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Molecular serotyping of *Salmonella* strains isolated from retail chicken meats by *in silico* derived multiplex PCR, determination of ESBL, and colistin resistance genes *mcr-1* to *-5*

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ABSTRACT

Salmonella spp. are zoonotic pathogenic agents that cause important infections in humans and animals. They are the most common foodborne pathogens after *Campylobacter* spp. worldwide. The aim of this study was to determine the phenotypic and genotypic extended-spectrum β-lactamase (ESBL) and colistin resistance of 67 *Salmonella* spp. isolated from retail chicken meats between May and December 2016, and stored in the culture collection of Ataturk University, Faculty of Veterinary Medicine, Department of Microbiology. The isolates were serotyped using multiplex polymerase chain reaction (mPCR). The serovar distribution of strains was 74.6% *S*. Infantis, 16.4% *S*. Enteritidis, 3.0% *S*. Arizonae, 3.0% *S*. Dublin, 1.5% *S*. Gallinarum and 1.5% *S*. Indica. Of the 67 strains, 20 (29.9%) were ESBL-positive. The main types of β-lactamase identified were $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-8-25}}$, bla_{SHV} and bla_{TEM}. Four isolates were found to be phenotypically colistin-resistant. These isolates did not carry mobilised colistin resistance (*mcr*) genes 1 to 5. In this study, both genotypically ESBL-producing and phenotypically colistin-resistant *Salmonella* strains were found. We revealed that ESBL-producing *Salmonella* strains have dramatically increased over the years, especially when compared with previously reported chicken meat *Salmonella* strains in Turkey. The increase in *Salmonella* strains, particularly ESBL producers and the colistin resistant, is of great concern for selected antimicrobial therapy in human infections. Hence, epidemiological information and monitoring systems are extremely important in controlling *Salmonella* infections in public health services.

Key words: colistin; ESBL; Retail chicken meat; *Salmonella* spp.; serotyping

Introduction

Salmonella spp. are zoonotic pathogenic agents that cause serious infections in humans and animals (NIDAULLAH et al., 2016; SAHAN et al., 2016; BARAN et al., 2019). The global increase in the consumption of poultry products has led to the need for the production of food of higher quality and uncontaminated by foodborne pathogens. According to European Union data, a total of 91,662 cases of human *Salmonella* were reported in 2018. Salmonellosis is the second most common zoonotic

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disease after campylobacteriosis. It is also known that *Salmonella* spp. are mostly isolated from poultry and egg-containing products, which are therefore among the main sources of salmonellosis. Due to the high prevalence of *Salmonella* species in poultry, control programs are applied to poultry in Turkey, as well as in many other countries (SAHAN et al., 2016; EFSA and ECDC, 2018).

Salmonella spp. is known to include more than 2,500 serovars. *Salmonella enterica* subsp. *enterica* includes approximately 1,500 serovars, many of which can infect humans and animals. The identification of these serovars is based on the conventional detection of flagellar (H-antigens) and surface antigens (O-antigens). The Kauffmann-White scheme has long been used as the standard serotyping method. This serotyping process is expensive, labour-intensive and time-consuming, often requiring three or more days for experienced staff to produce results (LEADER et al., 2009; BORAH et al., 2017). Consequently, polymerase chain reaction (PCR)-based serotyping methods, which are easier, faster and time-saving, have recently been used by several researchers (ARRACH et al., 2008; LEVY et al., 2008; BORAH et al., 2017).

Multidrug-resistant *Salmonella* spp. have been detected after excessive use of antibiotics and feed additives in animal breeding (BASARAN KAHRAMAN et al., 2016). Some *Salmonella* serovars are more prone to developing multidrug resistance (MDR) than others, and thus infect humans via the food chain. The success rate of treatment decreases in MDR bacterial infections (VAN DUIJKEREN et al., 2003; ATA et al., 2015; KIRKAN et al., 2017). The wide spread of Gram-negative bacteria, such as extended-spectrum β-lactamase (ESBL)-producing bacteria, causes serious infections worldwide. ESBL-producing bacteria usually acquire resistance to penicillin, cephalosporin and aztreonam. Most multidrug-resistant ESBL-producing bacteria are isolated from food-producing animals (LYNCH et al., 2013). Between 2003 and 2007, *Salmonella* strains of ESBL-producing $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, bla_{TEM20}, $bla_{\text{TEM-52}}$ genes were recovered from chicken in Germany (RODRIGUEZ et al., 2009). In China, *Salmonella* strains from retail chickens harbored ESBL genes $bla_{\text{CTX-M}}$, bla_{CMY}, $bla_{\text{OXA-1}}$, bla_{TEM} between 2007 and 2012 (QIAO et al., 2018). In the USA, *S*. Infantis was found harbouring the $bla_{\text{CTX-M-65}}$ ESBL gene in retail chicken meat in 2014 (BROWN et al., 2018), and ESBL-positive *Salmonella* strains harboring the $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CMY-2}}$ genes from chicken and turkey meat were identified in Brazil between 2008 and 2015 (MOURA et al., 2018). In Turkey, whereas ARSLAN and EYI (2010) detected negative results in chicken meat *Salmonella* strains in 2010, and ATA et al., (2015) and BABACAN and KARADENIZ (2019) reported ESBL-producing *Salmonella* strains.

ESBL-producing *Salmonella* strains may be also resistant to colistin, which has increased interest in antimicrobial resistance. Plasmid-mediated mobilised colistin resistance (*mcr*) genes pose a threat to public health, as they can be transmitted through horizontal gene transfer, and have the potential to spread globally. Therefore, a gene reference that can be used to screen for plasmid-mediated colistin resistance is essential to developing effective control strategies (CARROLL et al., 2019). The aim of this study was to serotype *Salmonella* spp., previously isolated from retail chicken meats, using multiplex PCR (mPCR), and to identify ESBL-producing and colistin-resistant serovars phenotypically and genotypically.

Materials and methods

Isolates. This study used 67 *Salmonella* spp. strains that had been isolated from retail chicken meats between May and December 2016 in our previous study (BARAN et al., 2019) and stored in the culture collection of Ataturk University, Faculty of Veterinary Medicine, Department of Microbiology. The isolates, stored at -80 °C, were collected on a loop, spread on a Mueller-Hinton Agar (MHA) surface and incubated at 37 °C for 24 hours.

Multiplex PCR-based serotyping. Multiplex PCR conditions and primers for the serotyping of isolates were used as described previously (BORAH et al., 2017). In brief, to extract genomic DNA, a loopful of bacterial colonies harvested from agar plates was suspended in 0.5 ml sterile water, heated

at 95 °C for 10 min and centrifuged at 5,000 rpm for 5 min at 4 °C. Four primary master mixes (10x) containing 4µM of each primer were then prepared. Each PCR amplification was performed in a volume of 25 µL containing 18.5 µL distilled water, 2.5 µL 10x PCR buffer, 1.0 µL template DNA, 2.5 µL primer master mix and 0.5 µL Taq DNA polymerase (Qiagen Inc., Germantown, MD, USA). The PCR conditions were 94 °C for 3 min, 30 cycles of 94 °C for 15 sec, 50 °C for 30 sec, 72 °C for 25 sec, and 72 °C for 5 min. The PCR products were separated by 2% agarose gel electrophoresis. The results were recorded manually according to the expected amplicon size. *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028) was used as a positive control.

Antimicrobial susceptibility test. Detection of ESBL-producing *Salmonella* spp. was determined by the Kirby-Bauer disc diffusion method, using commercial discs (Himedia, Bombay, India) on Mueller-Hinton Agar (MHA), according to the Clinical Laboratory Standards Institute (CLSI, 2017). The antimicrobial discs contained aztreonam (30 µg), ceftriaxone (30 µg), cefepime (30 µg), cefpodoxime (10 µg), cefoxitin (30 µg), ceftazidime (30 µg) and cefotaxime (30 µg). The confirmatory test was determined by the double-disc synergy test using cefotaxime (30 µg) and ceftazidime (30 µg), and in a combination of ceftazidime-clavulanic acid (30/10 µg) and cefotaxime-clavulanic acid (30/10 µg), as recommended by the CLSI (2017). After 16-18 hours of incubation at 35 °C, a ≥5 mm increase in the zone diameter of the ceftazidime-clavulanic acid disc and the ceftazidime disc alone, and/or a ≥5 mm increase in the zone diameter of the cefotaxime-clavulanic acid disc and the cefotaxime disc alone were considered as ESBL-producing *Salmonella* strains.

Detection of colistin resistance. The evaluation of the isolates' phenotypic antimicrobial resistance to colistin was performed using the broth microdilution method (EUCAST, 2017). Cation-adjusted Mueller-Hinton broth (Oxoid, Hampshire, UK) was used in order to determine the minimum inhibitory concentration (MIC). Antibiotics diluted in appropriate concentrations (128-0.125 µg/mL) were distributed to 50 µL microplates. A bacterial suspension was prepared at a density of 0.5 McFarland with a 24 h bacterial culture in tryptic soy broth (TSB), diluted to 1:100 with TSB and distributed to all wells in volumes of 50 µL. The total amount of liquid in each well was 100 µl. The last well containing the suspension of media and bacteria was used as a negative control. The microplate was sealed and allowed to incubate for 24 h at 37 °C. At the end of the incubation, the lowest antimicrobial concentration without bacterial growth was recorded as the MIC value. Isolates were resistant to colistin at >2 µg/mL (ADIGUZEL et al., 2018; EUCAST, 2019).

Genotypic characterization. Genomic DNA extraction was performed, as previously mentioned, in the mPCR-based serotyping section. The PCR amplifications were performed in a total volume of 15 µL solution containing 2 µl template DNA, 3 µl 1×PCR buffer, 0.25 mM $MgCl_2$, 200 µM dNTP (each), 10 pmol of each primer and 1.25 U of Taq polymerase (Vivantis Technologies, Selangor Darul Ehsan, Malaysia). To screen for the presence of ESBLs in *Salmonella* spp. isolates, multiplex PCR analysis was performed for simultaneous detection using bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8\text{-}25}$, and $bla_{CTX\text{-}M\text{-}9}$ group primers, as previously reported by LE et al., (2015).

Mobilised colistin resistance (*mcr*) genes 1 to 5 were investigated by mPCR. Each PCR amplification was performed in a volume of 25 µl containing 12.5 µl of DreamTaq PCR master mix (Thermo Scientific, Grand Island, NY, USA), 5.5 µl distilled water, 2.0 µl template DNA, 0.5 µl of each primer (10 pmol) and 2 µl target DNA, following the cycling conditions described previously (REBELO et al., 2018; BOROWIAK et al., 2017; XAVIER et al., 2016).

Statistical analysis. The statistical analysis was performed using SPSS statistics 20 (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA). To compare between phenotypical colistin resistance and ESBL resistance genes of the individual *Salmonella* serovars, Pearson probability value (P value) was calculated using the Chi-square test. A P-value less than 0.05 was considered statistically significant.

Results

To determine the molecular serotyping of *Salmonella* strains by *in silico* derived amplification, strains were searched by mPCR. The mPCR results showed that 67 strains were determined to be: 74.6% *S.* Infantis (n = 50), 16.4% *S.* Enteritidis (n = 11), 3.0% *S.* Arizonae (n = 2), 3.0% *S.* Dublin (n = 2), 1.5% *S.* Gallinarum (n = 1) and 1.5% *S.* Indica (n = 1).

All phenotypically resistant isolates were screened for ESBL resistance phenotypically and genotypically. One *S.* Indica, one *S.* Arizonae, three *S.* Enteritidis, and 38 *S.* Infantis serovars were negative for phenotypic β-lactam and colistin resistance. Of the 67 *Salmonella* strains, 20 (29.9%) were identified as ESBL-producing (Table 1). β-lactam resistance coding $bla_{\text{CTX-M-8-25}}$ (n = 19) and bla_{TEM} (n = 18) were the most predominant β-lactam genes (Fig. 1). In addition, other β-lactamase genes $bla_{\text{CTX-M-1}}$ and bla_{SHV} were detected in one and 12 strains, respectively. Groups $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-9}}$ were not detected in ESBL-producing *Salmonella* strains.

The MIC of colistin for 67 *Salmonella* strains are shown in Table 1. The MIC values ranged from 0.5 - 16 µg/mL. Four *Salmonella* strains (6%), that is, *S.* Infantis, *S.* Arizona, *S.* Gallinarum, and *S.* Enteritidis, had phenotypical colistin resistance (>2 µg/mL). PCR screening for *mcr-1* to *-5* genes in the four colistin resistant strains showed that none of them harbored *mcr-1* to *-5* genes. Furthermore, phenotypic colistin-resistant serovars were found to be ESBL-negative. The incidences of phenotypic colistin resistance and ESBL resistance genes was significantly different in *Salmonella* serovars by the Pearson Chi-square test (Table 1).

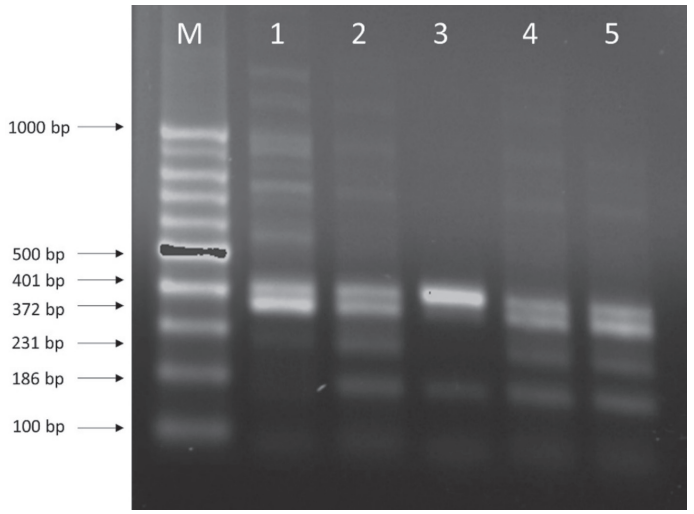

Fig. 1. The result of multiplex polymerase chain reaction of extended-spectrum beta-lactamase producing positive *Salmonella* strains. M - Marker, 1-5 - *Salmonella* spp. strains, 186 bp; $bla_{\text{CTX-M-8-25}}$, 231 bp; bla_{SHV}, 372 bp; bla_{TEM}, 401 bp; 16sRNA.

Table 1. Distribution of *Salmonella* serovar carrying ESBL-produced genes and phenotypical colistin resistance

Salmonella Serovars	Colistin resistance range (µg/mL)					ESBL-producing genes	*mcr* genes	P value
	0.5	1	2	4	16			
S. Arizonae (n = 2)	1	-	-	1	-	-	-	-*
S. Dublin (n = 1)	1	-	-	-	-	*CTX-M-8/25, SHV, TEM*	-	-*
S. Dublin (n = 1)	1	-	-	-	-	*CTX-M-8/25, TEM*	-	

*The Chi-square test was not performed because of expected cell less than one

Table 1. Distribution of *Salmonella* serovar carrying ESBL-produced genes and phenotypical colistin resistance (continued)

Salmonella Serovars	Colistin resistance range (µg/mL)					ESBL-producing genes	*mcr* genes	P value
	0.5	1	2	4	16			
S. Enteritidis (n = 5)	4	1	-	-	-	*CTX-M-8-25, SHV, TEM*	-	0.026691
S. Enteritidis (n = 2)	2	-	-	-	-	*CTX-M-8-25, TEM*	-	
S. Enteritidis (n = 4)	2	-	1	1	-	-	-	
S. Gallinarum (n = 1)	-	-	-	-	1	-	-	-*
S. Indica (n = 1)	1	-	-	-	-	-	-	-*
S. Infantis (n = 8)	8	-	-	-	-	*CTX-M-8-25, SHV, TEM*	-	< 0.00001
S. Infantis (n = 2)	2	-	-	-	-	*CTX-M-8-25, SHV*	-	
S. Infantis (n = 1)	1	-	-	-	-	*CTX-M-1, TEM*	-	
S. Infantis (n = 39)	36	1	1	1	-	-	-	
TOTAL (n = 67)								

*The Chi-square test was not performed because of expected cell less than one

Discussion

Poultry asymptomatically carries *Salmonella* spp. in the gastrointestinal tract. Cross-contamination commonly occurs during slaughtering. Once contamination occurs in the slaughtering line, its adverse effects are spread throughout the entire process. The absence of separate chilling conditions after the slaughter process promotes the growth of *Salmonella* spp. in chicken meat. Inadequate hygiene rules in slaughterhouses and careless workers are additional factors that facilitate the spread of *Salmonella* strains. The antimicrobial resistance of *Salmonella* serovars in retail chicken meat in Turkey is vitally important because of the potential risk of infections in humans. Therefore, continuous monitoring of antimicrobial-resistant strains is necessary.

Advances in genomic sequence analysis have led to the emergence of alternative methods, such as PCR, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS), for the identification of *Salmonella* serovars. One of these methods is the in silico derived multiplex PCR amplification method, which allows typing by comparing multiplex PCR-based genome typing (MPGT) codes obtained from different mPCR combinations with predetermined serovars (BORAH et al., 2017). In this study, *Salmonella* serotypes were determined using quadruplex mPCR according to previously confirmed MPGT codes.

Salmonella Typhimurium and *Salmonella* Enteritidis have previously been reported as the most commonly isolated serovars from chicken meat (WAJID et al., 2019a; YANG et al., 2010). This study found that *S.* Infantis and *S.* Enteritidis serovars were the most common. Other studies have found *S.* Saintpaul, *S.* Brancaster, *S.* Albany, and *S.* Stanley in Singapore (ZWE et al., 2018), *S.* Corvallis and *S.* Albany in Vietnam (NGUYEN et al., 2016), *S.* Enteritidis in China and Egypt (ABD-ELGHANY et al., 2015; LI et al., 2017), and *S.* Infantis and *S.* Enteritidis in Turkey (SAHAN et al., 2016). The distribution of serovars obviously varies depending on geographical region.

The widespread, excessive and long-term use of antimicrobial agents can lead to resistance in many bacteria, including *Salmonella*. In addition, the increase in the prevalence of multidrug-resistant *Salmonella* strains has become a worrying phenomenon both in Turkey and globally (ABBASOGLU and AKCELIK, 2011;

AVSAROGLU et al., 2007; NÓGRÁDY et al., 2012). Beta-lactam antibiotics are especially preferred for the treatment of invasive *Salmonella* infections. In contrast to our findings, low levels of ESBL-producing *Salmonella* strains have previously been reported in Turkey (ATA et al., 2015; BABACAN and KARADENIZ, 2019). This study shows that ESBL-producing resistance genes are carried horizontally and have increased sharply over the years.

Many countries have experienced an increase in *S*. Infantis isolates in poultry and cases of human salmonellosis (ABBASOGLU and AKCELIK, 2011; NÓGRÁDY et al., 2012; WAJID et al., 2019b), mainly sourced from chicken farms and chicken meat (EFSA and ECDC, 2018). NÓGRÁDY et al., (2012) revealed the genetically close association of *S*. Infantis isolated from human and broilers. ESBL-producing genes such as bla$_{CTX\text{-}M\text{-}1}$, bla$_{CTX\text{-}M\text{-}8\text{-}25}$, bla$_{TEM}$, and bla$_{SHV}$, which are important for human infections, were found in this study. In contrast, *bla*$_{\text{CTX-M-2}}$ and *bla*$_{\text{CTX-M-9}}$ were not detected in any isolate in this study. Among ESBL-producing genes, previous studies found that the most prevalent were *bla*$_{\text{SHV}}$ (7.4%) and *bla*$_{\text{TEM}}$ (1.8%) (WAJID et al., 2019b), *bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{CTX-M-15}}$ (11.3%), *bla*$_{\text{SHV-12}}$ (7.5%) and *bla*$_{\text{OXA-1}}$ (3.7%) (AHMED et al., 2014). Another study reported that *bla*$_{\text{TEM}}$ (54.17%), *bla*$_{\text{SHV}}$ (35.42%), *bla*$_{\text{CTX-M-1}}$ (27.08%), *bla*$_{\text{CTX-M-2}}$ (47.92%) and *bla*$_{\text{CTX-M-9}}$ (62.5%) genes were positive, while *bla*$_{\text{CTX-M-8-25}}$ was negative (REN et al., 2017).

Plasmid-mediated colistin resistance was detected in various *Salmonella enterica* serovars in previous studies (LIMA et al., 2019). To date, nine different *mcr* gene homologues have been identified (CARROLL et al., 2019). In this study, only *mcr*-1 to 5 genes were investigated. *Salmonella* Typhimurium was found to be the most common serovar bearing *mcr* genes (LIMA et al., 2019). CARFORA et al., (2018) reported that four *S*. Infantis serovars carried the *mcr-1.1* gene variant. In this study, none of the four isolates with phenotypic colistin resistance were found to carry *mcr* genes. These isolates were thought to be one of the other *mcr* gene variants not investigated in this study.

Conclusion

It should be remembered that the sources of salmonellosis in the food chain and the ways of transmission are inseparably linked to the risks of infection in humans. In this study, the detected high frequency of the most important zoonotic *Salmonella* serovar, Infantis, is of public health significance. We revealed that the presence of ESBL-producing *Salmonella* strains has dramatically increased during the years, especially when compared with chicken meat *Salmonella* strains previously reported in Turkey. The increase in *Salmonella* strains, particularly ESBL producers and the colistin resistant, is of great concerns for selected antimicrobial therapy in human infections. Hence, epidemiological information and monitoring systems are extremely important in controlling *Salmonella* infections in public health services.

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SAŽETAK

Salmonella spp. zoonotski su patogeni koji uzrokuju infekcije u ljudi i životinja. Na globalnoj su razini najčešći patogeni koji se prenose hranom poslije bakterija *Campylobacter* spp. Cilj ovog istraživanja bio je fenotipski i genotipski odrediti β-laktamaze proširenog spektra (ESBL) i otpornost na kolistin 67 sojeva bakterija *Salmonella* spp. izoliranih iz pilećeg mesa za prodaju od svibnja do prosinca 2016. i pohranjenih u kulturama na Odsjeku za mikrobiologiju Fakulteta veterinarske medicine na Sveučilištu Ataturk. Izolati su serotipizirani primjenom višestruke lančane reakcije polimerazom (mPCR). Distribucija serovara bila je: 74,6 % za *S*. Infantis, 16,4 % za *S*. Enteritidis, 3,0 % za *S*. Arizonae, 3,0 % za *S*. Dublin, 1,5 % za *S*. Gallinarum i 1,5 % za *S*. Indica. Od 67 sojeva njih je 20 (29,9 %) bilo pozitivno na ESBL. Glavni identificirani tipovi β-laktamaza bili su: $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-8-25}}$, bla_{SHV} i bla_{TEM}. Četiri izolata bila su fenotipski otporna na kolistin. Ovi izolati nisu sadržavali aktivne gene rezistencije na kolistin (mcr) 1 do 5. U ovom su istraživanju nađeni i sojevi salmonele koji su genotipski proizvodili ESBL i sojevi fenotipski otporni na kolistin. Sojevi salmonele koji proizvode ESBL s godinama su se znatno povećali, posebno u usporedbi s prethodnim izvješćima o sojevima salmonele u mesu pilića u Turskoj. Porast sojeva salmonele, osobito onih koji proizvode ESBL i onih koji su otporni na kolistin, zabrinjava zbog antimikrobnog liječenja infekcija u ljudi. Zbog toga su u kontroli infekcija salmonelama u javnome zdravstvu iznimno važni epidemiološki podaci i sustavi praćenja.

Ključne riječi: kolistin; ESBL; pileće meso za prodaju; *Salmonella* spp.; serotipizacija

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