Original Article

Antibiotic susceptibility and prevalence of foodborne pathogens in poultry meat in Romania

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Abstract

Introduction: The occurrence of pathogenic strains in poultry meat is of growing concern in Romania. Another problem found on a global level is the continuous increase of antimicrobial resistance in bacteria isolated from food. This study aimed to evaluate the prevalence of pathogenic bacteria in poultry carcasses obtained in Romania in 2012–2013 and to reveal the most prevalent patterns of antimicrobial resistance in the isolated strains.

Methodology: A total of 144 broiler chicken carcasses were evaluated according to classical microbiological methods. The DNA was extracted from the bacterial colonies and the resistance genes were identified by PCR.

Results: In 2012, 47.2% of the samples revealed at least one of the following bacteria: *Campylobacter jejuni* (9.72%; n = 7), *Salmonella enterica* serotype Enteritidis (4.17%; n = 3), *Listeria monocytogenes* (15.28%; n = 11), and *Escherichia coli* (16.67%; n = 12). In 2013, the number of positive samples of pathogenic bacteria decreased, although *Campylobacter jejuni* was isolated in a higher percentage (20.8% *vs*. 9.72%). The percentage of multidrug-resistant (MDR) bacteria was high (23%); the most prevalent pattern included resistance to tetracycline, sulfonamides, and quinolones/fluoroquinolones. All the resistant *Salmonella* and *E. coli* strains were tested for the presence of characteristic resistance genes (*Kn, bla*$_{TEM}$*, tetA, tetB, tetG, DfrIa, aadA1a, Sul*) and revealed that these isolates represent an important reservoir in the spread of this phenomenon.

Conclusions: Our findings suggest that Romania urgently needs an integrated surveillance system within the entire chain, for drug-resistant pathogens isolated from poultry meat.

Key words: poultry; meat; pathogen; gene; resistance.

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Introduction

Poultry meat is a good source of animal protein, appealing to consumers very easily due to its sensorial attributes. In terms of safety, poultry meat ranks first or second in food associated with disease in Australia, Canada, and England, while in the United States it is considered a prevalent food vehicle of reported foodborne disease outbreaks [1]. Contamination can occur at several points throughout the processing operation, the most incriminating factors being the temperature abuse and improper handling or preparation [2]. Processed raw poultry meat naturally harbors bacteria, but most of these bacteria are responsible for the spoilage of poultry meat, and thus are non-pathogenic to humans [3]. However, many studies have shown that poultry products can harbor bacteria capable of causing human diseases [4].

Each country and region has its own unique food safety problems related to culture, climate, and economic status, but some bacteria are common to all poultry meat produced, regardless of the area. The most frequent outbreaks associated with consumption of contaminated poultry are caused by *Salmonella* spp., *Staphylococcus aureus*, and occasionally by *Bacillus cereus* and psychotropic pathogens such as *Aeromonas hydrophila*, *Listeria monocytogenes*, and *Yersinia enterocolitica* [4]. *Campylobacter* spp. is not usually connected with outbreaks, due to erroneous diagnosis or the difficulties in detection and isolation of the pathogen [5]. In light of their importance, the Food and Agriculture Organization of the United

Nations (FAO) and the World Health Organization (WHO) have established risk assessments on *Salmonella* and *Campylobacter* in broiler chickens [6-8].

Another public health concern is associated with the increased incidence of antibiotic-resistant strains isolated from poultry meat [9].

Due to the widespread use of antimicrobials in chicken growth units, the development of resistant strains that can infect humans via the food chain has increased [10]. Although in Romania, data regarding foodborne diseases associated with resistant pathogenic strains in poultry is lacking, it is considered to be significant.

The aim of this research was to assess to what extent the most popular foodborne pathogens are prevalent in poultry meat produced in Romania. The study of the antibiotic-resistant properties of these pathogens was another goal of this work.

Methodology

Collection, isolation, and identification of bacteria

A total of 144 broiler chicken carcasses were collected from two slaughtering units in Transylvania between 2012 and 2013. Each month, six samples (n = 72/year) were evaluated. The isolation of the pathogenic microorganisms present on the surfaces of the carcasses was made based on the standardized methods in conformity with the regulation (EC) 1441/2007 [11]: the identification of *Salmonella* spp. (SR EN ISO 6579/2003 AC/2006) [12], the identification of *Campylobacter jejuni/coli* (SR EN ISO 10272/2006) [13], the identification of *Listeria monocytogenes* (SR EN ISO 11290/2000 A1/2005) [14], the identification of *Echerichia coli* (SR ISO 16649/2007) [15], and the identification of *Yersinia enterocolitica* (SR EN ISO 10273/2003) [16]. Briefly, for the isolation of *Salmonella* spp., XLD (Oxoid, Frankfurt, Germany) and Rambach (LabM, England) media were used; the inoculated plates were incubated afterwards at 37°C. *Campylobacter jejuni/coli* were isolated on Columbia media (Oxoid) supplemented with blood, in complete anaerobic conditions and incubated at 41°C. *Listeria monocytogenes* was detected using the ALOA and Oxford media (LabM) and incubated at 37°C. For *E. coli* isolation, TBX media (Oxoid) was used, incubated at 44°C. For *Yersinia enterocolitica*, CIN media (Oxoid) was used, followed by incubation at 32°C.

Determination of minimum inhibitory concentrations (MICs)

The antimicrobial susceptibility testing was performed by agar dilution method on Muller-Hinton agar (Oxoid). All pathogenic strains were tested for sensitivity to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphmethoxazole (Su), oxytetracycline (T), kanamycin (Km), and ofloxacin (O). The resistance breakpoints were those established by the Clinical and Laboratory Standards Institute (CLSI) [17]. The interpretation was performed according to the following MIC ranges: 0.5–128 g/mL for ampicillin; 0.5–256 g/mL for chloramphenicol; 1–512 g/mL for streptomycin; 16–512 g/mL for sulphamethoxazole; 1–512 g/mL for oxytetracycline; 0.5–512 g/mL for kanamycin; and 0.25–64 g/mL for ofloxacin.

For *Campylobacter jejuni*, the MICs of tetracycline, ciprofloxacin, nalidixic acid, erythromycin, chloramphenicol, gentamicin, and amoxicillin-clavulanic acid were determined by the E-test method (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. The breakpoints were those recommended by CLSI for *C. jejuni/C. coli* [17], except for nalidixic acid, chloramphenicol, gentamicin, and amoxicillin-clavulanic acid, for which the breakpoints for *Enterobacteriaceae* were used [17].

Bacterial DNA preparation

The bacterial DNA extraction followed the basic steps previously described by Yang *et al*. [18] with a few particularities. Briefly, 150 µL of CHELEX (10%) reactive (Sigma-Aldrich, Steinheim, Germany) was added in Eppendorff tubes (1.5 mL) (RatioLab, Dreieich, Germany). The tubes were subjected to UV sterilization in a microbiological laminal flow class II to remove any possible contaminants from the manipulation performed earlier. One to two colonies were harvested with a sterile microbiological loop and immersed in the CHELEX reactive. The following extraction temperatures were used: 57°C for 30'; 94°C for 5'. The last step included a high-speed centrifugation (14,000 rot/min.) for 1 minute.

Detection of resistance genes

Detection of antimicrobial resistance genes was performed by polymerase chain reaction (PCR).

The PCR reaction mix (25 µL) comprised 1X PCR green buffer, 2.5 mM MgCl2, 5 pmol of each primer, dNTPs each at 200 µM, 2.5 U of Taq DNA polymerase (Promega, Madison, USA), and 100 ng of

genomic DNA. PCR was performed under the following conditions: 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 50°C–68°C for 30 seconds, 72°C for 1 minute, and a final extension step of 72°C for 7 minutes. The PCR primers used are listed in Table 1.

Statistical methods

OriginPro (software version 8.5, Origin Lab Institute, NorthHampton, USA) was used for the analysis of variance (ANOVA) one-way and the least significant difference test. These tests were performed to evaluate the results obtained in the total bacterial load and to evaluate the prevalence of resistance genes. Tukey post-hoc means comparison and Levene's test for equal variance were also included. Differences were considered significant at a pvalue lower than 0.05.

Results

Bacterial isolation and prevalence of pathogens

Following the analyses made during the year 2012, 54.2% (n = 39) of the total samples (n = 72) did not reveal the presence of pathogens (Figure 1), while 45.8% (n = 33) revealed at least one of the following bacteria: *Campylobacter jejuni* (9.72%; n = 7), *Salmonella* Enteritidis (4.17%; n = 3), *Listeria monocytogenes* (15.28%; n = 11), and *E. coli* (16.67%; n = 12) (Figure 1).

In 2013, the prevalence of pathogens decreased (43.05%; n = 31), with no significant differences compared to the year 2012 ($p > 0.05$) (Figure 2).

Yersinia enterocolitica bacteria was not found in the samples analyzed during 2012–2013.

Characterization of resistance genes and antimicrobial susceptibility profiles

All of the pathogenic isolates showed markedly high resistance rates to the antibiotics tested. The isolates that showed resistance to more than three antibiotics were classified as multidrug resistant (MDR).

In the case of *Salmonella* Enteritidis isolates (n = 5), only one showed MDR, the pattern revealing resistance to tetracycline, nalidixic acid, and cefotaxime.

Table 1. Polymerase chain reaction and primer conditions

Bacteria	Gene or region	Primer sequence	Reference	Size (bp)	T_a (°C)
Salmonella spp.	*aadA1a*	F-GTGGATGGCGGCCTGAAGCC R-AATGCCCAGTCGGCAGCG	Guerra *et al*. (2001) [32]	526	68
	Kn	F-ACTGGCTGCTATTGGCGA R-CGTCAAGAAGGCGATAGAAGG	Frana *et al*. (2001) [33]	525	55
	bla_{TEM}	F-GCACGAGTGGGTTACATCGA R-GGTCCTCCGATCGTTGTCAG	Carlson *et al*. (1999) [34]	310	68
	tetA	F-GCTACATCCTGCTTGCCT R-CATAGATCGCCGTGAAGA	Carlson *et al*. (1999) [34]	210	52
	tetB	F-TTGGTTAGGGGCAAGTTTTG R-GTAATGGGCCAATAACACCG	Carlson *et al*. (1999) [34]	659	52
	tetG	F-GCTCGGTGGTATCTCTGCTC R-AGCAACAGAATCGGGAACAC	Carlson *et al*. (1999) [34]	468	59
E. coli	*aadA1a*	F-GCAGCGCAATGACATTCTTG R-ATCCTTCGGCGCGATTTTG	Saenz *et al*. (2004) [35]	680	60
	bla_{TEM}	F-ATTCTTGAAGACGAAAGGGC R-ACGCTCAGTGGAACGAAAAC	Belaaouaj *et al*. (1994) [36]	1150	60
	TetA	F-GTAATTCTGAGCACTGTCGC R-CTGCCTGGACAACATTGCTT	Guardabassi *et al*. (2000) [37]	600	50
	TetB	F-CTCAGTATTCCAAGCCTTTG R-CTAAGCACTTGTCTCCTGTT	Guardabassi *et al*. (2000) [37]	416	57
	DfrIa	F-GTGAAACTATCACTAATGG	Navia *et al*. (2003) [38]	474	55
	Sul	F-TGGTGACGGTGTTCGGCATTC R-GCGAGGGTTTCCGAGAAGGTG	Saenz *et al*. (2004) [35]	789	63
	Sul2	F-CGGCATCGTCAACATAACC R-GTGTGCGGATGAAGTCAG	Saenz *et al*. (2004) [35]	722	50
	Sul3	F-CATTCTAGAAACAGTCGTAGTTCG R-CATCTGCACGTAACCTAGGGCTTTGGA	Saenz *et al*. (2004) [35]	990	51

F: forward; R: reverse; T_a: annealing temperature

Figure 1. Prevalence of pathogenic bacteria identified in poultry carcasses in 2012 (n = 72)

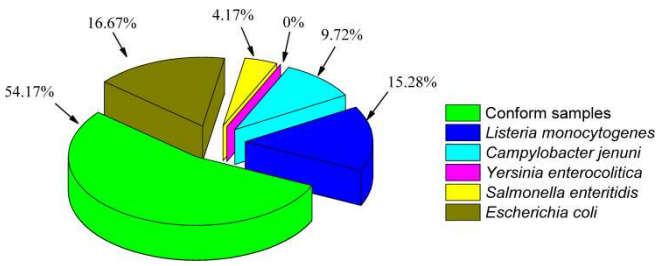

Figure 2. Prevalence of pathogenic bacteria identified in poultry carcasses in 2013 (n = 72)

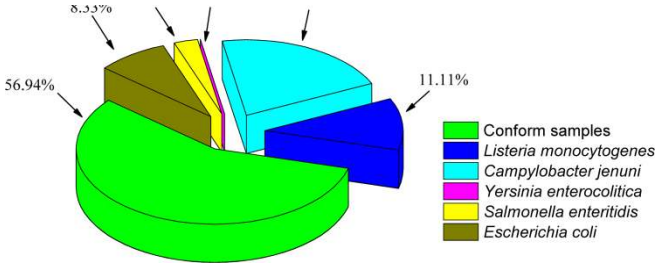

Table 2. Antimicrobial resistance of pathogenic isolates from chicken carcasses

Antibiotic	***Salmonella enteritidis* (n = 5)**	***E. coli* (n = 18)**	***Campylobacter jejuni* (n = 22)**	***Listeria monocytogenes* (n = 19)**
β-lactams	2 (40%)	5 (27.7%)	1 (4.54%)	2 (10.52%)
Ampicillin	0	5 (27.7%)	1 (4.54%)	2 (10.52%)
Cefotaxime	1 (20%)	0	0	0
Ceftazidime	1 (20%)	0	0	0
Aminoglycosides	1 (20%)	2 (11.11%)	0	0
Kanamycin	0	0	0	0
Streptomycin	1 (20%)	2 (11.11%)	0	0
Sulfonamides	1 (20%)	4 (22.22%)	3 (13.63%)	1 (5.26%)
Sulfamethoxazole	1 (20%)	4 (22.22%)	3 (13.63%)	1 (5.26%)
Sulfamethoxazole/trimethoprim	1 (20%)	4 (22.22%)	3 (13.63%)	1 (5.26%)
Quinolones and fluoroquinolones	2	10 (55.5%)	2 (9.09%)	4 (21.05%)
Nalidixic acid	2 (40%)	8 (44.4%)	2 (9.09%)	2 (10.52%)
Ciprofloxacin	0	6 (33.33%)	2 (9.09%)	2 (10.52%)
Tetracycline	2 (40%)	12 (66.66%)	7 (31.81%)	6 (31.57%)
Chloramphenicol	0	4 (22.22%)	2 (9.09%)	4 (21.05%)

The penicillin resistance of the three *Salmonella* isolates was suspected given the fact the bla_{TEM} gene was detected in these isolates. This gene is normally incriminated in the resistance to ampicillin and ticarcillin and also to cephalosporins such as cefalothin and cefazolin. Surprisingly, all of the *Salmonella* isolates detected were susceptible to ampicillin, and only two isolates showed resistance to cefotaxime and ceftazidime (Table 2). The streptomycin-resistant isolate carried the *aadA1* gene and tested positive also for sulphmethoxazole. Both *Salmonella* isolates that were resistant to tetracycline (40%) had amplification reaction with *tetA* primers, while there were no amplification products detected when *tetB* and *tetG* primers were employed. No isolates tested carried the *Kn* gene, which normally confers resistance to kanamycin.

Multiple resistances to antibiotics in *E. coli* isolated from poultry carcasses were also found. Overall, resistance was most frequently observed to tetracycline (66.66%), quinolones and fluoroquinolones (55.5%), ampicillin (27.7%), sulfmethoxazole (22.22%), trimethoprim/sulfmethoxazole (22.22%), and chloramphenicol (22.22%). Regarding the presence of resistance genes, all of the *E. coli* isolates tested positive to at least one. The isolates resistant to tetracycline tested positive to *tetA* gene (66.6%), and five of them (27.7%) also contained the beta-lactamase bla_{TEM} gene. Genes that are responsible for resistance to sulfonamides (*Sul, Sul1*) and trimethoprim (*DfrIa*) were also detected (22.22%). It is important to mention that 52% of the *E. coli* isolates were MDR. The most common resistance profile of MDR isolates was tetracycline, sulfonamide, and trimethoprim.

A large percentage (60%) of the *Campylobacter jejuni* isolates (n = 22) proved to be susceptible to antibiotics. Nine of the 22 isolated strains showed MDR, the most prevalent resistance pattern being tetracycline, sulphmethoxazole, and sulfmethoxazole/trimethoprim. All the isolates that were resistant to sulfonamides were also resistant to tetracycline. Nalidixic acid and ciprofloxacin resistance was detected in 9% of the samples associated with tetracycline resistance. Only one sample was resistant to ampicillin (4.54%) and found in association with tetracycline and ciprofloxacin resistance.

Listeria monocytogenes isolates (n = 19) were found to be susceptible especially to aminoglycosides. The most prevalent resistance pattern was found for tetracycline and chloramphenicol.

Discussion

The increased level of pathogenic strains isolated from poultry meat obtained in Romania is a great reason for concern. Another problem identified in our study is the high number of bacteria resistant to antibiotics, which shows that preventive medications are still given to broilers in order to reduce mortality. The recognition of the dangers of antibiotic resistance prompted the ban on sub-therapeutic antibiotic usage in Europe [20]. The application of antibiotics not only increases the resistance in pathogenic strains but also leads to resistance in the endogenous flora of humans and animals [21]. Following the consumption of poultry meat, MDR bacterial strains may spread to the human population, which will lead to the transfer of genes coding for resistance [22]. The dissemination pathways of bacterial resistance from animals to humans were described earlier by Hummel *et al*. [23]. Levey *et al.* [24] also confirmed that in chickens fed tetracycline, the transfer rate of tetracycline resistance genes between *Escherichia coli* strains from chicken to chicken and from chicken to human was higher.

In the case of *Salmonella* Enteritidis, the percentage of positive samples was lower, but not in a significant way ($p > 0.05$). A significant decrease in prevalence was noticed at *Listeria* spp. bacteria (15.3% *vs*. 11.1%). A concerning fact was the consistent increase of *Campylobacter jejuni* (20.8% *vs*. 9.72) prevalence ($p < 0.05$). The results obtained were similar to those reported by the European Food Safety Authority (EFSA) in 2007–2011 [19].

E. coli, *Campylobacter* spp., *Salmonella* spp., and *Listeria* spp. are frequent contaminants of food of animal origin, having been recovered in a total of 32% (n = 46) samples in this study; most of the isolates had a multi-resistant phenotype. The presence of genes in *Salmonella* Enteritidis and *E. coli* isolates that confer resistance to some antimicrobial agents (sulfonamides, ampicillin, tetracycline) were especially high (34% to 54%), indicating that these isolates originating from poultry meat could be a serious reservoir of antimicrobial resistance.

This report also shows that 23% of the strains were resistant to at least three antimicrobial agents tested, suggesting that preventive antibiotics were given to broilers. These results are in agreement with recent investigations that showed a high prevalence of multidrug-resistant bacteria in poultry carcasses [25-30]. To our knowledge, this is the first detailed report concerning the prevalence of pathogenic bacteria and the characterization of various resistance genes in Romania.

The significant usage of quinolones, enrofloxacin, and tetracycline in poultry production in our country has lead, as shown in our study, to the emergence of quinolone-resistant and tetracycline-resistant *Campylobacter jejuni*, *E. coli*, *Salmonella* Enteritidis, and *Listeria monocytogenes*. It is therefore important to examine tetracycline and also ampicillin resistance markers, as they are associated with transposable, multidrug-resistant elements [31]. Penicillins and trimethoprim-sulfamethoxazole are antimicrobial agents commonly used, which explains the presence of the incriminated resistance genes in the isolated pathogenic strains. The high resistance to sulfmethoxazole in *E. coli* and *Campylobacter jejuni* isolates was not surprising, since sulfmethoxazole (in combination with trimethoprim) is widely used in Romania, both in human and veterinary medicine.

Conclusions

This study indicates that in Romania, there is an increasing emergence of antibiotic resistance among pathogenic bacterial strains in poultry. This fact is supported by the detection of various resistance genes in *E. coli* and *Salmonella* Enteritidis isolates. In order to diminish contamination rates by resistant pathogenic bacteria in poultry meat, it is critical that risk reduction strategies are used throughout the food chain. These findings call for integrated efforts to promote the appropriate use of antimicrobials in food animals and for the implementation of a surveillance system of drug resistance in bacterial pathogens in Romania.

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