos antimikrobinio jautrumo tyrimų komiteto (EUCAST) duomenų bazėje. Nustatyta, jog dažniausiai tirtosios *E. coli* padermės pasižymėjo atsparumu streptomicinui (100 proc.), ampicilinui (60 proc.), nalidikso rūgščiai (50 proc.), ciprofloksacinui (47 proc.) ir tetraciklinui (45 proc.). Atsparių amikacinui padermių nenustatyta. Nustatytas retas atsparumas cefoksitinui (2 proc.), ceftiofurui (7 proc.), chloramfenikoliui (10 proc.), amoksicilino ir klavulano rūgšties kombinacijai (15 proc.). Mažiausios slopinamosios koncentracijos, didesnės nei plokštelėse esančios antimikrobinių medžiagų koncentracijos, nustatytos visoms tirtoms medžiagoms, išskyrus amikaciną. Rasta daug *E. coli* padermių, kurioms inaktyvuoti reikėjo didelio MSK ampicilino, nalidikso rūgšties, sulfonamidų ir tepraciklino kiekio. Tyrimų duomenys rodo potencialią riziką vartotojams (užsikrėsti atspariomis bakterijomis ar jų perduodamais atsparumo veiksniais per maisto gamybos grandinę), nes šios antimikrobinės medžiagos naudojamos tiek žmonių, tiek ir veterinarinėje medicinoje.

Raktažodžiai: antimikrobinis atsparumas, *E. coli*, kepenėlės, vištos.

Introduction. The use of antibiotics and other antimicrobial agents throughout the food chain contributes to the growth of resistant bacteria that could be passed directly to humans. The emergence and spread of resistant bacterial strains like *Campylobacter* spp., *Escherichia coli* and *Enterococcus* spp. from poultry products to consumers put humans at risk to the strains of bacteria that resist antibiotic treatment (Apata, 2009; Hammerum et al., 2010; Ruzauskas *et al*., 2010-2). Commensal microbiota plays an important role as a reservoir of resistance determinants (Blanc *et al*., 2006; Scott, 2002). Antimicrobial resistance in commensal strains of *E. coli* may play an important role in the ecology of resistance and clinical infectious diseases (Winokur *at al*., 2001). Transmission of resistance genes from normally nonpathogenic species to more virulent organisms within the animal or human intestinal tract may be an important mechanism for acquiring clinically significant antimicrobial-resistant organisms. Fecal-oral and food-borne transmission of *E. coli* as well as resistance determinants are well documented (Bartolini *et al*., 2006; Bates *et al*., 2004).

Inappropriate food processing is one of the most important reasons of passing bacteria on to humans from contaminated food products. For instance, using of not separate cutting boards for vegetables and raw animal products could easily lead to ingestion of bacteria (Ak *et al*., 1994). Some studies prove that there is no difference on surviving susceptible or resistant bacteria on such surfaces (Neely and Maley, 2000).

Chicken is the main poultry source produced by large national manufacturers in Lithuania. Whole body and separate parts (wings, drumsticks, liver, hearts) of chickens are sold. Raw meat products could be an important source of contamination of humans by resistant bacteria (Sorum and L'Abee-Lund, 2002).

Different antimicrobial agents are still widely used in poultry production (Apata, 2009; Islam *et al*., 2008; Nonga *et al*., 2009). By this reason poultry are increasingly being associated with carriage of multiresistant organisms (Joseph *et al*., 2001). The same classes of antibiotics are used for human and animal treatment, thus surveillance of antimicrobial resistance in animals and food products are underlying (Heuer *et al*., 2006).

The data on the antimicrobial resistances of bacteria that contaminate food are still insufficient. Routine testing of susceptibility of pathogenic bacteria are performed in different laboratories within the country however, no testing on commensals are performed on a regular base. Testing of commensals should be prioritized as they usually do not cause any disease. By this reason commensal bacteria often left uncontrolled and that helps them to survive in different conditions (Ruzauskas *et al*., 2009-3).

Food contamination with pathogenic and commensal bacteria occurs during food processing operations. Intestinal contents and feces of broilers harbor huge quantity of *E. coli*. In slaughterhouses contamination with spilled gut contents occurs during evisceration (Borck and Pedersen, 2005). Inappropriate handlings, especially in retail markets have impact on cross-contamination of bacteria in raw meat products. Resistance profile in bacteria in raw food products could reflect the susceptibility situation in animal farms and also the consumption of antimicrobial agents within the country.

The objective of this study was to estimate prevalence and the antimicrobial resistance of *E. coli* that contaminates raw chicken liver as one of the most popular poultry sub-product sold in retail markets.

Material and methods

Place and sampling. Investigations were carried out from January till December in 2009 in Lithuania. The samples were obtained from retail markets in biggest (Vilnius, Kaunas, Klaipėda, Šiauliai, Panevėžys and Alytus) as well as in smaller (Marijampolė, Kaišiadorys, Birštonas) towns. Fresh raw liver of chickens were randomly selected however, samples were taken only from the national producers. One sample (separate liver) was taken from one marketing site. A total of 240 samples were collected with amount of 20 samples in each month. One randomly selected colony (isolate) from one sample after identification was used for further testing.

Isolation and identification of *E. coli*. Each product was shaken manually with 100 ml Brain Heart Infusion Broth (CM1135, Oxoid) in a technically clean plastic bag for 1 min. Aliquots of 100 µl were plated on MacConkey Agar (CM0007, Oxoid). Inoculated MacConkey Agar plates were incubated at +37 $^{\circ}$C for 24 hours. After incubation MacConkey Agar plates were screened for presumptive colonies. Each selected isolate was identified using Microbact 24E identification system (MB1131A, Oxoid). Microbact 2000 (Oxoid) software was used for assessment of the results. As a control strain *Escherichia coli* ATCC 25922 from American Type Culture Collection was used.

Susceptibility testing. The MICs (Minimum Inhibitory Concentrations) of 14 antimicrobial agents were determined for each of the isolates using the broth microdilution method with custom-made microtitre plates (CMV1AGNF, Trek Diagnostic Systems, Inc., Westlake, Ohio). The antimicrobial agents and test ranges (2-fold dilution series) were as follows: ampicillin, 1 to 32 µg/ml; amikacin, 0.5 to 64 µg/ml; amoxicillin/clavulanic acid, 1/0.5 to 16/8 µg/ml; chloramphenicol, 2 to 32 µg/ml; ciprofloxacin, 0.015 to 4 µg/ml; trimethoprim/sulfamethoxazole, 0.12/2.38 to 4/76 µg/ml; cefoxitin, 0.5 to 32 µg/ml; gentamicin, 0.25 to 16 µg/ml; kanamycin, 8 to 64 µg/ml; nalidixic acid, 0.5 to 32 µg/ml; sulphisoxazole, 16 to 256 µg/ml; streptomycin, 32 to 64 µg/ml; tetracycline, 4 to 32 µg/ml; and ceftiofur 0.12 to 8 µg/ml. Fifty microlitres of a culture suspension in Mueller-Hinton broth containing $5x10^8$ CFU of each isolate/L was inoculated into microtitre plates containing the test antimicrobial agents and incubated at 37°C for 18 h ± 1 h in ambient air. *E. coli* strain ATCC 25922 was used as quality control. The plates were removed and read manually for growth to score the MIC determinations by using EUCAST cut-off values (Table 1) (EUCAST 2010). Additional testing with 16 µg/L concentration of streptomycin was performed using broth dilution method for the possible interpretation of susceptibility to streptomycin as the plates did not contain streptomycin concentration below EUCAST cut-off value.

Statistical analysis. Statistical analysis was performed using statistical package Instat (GraphPad Software). The reliability of the differences of arithmetical average was determined by Student‘s t-test. Data were considered as reliable in case of $p \leq 0.05$

Results. One hundred samples (41.7%) were found to be positive for the presence of *E. coli* from total 240 chicken liver tested. The same quantity of *E. coli* isolates were purified and tested for antimicrobial susceptibility.

In-vitro susceptibility of the isolated strains varied according to the antimicrobial agents tested. As it could bee seen from the results (Table 1) *E. coli* spread in food sub-products of poultry origin demonstrated frequent resistances to the most extensively used antimicrobials including aminopenicillins, (fluoro)quinolones and tetracycline. Resistance to the 3-d generation of the cephalosporins that are exlusively important in human medicine was also recorded. Seven percent of all tested isolates demonstrated decreased susceptibility to ceftiofur. All of the tested strains demonstrated resistance to streptomycin. The most frequent resistant characteristics were resistances to ampicillin (60%), nalidixic acid (50%), ciprofloxacin (47%) and tetracycline (45%). No resistant strains were found to amikacin. Relatively law percentage of resistant strains was recorded to cefoxitin (2%), chloramphenicol (10%) and amoxicillin/clavulanic acid (15%).

Table 1. **Distribution of resistances of *E. coli* isolated from chicken liver**

Antimicrobial	Cut-off values (EUCAST)	Number of resistant strains, % (MIC's above cut-off values)
Amikacin	>8	0
Ampicillin	>8	60
Amoxicillin/clavulanic acid	>8/4	15
Chloramphenicol	>16	10
Ciprofloxacin	>0,032*	47
Trimethoprim/sulfamethoxazole	>2/38	23
Cefoxitin	>16	2
Gentamicin	>2	20
Kanamycin	>8	35
Nalidixic acid	>16	50
Sulphisoxazole	>256	41
Streptomycin	>16	100
Tetracycline	>8	45
Ceftiofur	>1	7

** - cut off value 0.03 was used in this study, as it was the dilution in plates*

MIC's of the each antimicrobial agent varied in a wide range, depending on antimicrobial (Table 2). Highest concentrations required for inhibition of the tested strains were recorded to ampicillin (53% of the isolates ≥64 mg/L), nalidixic acid (49% of the isolates ≥64 mg/L), tetracycline (42% of the isolates ≥64 mg/L) and sulphisoxazole (41% of the isolates ≥512 mg/L). The obtained results demonstrated highest resistances of commensal *E. coli* spread in chicken liver to the highly important antimicrobials for humans including aminopenicillins, quinolones, tetracyclines and sulphonamids. Statistical comparison of resistances between separate isolates to different antimicrobials demonstrated link between resistances to nalidixic acid, ciprofloxacin and tetracycline: 43 isolates from 45 resistant to tetracycline were also resistant to (fluoro)quinolones.

Table 2. **MIC distributions among *E. coli* isolates**

Antimicrobial agents	MIC distribution (%) (mg/l)															
	0.015	0.03	0,06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Streptomycin											0	68	5	27		
Ampicillin							6	17	10	7	3	4	53			
Kanamycin										65	9	15	3	8		
Trimeto/sulfa				61	10	5	1	0	0	23						
Sulphisoxazole											17	15	17	8	2	41
Ceftiofur				1	32	54	6	2	2	3						
Nalidixic acid						11	2	25	10	1	1	1	49			
Gentamicin					25	18	33	4	1	0	1	18				
Ciprofloxacin	39	14	4	2	12	6	3	3	3	14						
Amoxi/clav						22	0	7	16	40	12	3				
Tetracycline									54	1	1	2	42			
Chloramphenicol								20	43	23	4	2	8			
Amikacin						15	16	19	45	5	0	0	0			
Cefoxitin						4	1	18	55	16	4	1	1			

Green cells – MICs below cutt-off value (epidemiologically susceptible)
Red cells – MICs above cutt-off value (epidemiologically resistant)

Discussion. The primary habitat of *Escherichia coli* is the vertebrate gut, where it is the predominant aerobic organism, living in symbiosis with its host (Tenaillon *et al*., 2010). These bacteria could easily contaminate food products during evisceration at the process of slaughtering (Abu-Ruwaida *et al*., 1994). Meat inspection on the bacterial contamination is limited to pathogenic microbiota – *Salmonella enterica,* VTEC and *Campylobacter* spp. Commensal bacteria such as *E. coli* and *Enterococcus* are left uncontrolled. However, these bacteria of food-producing animals are considered an important reservoir of antibiotic resistance (Knezevic and Petrovic, 2008).

In our previous study the status of the antimicrobial susceptibility of bacteria genus *Enterococcus* on poultry farms was investigated in Lithuania. Resistances to tetracyclines, macrolides and fluoroquinolones were common among *E. faecalis* and *E. faecium*. No resistant strains to linezolid and glycopeptides were detected, however, resistances to quinupristin/dalfopristin and tigecycline was found (Ruzauskas *et al*., 2009-2). Resistance of bacteria that are spread in foodstuffs indicates the necessity of examination and control of raw food products. To our knowledge, no studies according resistance of commensal bacteria spread in food products intended for humans have been performed by other investigators in Lithuania.

During this study, obtained results demonstrated frequent contamination of poultry liver with *Escherichia coli.* One hundred positive samples (42 %) from total 240 tested were positive for *E. coli*. Contamination frequency was very similar as it was found in retail poultry meat, on the surface of eggs or from hand washing fluid of chicken handlers i.e. results of the study obtained by other authors (Schroeder *et al*., 2003; Akond *et al*., 2009). One hundred of isolates were randomly selected for susceptibility testing: one strain of *E. coli* from one positive sample. These strains were obtained from the different counties within the country.

Obtained results demonstrated frequent resistances to certain antimicrobial agents of different classes, particularly aminoglycosides, aminopenicillins and fluoroquinolones. All these classes of antimicrobial agents are recognized to be critically important antimicrobial agents for humans. Frequent resistance to fluoroquinolones of isolated *E. coli* from animals was recorded in Lithuania previously, with incidence of 34-50 % (Ruzauskas *et al.*, 2009-1; Ružauskas *et al.*, 2010-2). These findings in a poultry products demonstrate possible link between spread of commensal bacteria in live animals and animal products after slaughtering. Cephalosporins of 3-d generation are not authorized for poultry however, 7 % of the tested isolates demonstrated resistance to ceftiofur. That might be associated with resistance (or bacterial) transfer from other species of animals or man to poultry.

Resistance situation of commensal bacteria obtained from food-producing animal species can reflect the resistance status in live animals and consumption of antibiotics in animal husbandry. The obtained results demonstrated that bacteria spread in food products were resistant to antibiotics that are widely used for human and animal treatment. It means that *E. coli* from poultry origin are potentially hazardous to humans from the point of antimicrobial resistance. The status of the antimicrobial susceptibility of *E. coli* isolated from poultry origin obtained by other authors depended on country. For instance, in the USA among non-toxigenic *E. coli* isolates resistances were observed to tetracycline (59%), streptomycin (44%), sulfamethoxazole (44%), cephalothin (38%), and ampicillin (35%). Fewer isolates were resistant to gentamicin (12%), nalidixic acid (8%), chloramphenicol (6%), ceftiofur (4%), and ceftriaxone (1%), and all were susceptible to ciprofloxacin (Schroeder *et al*., 2003). In Denmark the most frequent resistances of indicator *E. coli* from chickens were recorded to sulfonamides (18%), ciprofloxacin (14%), ampicillin (11%), nalidixic acid (11%), tetracycline (8%) and streptomycin (7%) i.e. the same antimicrobial agents as it was recorded in our study, however, with much more less quantity of resistant isolates (DANMAP, 2007). In Sweden resistances of *E. coli* isolated from chicken samples were found to be the most common to quinolones and fluoroquinolones (7%) followed by resistances to sulphonamides, ampicillin, streptomycin and tetracycline (3-6%).

Extended series dilutions of antimicrobial agents were used in the plates, however, MIC's values above dilution ranges were found to all antimicrobial agents except amikacin. The biggest numbers of resistant strains that demonstrated resistances to the highest concentrations of antimicrobial agents were found to ampicillin, nalidixic acid, sulphisoxazole and tetracycline. Tetracycline is an old, inexpensive antibiotic and have very broad spectrum of activity. By this reason it is very widely used in veterinary medicine within the community. Aminopenicillins and sulphonamides are also widely used. Resistances of enteric bacteria to quinolones became very problematic after enrofloxacin as generic drug became available (Ružauskas *et al*., 2009-1). Statistical comparison of resistances between separate isolates to different antimicrobials demonstrated link between resistances to (fluoro)quinolones and tetracycline however, it could not be stated that is due co-resistance. In addition, performing of the statistical analysis was complicated by limited concentrations of antimicrobials in plates as some results were interpreted without natural numbers but using sign $\geq$ for concentration expressing.

The association between resistance and usage of antimicrobial agents are described by different authors (Bergman *et al.,* 2009). Antibiotics that are intended for use exclusively in human medicine are still effective against bacteria spread in animal origin and should be kept for human use in future. That is the main measure in curing of humans infected with resistant bacteria. Nevertheless prophylactic measures should be prioritized and should be implemented at all stages of food processing. Personal hygiene during marketing is also very important since resistant bacteria could easily spread among salespersons and consumers. Commensal microbiota should be treated as indicator of selection pressure by antimicrobial use and should be tested for antimicrobial resistance as well as pathogenic bacteria. This suggestion is in coincidence with the opinion of some other authors (Akwar *et* *al*., 2008; Kikuvi *et al*., 2007; Knezevic and Petrovic 2007; Mathai *et al*., 2008).

Conclusion. *E. coli* often contaminates raw poultry liver as food sub-product: positive samples were found in 42% from total 240 samples tested in retail markets. These bacteria are often resistant to different classes of antimicrobials, including those are critically important for humans – aminoglycosides, aminopenicillins and fluoroquinolones. The most frequent resistances were demonstrated to streptomycin (100 %), ampicillin (60%), nalidixic acid (50%), ciprofloxacin (47%) and tetracycline (45%).

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