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Antimicrobial susceptibility testing of *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from chicken meat originating from markets and family farms

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ABSTRACT
Antimicrobial resistance is one of the major threats to public health affecting humans, animals, and the environment. Potential transmission of multidrug-resistant zoonotic pathogens from chicken meat to humans is of particular concern. The aim of our research was to determine the antibiotic resistance patterns in *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from chicken meat originating from markets and family farms in Novi Sad, Serbia. This study included 21 bacterial strains isolated from 31 chicken meat sample. For the antimicrobial susceptibility testing of the bacterial isolates the disc diffusion method was used according to EUCAST guidelines. Highest resistance was observed to ampicillin (66.66%), followed by amoxicillin with the addition of clavulanic acid (42.86%), and sulfamethoxazole with trimethoprim (19.05%), with the lowest resistance to ceftriaxone and gentamicin (4.76%). No isolates were resistant to cefuroxime and ciprofloxacin. Extended-spectrum β -lactamases (ESBL) were detected in only one sample (4.76%) identified as *K. pneumoniae*. Multidrug-resistant strains were not found. Considering the small number of samples included in this research, further studies are needed with a larger number of samples and other classes of antibiotics in order to gain a clearer picture on the abundance and prevalence of antibiotic-resistant enteric bacteria isolated from food od animal origin for human consumption in Serbia.

KEY WORDS: antimicrobial resistance, *Escherichia*, *Klebsiella*, chicken meat

Introduction

Antimicrobial resistance (AMR) represents a growing global concern and threat to public health and food safety. The AMR carries a huge risk of returning to the pre-antibiotic era if left untreated (Nhung et al., 2017; FAOSTAT 2021). Therefore, the World Health Organization (WHO) adopted the "One Health" approach in 2017, which states that human health, animal health and environment are closely connected. Also, the WHO emphasizes that the "One Health" approach is especially important when it comes to antibiotic resistance, food safety and zoonosis control. A large number of studies have found that the use of antibiotics in animals, especially in subtherapeutic doses, affects the microbiome of animals, which further affects the environment (Xiong et al., 2018).

Resistant strains can be transmitted from animals to humans by consuming food of animal origin, including meat, but also by direct contact with animals (Hoelzer et al., 2017). Food products of animal origin, especially chicken meat, are considered as a possible source of human exposure to bacteria resistant to antibiotics. Microbiological contamination of meat occurs during slaughter and processing of carcasses, through fecal contents and / or contaminated equipment from the slaughterhouse (Projahn et al., 2019).

The bacteria of the normal biota of the gut, such as f. *Enterobacteriaceae*, can easily acquire and transfer resistance genes. These commensal bacteria, which represent a reservoir of resistance genes for pathogenic bacteria, can thus be used as indicators of changes in antimicrobial resistance (Caprioli et al., 2000). Extended-spectrum β-lactamases are responsible for resistance to most β-lactam antibiotics (e.g. penicillins and cephalosporins) and are predominantly found among *Enterobacteriaceae* leading to the spread of resistance genes and to serious infections. Extended-spectrum β-lactamases producing *Escherichia coli* and *Klebsiella pneumoniae* are of essential importance in both human and veterinary medicine.

Escherichia coli, a member of the family *Enterobacteriaceae*, whose pathogenic strains are among the main causes of alimentary infections, is present in the gastrointestinal tract of poultry, other domestic and wild animals, as well as of humans. During slaughter, intestinal strains easily reach poultry carcasses and as a result poultry meat is often contaminated with multidrug-resistant *E. coli*

(Van den Bogaard et al., 2001). The increasing incidence of multidrug-resistant resistant *E. coli* strains has resulted in significant morbidity and mortality in humans (Rahman et al., 2020).

Klebsiella pneumoniae is an opportunistic pathogen associated with severe nosocomial infections accompanied by signs of septicemia and pneumonia, but also associated with urinary tract infections in humans (Fielding et al., 2012). In addition to nosocomial infections, *K. pneumoniae* also spreads through food products such as seafood, frozen foods and fresh chicken meat, can produce enterotoxins, and is therefore also considered a potential alimentary pathogen (Hayati et al., 2019). However, a small number of studies have evaluated the links between animal and human isolates of *K. pneumoniae*.

The aim of the study was to isolate and identify strains of *Escherichia coli* and *Klebsiella pneumoniae* from chicken meat, as well as to determine their antibiotic resistance patterns.

Material and methods

A total of 31 samples of chicken meat weighing 5 g, of which 17 originated from the market, and 14 from family farms from the territory of Novi Sad were taken. All samples were collected separately in sterile plastic dishes containing peptone water, and within four hours were transported to the Laboratory for Microbiology of the Department of Veterinary Medicine at the Faculty of Agriculture in Novi Sad for bacteriological cultivation.

Isolation and biochemical identification

Primary isolation was performed on a selective nutrient medium Endo agar (Biolife Italiana, Milan, Italy). The nutrient medium was previously prepared according to the manufacturer's instructions and poured (20 ml each) into sterile, plastic Petri dishes. After incubation at 37 °C for 24 hours, two types of colonies were chosen for further processing. The first type as single, small, smooth, round colonies 1 mm in diameter with a metallic green shine that phenotypically correspond to *E. coli* species, and the second type as large, pink, mucoid colonies that phenotypically correspond to *K. pneumoniae*.

In order to obtain a pure culture, subcultivation was performed on a differential medium, HiCrome Universal Differential agar (HiMedia Laboratories, Mumbai, India). The medium was previously prepared according to the manufacturer's instructions and poured (20 ml each) into sterile, plastic Petri dishes. Based on the color of the colonies listed in the manufacturer's instructions, individual purple colonies phenotypically corresponding to *E. coli* and blue-green mucoid colonies phenotypically corresponding to *K. pneumoniae* were selected.

The identification of bacteria was based on biochemical testing that included test of indole production, motility and H_2S production on SIM medium (HiMedia, Mumbai, India), citrate utilisation test on Simmon's citrate agar (HiMedia, Mumbai, India) and double sugar fermenation test and H_2S production on Kligler iron agar (Biolife Italiana, Milan, Italy) (Scheutz and Strockbine, 2015).

E. coli isolates were considered to be indole positive, MR positive, VP negative, citrate negative, which ferment lactose and glucose to acids in the presence of gas and without H2S production.

K. pneumoniae isolates were considered to be indole negative, MR positive, VP negative, citrate negative, which do not ferment lactose, ferment glucose to acids without gas and without H2S production.

Antimicrobial susceptibility testing

For the antimicrobial susceptibility testing of the bacterial isolates, the disc diffusion method was used according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST, 2017a). Müller-Hinton agar (HiMedia, Mumbai, India) was prepared according to the manufacturer's instructions and stored in Petri dishes. The inoculum was prepared using the direct colony suspension method to make a suspension of the organism in saline to the density of the McFarland 0.5 turbidity standard, approximately corresponding to 1-2 x 10^8 CFU/ml for *Escherichia coli* and *Klebsiella pneumonia.* Prior to inoculation, the agar plates were kept at room temperature. The inoculum suspension was used within 15 minutes of the preparation by dipping a sterile cotton swab into the suspension. To avoid over-inoculation of Gram negative bacteria, the excess of fluid was removed by pressing and turnig the swab against the inside of the tube. The inoculum was spread evenly over the entire agar surface ensuring that there are no gaps between the streaks. Antimicrobial disks (Bioanalyse, Ankara, Turkey) were applied within 15 minutes of inoculation, after which the agar plates were incubated for 16-20 hours at 35+/-1°C in air. Antimicrobial susceptibility of the bacterial isolates was tested for the following classes of antibiotics: penicillins (ampicillin 10 µg, amoxicillin with clavulanic acid 20+10 µg), cephalosporines (cefuroxime 30 µg, ceftriaxone 30 µg), macrolides (gentamicin 10 µg), sulfonamides (sulfamethoxazole with trimethoprim 1,25+23,75 µg) and

quinolones (ciprofloxacin 5 µg). After the incubation the inhibition zones of bacterial growth around the applied discs were mesured and interpreted according to the EUCAST guidelines (EUCAST, 2017b).

Results and discussion

From a total of 31 samples of chicken meat originating from family farms and markets, 11 (35.48%) strains were identified as *Escherichia coli*, and 10 (32.26%) as *Klebsiella pneumoniae* based on biochemical properties. Based on the results of several studies conducted in Nepal, researchers reported different values of prevalence of *E. coli* in chicken meat, 98% (Bajagai,, 2012), 70% (Thanigaivel and Anandhan, 2015), 4.8% (Shrestha et al. , 2017), even up to 100% (Gautam et al., 2019). The reason for this huge difference may be due to the use of contaminated water during slaughter, carcass washing and other handling procedures, and also because *E. coli* strains are common inhabitants of the intestinal tract of animals and humans (Cabral, 2010). The high prevalence of *E. coli* in chicken meat (87.6%) is also stated by the authors of a study conducted in Arizona (Davis et al., 2018), as well as in Ghana (80%) (Adzitey et al., 2020), and a slightly lower prevalence was determined by a study conducted in India (78%) (Hussain et al., 2017), which is significantly more than the results obtained in this study (35.48%). *Klebsiella spp.* (Gautam et al., 2019) had the lowest prevalence (8.64%), compared to other strains isolated from chicken meat, which is similar to the results from Egypt (10.66%) (Al-Mutairi 2011), but less compared with the results of a study in Oklahoma (30%) (Kim et al., 2005), as well as within this study (32.26%).

In this study, antimicrobial resistance was observed in 15 (71.43%) out of 21 tested isolates, whereas six (28.57%) isolates originating from family farms and identified as *K. pneumoniae*, showed sensitivity to all used antibiotics. Antimicrobial resistance was noted for 5 out of 7 tested antibiotics.

The results of the antibiogram are shown in Table 1.

Table 1.
Patterns of antibiotic resistance of bacterial isolates based on origin
Pregled antibiotske rezistencije bakterijskih izolata prema poreklu

Antibiotic	***Escherichia coli***			**Klebsiella pneumoniae**			**total samples**
	FF*	M**	total *E. coli*	FF	M	total *K. pneumoniae*	
AMC[1]	2	4	6	1	2	3	**9**
AMP[2]	2	9	11	1	2	3	**14**
CXM[3]							
CTR[4]				1		1	**1**
GN[5]		1	1				**1**
TSX[6]	2	1	3		1	1	**4**
CIP[7]							

*(Family farm); **(market); [1](amoxicillin+clavulanic acid); [2](ampicillin); [3](cefuroxime); [4](ceftriaxone); [5](gentamicin); [6](sulfamethoxazole+trimethoprim); [7](ciprofloxacin)

Highest resistance was observed to ampicillin (66.66%), followed by amoxicillin with the addition of clavulanic acid (42.86%), and sulfamethoxazole with trimethoprim (19.05%), with the lowest resistance to ceftriaxone and gentamicin (4.76%). No isolates were resistant to cefuroxime and ciprofloxacin. All *E. coli* isolates showed resistance to ampicillin, out of which 65% were *E. coli* strains of market origin. The highest prevalence of resistance to amoxicillin was also observed in *E. coli* isolates from markets (45%). Only one isolate of *K. pneumoniae* originating from a family farm showed resistance to ceftriaxone, while only one isolate of *E. coli* from markets showed resistance to gentamicin.

Researchers from Nepal reported 100% resistance of *E. coli* isolates to ampicillin and 3.71% to ciprofloxacin, as well as 100% sensitivity to gentamicin and ceftriaxone (Gautam et al., 2019). However, other researchers in Nepal (Saud et al., 2019) found resistance to gentamicin (24.2%), which is contrary to the previous study. The higher prevalence of gentamicin-resistant *E. coli* isolates (28.3%) was also reported in Nigeria (Adeyanju and Ishola, 2014), which is higher than the results obtained in our study (4.76%).

On the other hand, all isolates of *Klebsiella spp.* showed sensitivity to ceftriaxone, resistance to ampicillin (85.71%), ciprofloxacin (14.29%) and gentamicin (28.57%) in Nepal (Gautam et al., 2019), in contrast to the results of our study where only one *K. pneumoniae* strain resistant to ceftriaxone was found (10%), three resistant to ampicillin (30%), while all *K. pneumonia* isolates showed sensitivity to ciprofloxacin and gentamicin. Significantly higher prevalence of resistant strains of *Klebsiella* spp. was noted in Oklahoma, with 100% resistance to ampicillin and ceftriaxone (Kim et al., 2005), as well as in Arizona (Davis et al., 2015), which is also in contrast with the results obtained in our study.

Extended-spectrum β-lactamases were noted in only one sample (4.76%) identified as *K. pneumoniae* (resistant to ampicillin and ceftriaxone), resistant to the representatives of penicillin and cephalosporin classes of antibiotics. According to a study conducted in the Netherlands, 76.8% of tested chicken meat samples contained ESBL-producing *E. coli* and 7.7% contained *Klebsiella* spp. which produces ESBL. The emergence of the ESBL gene in poultry has been associated with the use of third-generation cephalosporins (especially ceftiofura) in chickens (Overdevest et al., 2011). Studies conducted on farms in Belgium (Smet et al., 2008) and Portugal (Machado et al., 2008) also underlined poultry as a significant reservoir of ESBL genes. Results of a study from Spain showed that 67% of retail chicken meat samples possessed ESBL or ESBL-like resistance genes (Doi et al., 2010). In the United Kingdom, ESBL genes were reported in 37% of samples (Warren et al., 2008) in a study of imported raw chicken, while only one ESBL strain (4.76%) identified as *K. pneumoniae* was found in our study, whereas *E. coli* producing ESBL was not found in one sample.

Multidrug resistance (MDR), defined as resistance to at least one antibiotic from at least three classes of antibiotics, was not found. In contrast to these results, in a study from Senegal, all tested *E. coli* strains (100%) showed resistance to one or more antibiotics (Fofana et al., 2006). Cases of MDR in *K. pneumoniae*, particularly from the extended-spectrum β-lactamase group, have been reported in strains isolated from a poultry slaughterhouse in China, where 96.7% of *K. pneumoniae* isolates were resistant to more than three types of antibiotics, which is significantly higher compared to the prevalence of ESBL strains identified in our study, in which only one ESBL strain was found (4.76%) identified as *K. pneumoniae* (Wu et al., 2016). A similar trend of resistance was observed in *K. pneumoniae* strains from chicken samples in South Africa, where multidrug-resistant *K. pneumoniae* was identified in almost 40% of the total number of isolates (Fielding et al., 2012), in contrast to the results obtained in our study, where MDR was not found in any of the tested isolates.

Conclusion

Monitoring of antibiotic consumption in animals, as well as a system for monitoring antimicrobial resistance of bacteria in animals in accordance with European Union guidelines was not established in the Republic of Serbia until 2018 (Krnjaić et al., 2018). Although the Government of the Republic of Serbia has adopted a National program for the control of bacterial resistance to antibiotics for the period 2019-2021, data on this issue are still very scarce. To our knowledge, no research has been done so far that indicates the presence of resistant strains of *Escherichia coli* and *Klebsiella pneumoniae* in chicken meat in Serbia.

Considering the small number of samples included in this research, further studies are needed with a larger number of samples and other classes of antibiotics in order to gain a clearer picture on the abundance and prevalence of antibiotic-resistant enteric bacteria isolated from food of animal origin for human consumption in Serbia.

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Ispitivanje antimikrobne rezistencije sojeva *Escherichia coli* i *Klebsiella pneumoniae* izolovanih iz pilećeg mesa poreklom iz marketa i porodičnih gazdinstava

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SAŽETAK
Antimikrobna rezistencija predstavlja jednu od glavnih pretnji javnom zdravlju koja utiče na ljude, životinje i životnu sredinu. Od posebnog značaja je potencijalni prenos multirezistentnih zoonotskih patogena sa pilećeg mesa na ljude. Cilj našeg istraživanja je bio da se utvrdi prisustvo rezistentnih sojeva *Escherichia coli* i *Klebsiella pneumoniae* izolovanih iz pilećeg mesa poreklom iz marketa i porodičnih gazdinstava sa teritorije Novog Sada. Ova studija je obuhvatila 21 bakterijski soj izolovan iz 31 uzorka pilećeg mesa. Za ispitivanje antimikrobne osetljivosti bakterijskih izolata korišćen je disk difuzioni metod prema smernicama EUCAST-a. Najveća rezistencija zabeležena je prema ampicilinu (66,66%), zatim amoksicilinu sa klavulanskom kiselinom (42,86%) i sulfametoksazolu sa trimetoprimom (19,05%), dok je najmanja rezistencija zabeležena prema ceftriaksonu i gentamicinu (4,76%). Svi izolati pokazali su osetljivost na cefuroksim i ciprofloksacin. Beta-laktamaze proširenog spektra (ESBL) otkrivene su u samo jednom uzorku (4,76%) identifikovanom kao *K. pneumoniae*. Multirezistentni sojevi nisu pronađeni. S obzirom na mali broj uzoraka obuhvaćenih ovim istraživanjem, potrebna su dalja istraživanja sa većim brojem uzoraka i drugim klasama antibiotika kako bi se stekla šira slika o rasprostranjenosti i prevalenciji enteričnih bakterija rezistentnih na antibiotike izolovanih sa namirnica animalnog porekla za ishranu ljudi u Srbiji.

KLJUČNE REČI: antimikrobna rezistencija, *Escherichia*, *Klebsiella*, pileće meso

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