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RESEARCH ARTICLE

Presence and antibiotic resistance of *Salmonella* spp. isolated from chicken meat and giblets consumed in Konya, Turkey

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Konya'da tüketilen tavuk eti ve iç organlarında *Salmonella* spp. varlığı ve antibiyotik direnci

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Öz

Amaç: Bu çalışmada tüketime sunulan tavuk eti ve sakatatlarda *Salmonella* spp. ve iki önemli *Salmonella* serotipinin (S. Thyphimurium ve S. Enteritidis) varlığı ve izolatların antimikrobiyal direncinin belirlenmesi amaçlandı.

Gereç ve Yöntem: Araştırmada Konya ilindeki süpermarket ve kasaplarda tüketime sunulan tavukların karaciğer (n = 40), taşlık (n = 40), kalp (n = 30), deri (n = 30), baget (n = 10) ve kanat (n = 20) örnekleri klasik kültür tekniği ile analiz edildi. Şüpheli *Salmonella* spp. izolatlarının moleküler düzeyde doğrulanması amacıyla gerçekleştirilen PCR uygulamasında Inv-A gen bölgesine ait primerler kullanıldı. S. Thyphimurium ve S. Enteritidis'e ait gen bölgelerinin tespitinde ise *Flic-C* ve *IE-1* primerleri ile dupleks PCR (d-PCR) uygulandı.

Bulgular: Toplam 170 örneğin 43'ü (% 25.29) *Salmonella* spp. pozitif olarak tespit edildi. d-PCR sonuçlarına göre izolatların hiçbirinde S. Thyphimurium ya da S. Enteritidis saptanmadı. İzolatlarda klindamisin, oksasilin, teikoplanin (% 100), vankomisin (% 79.1), eritromisin (% 79.1), nalidiksik asit (% 65.1), penisilin G (% 60.5) sefalotin (% 48,8), sülfametoksazol-trimetoprim (% 37.2), tetrasiklin (% 37.2), ampisilin (% 23.3), kanamisin (% 18.6), kloramfenikol (% 11.6) amikasin, sefazolin, siprofloksasin ve gentamisine (% 4.7) direnç saptandı. Tüm izolatlar amoksisilin / klavulanik asit ve sefixime duyarlı bulundu.

Öneri: Araştırmada S. Enteritidis ve S. Typhimurium'un tespit edilmemiş olması halk sağlığı açısından olumlu kabul edilmiştir. Bunun yanısıra, yasal mevzuat açısından daha düşük insidense sahip patojen türlerin tespit edilmesine yönelik çalışmalara dikkat çekmenin önemli olduğu düşünülmektedir. Ayrıca sık rastlanan patojen türlerin tespit edilmemesine rağmen, tür düzeyinde tespit edilen izolatlardaki antibiyotik direnç sonuçları antibiyotik sörvelans veritabanı için önemli bulunmuştur.

Anahtar kelimeler: Antibiyotik direnç; tavuk; S. Enteritidis; S. Typhimurium; *Salmonella* spp.

Abstract

Aim: The present study was on the detection of *Salmonella* spp. and two important *Salmonella* serotypes (S. Thyphimurium and S. Enteritidis) in chicken meat and giblets and also determination of antimicrobial resistance of the isolates.

Materials and Methods: In this study, livers (n=40), gizzards (n=40), hearts (n=30), skins (n=30), drumsticks (n=10) and wings (n=20) were collected from supermarkets and butcher shops in Konya, Turkey. The samples were analyzed by Classical Cultural Technique. Molecular confirmation of the suspicious colonies was carried out using Inv-A gene-based PCR. *Flic-C* and *IE-1* primers were used by duplex PCR for S. Thyphimurium and S. Enteritidis respectively. Antibiotic resistance of the isolates was determined by the disk diffusion method.

Results: Forty-three (25.29 %) of 170 samples were positive for *Salmonella* spp. According to the d-PCR assay, neither S. Thyphimurium nor S. Enteritidis was not detected. The resistance to clindamycin, oxacillin, teicoplanin were evident 100 % and resistance to vancomycin (79.1 %), erythromycin (79.1 %), nalidixic acid (65.1 %), penicillin G (60.5 %) cephalothin (48.8 %), sulfamethoxazole-trimethoprim (37.2 %), tetracycline (37.2 %), ampicillin (23.3 %), kanamycin (18.6 %), chloramphenicol (11.6 %) amikacin, cephazoline, ciprofloxacin, gentamycin (4.7 %) was also detected. All isolates were susceptible to amoxicillin/clavulanic acid and cefixime.

Conclusion: The results indicated that S. Enteritidis and S. Typhimurium were not identified and it was considered satisfactory in terms of public health. It should be still important to note the studies to identify species with lower pathogenic incidences for legal legislation. Furthermore, even the most common pathogenic species cannot be detected, the results of antibiotic resistance in isolates were noteworthy for antibiotic surveillance database.

Keywords: Antibiotic resistance; chicken; S. Enteritidis; S. Typhimurium; *Salmonella* spp.

Introduction

Poultry meat consumption especially chicken and turkey meat have been increasing in recent years. It is preferred by consumers because poultry meat is more economical than red meat. Turkey ranks eighth in the world with 1.9 million tons of poultry meat production and has achieved approximately 660 million dollars in foreign exchange earnings from the export of about 337 thousand tonnes of poultry (BESDBIR 2016).

Salmonella spp. is a Gram-negative, short and small rod-shaped, facultatively anaerobic, non-sporeous and non-capsular species in the Enterobacteriaceae family, their optimum growth temperature is 35-37 °C and has motility except S. Pullorum and S. Gallinarum. They can ferment many carbohydrates except lactose, produce H_2S, reduce nitrate to nitrite and they are indole, urease negative. According to epidemiologic classification, *Salmonella* spp. are divided into three groups. These are; serotypes that infect only humans, serotypes that only infect animals and host non-specific serotypes. S. Typhi, S. Paratyphi A and S. Paratyphi C in the first group; S. Gallinarum (poultry), S. Dublin (cattle), S. Abortus-equi (horse), S. Abortus-ovis (sheep) and S. Choleraesuis (pig) in the second group and S. Enteritidis and S. Typhimurium are classified in the third group. The serotypes in the third group are responsible for foodborne infections which are pathogenic for both humans and animals (Erol 2007).

Given the efforts to reduce *Salmonella* contamination in poultry meat and products, it is emphasized that this development is relatively less effective in reducing the incidence of human salmonellosis. Poultry meat, eggs and, red meat are important tools for the transmission of salmonellosis, although there are other important sources as well (Tauxe et al 2010). Among the known foodborne pathogens, the leading *Salmonella* enterica serotypes remain important in the etiology of foodborne illnesses. In the United States, *Salmonella* is the most common cause of foodborne bacterial infections and is estimated to be responsible for millions of cases per year (Mead et al 1999).

Although there are many *Salmonella* serotypes originating from poultry meat, most of them are not responsible for human cases. For example, S. Kentucky is reported to be very rarely isolated from human diseases (0.1 % of human isolates), although it is one of the most common serotypes (17 % of obtained isolates) in broilers (Sarwari et al 2001). It is reported that a total of 120.760 human salmonellosis cases in the European Union countries in 2008 were derived from S. Enteritidis (58%), S. Typhimurium (21.9 %) and S. Infantis (1.1 %) (EFSA 2010). According to the Centers for Disease Control and Prevention (CDC 2017) there are up to 2500 *Salmonella* serotypes but only about 100 of them have a disease-causing effect in humans.

Besides this, S. Enteritidis and S. Typhimurium in the pathogenic *Salmonella* serotypes are considered to have higher pathogenicity than the other se-rotypes worldwide (Tauxe et al 2010). Hereby, further analy-sis for identification of *Salmonella* isolates obtained from human infections or from animal and environmental sources are stated to provide a more effective use of resources in the prevention of diseases.

The emergence of antibiotic resistance to multiple antibiotic agents has risen on a worrisome level worldwide. It is stated that not only patients and physicians but also global health donors, technical agencies, pharmaceutical companies and governments are the key factors on spreading the resistance (O'Brien 2002; Nugent et al 2010). In this context, international opinion leaders claim to improve the surveillance systems to include both humans and animal origins.

Antibiotic resistance of non-typhoid *Salmonella* agents is admitted as a problem worldwide. Although non-typhoid *Salmonella* infections generally do not need antimicrobial therapy, young, elderly or immunocompromised people may require treatment. Furthermore, ubiquiotous and zoonotic nature of the microorganism may provide a good reference for antibiotic resistance surveillance systems (Park et al 2002; Vo 2007).

In the present study, it was focused on the detection of *Salmonella* spp. and two important *Salmonella* serotypes based on their association with human disease and determination of antimicrobial resistance patterns from poultry meat and giblets widely consumed in Turkey.

Materials and Methods

Sample collection

In this study, a total of 170 packaged chicken meats and giblets (40 livers, 40 gizzards, 30 hearts, 30 skins, 10 drumsticks and 20 chicken wings meat) were analyzed. The samples were purchased between January 2015 to January 2017 from the butchers and supermarkets of Konya city in Turkey. Samples were brought to the laboratory under cold chain and analyzed within 2 hours.

Isolation and identification of Salmonella spp.

Isolation and identification of *Salmonella* spp. have been carried out by the method recommended by the ISO 6579:2002 + A1:2007 with slight modifications. For the pre-enrichment, 25 g of the samples were transferred to sterile stomacher bags and mixed with the addition of 225 ml Buffered Peptone Water in a stomacher for 2 min. and then incubated at 37 °C overnight. For selective enrichment, 0.1 ml of pre-enriched culture was added to 10 ml of Modified Rappaport Vassiliadis Broth (MRVB, Merck 107700) and incubated at 41.5 °C for 24-48 h. 0.1 ml from the culture was streaked onto Xylose

Table 1.The primer pairs used in this study

	Primers	Product Length	Reference
Salmonella spp.	F:GTGAAATTATCGCCACGTTCGGGCAA		
(*Inv-A*)	R:TCATCGCACCGTCAAAGGAACC	284 bp	Rahn et al 1992
S. Enteritidis	F:AGTGCCATACTT TTAATGAC		
(*IE-1*)	R:ACTATGTCGATACGGTGGG	316 bp	Wang and Yeh 2002
S. Typhimurium	F:CCCGCTTACAGGTGGACTAC		
(*Flic-C*)	R:AGCGGGTTTTCGGTGGTTGT	432 bp	Paiao et al 2013

Table 2. Distribution of the isolates in sample types

Sample type	Positive Samples	%
Liver (n=40)	7	17.5
Gizzard (n=40)	8	20
Heart (n=30)	0	0
Skin (n=30)	19	63.3
Drumstick (n=10)	0	0
Wing (n=20)	9	45
Total	43	

Lactose Tergitol 4 (XLT4, Merck 1.13919) Agar supplemented with XLT4 Selective Supplement. The plates were incubated at 37°C for 24 h. The black colored colonies grown on XLT4 Agar was subcultured to Nutrient Agar and Latex agglutination test, oxidase, catalase, Triple Sugar Iron Agar, Lysine Iron Agar, Gram staining were performed to confirm the suspected colonies. The positive isolates were stored at -20 ° C until the DNA isolation step.

DNA isolation

The isolates maintained at -20 ° C in 15 % Glycerin Brucella Broth was resuscitated by transferring to Tryptic Soy Broth (TSB) for DNA extraction. Once 500 µl of TSB was taken into nuclease-free Eppendorf tubes, it was centrifuged at 8000 g for 5 min. The supernatant was removed and then 200 µl of Tris-EDTA (TE) solution was added to the pellet. After vortexing the mixture vigorously, it was held in a heat block which was adjusted at 95 °C for 10 minutes and vortexed again then centrifuged for 5 min at 6000 g. The supernatant was transferred to a new nuclease-free tube and used for PCR analysis.

Conventional PCR for detecting Salmonella spp.

Following optimization of the PCR conditions, conventional PCR was performed. The gene primers used for *Salmonella* spp., S. Enteritidis and, S. Typhimurium detection are shown in Table 1. Following the confirmation of isolates by Inv-A gene for *Salmonella* spp. (Rahn et al 1992), duplex

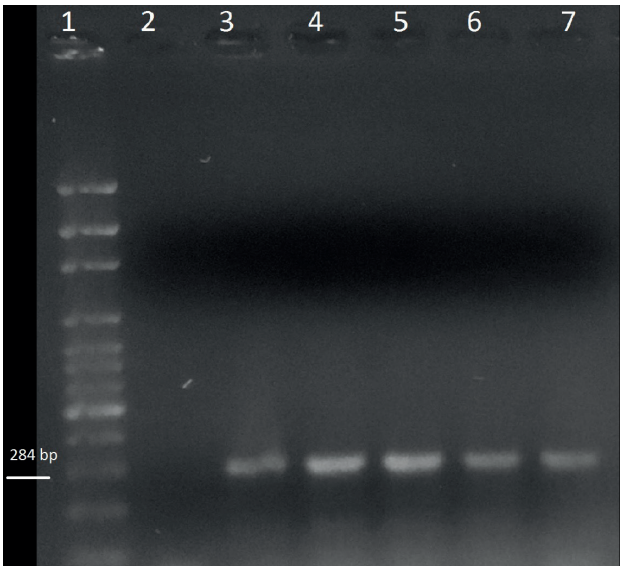

Figure 1. U.V. Transilluminator Image of Gel Electrophoresis of *Salmonella* spp. Positive Samples
1: 100 bp Ladder 2:NC 3:-7:Positive Samples

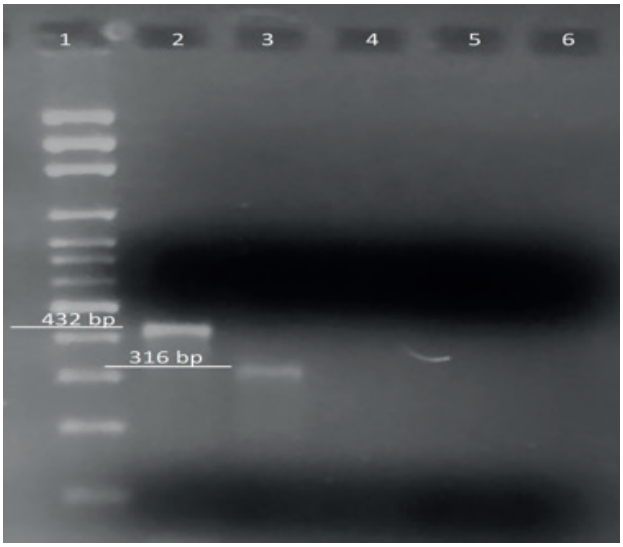

Figure 2. Gel Electrophoresis of Negative Samples
1:DNA Ladder (100 bp), 2:NC, 3:S. Enteritidis ATCC 13076, 3: S. Typhimurium 14028

PCR (d-PCR) assay was performed with *IE-1* (Wang and Yeh 2002) and *Flic-C* (Paiao et al 2013) genes for detection of

Table 3. Antibiotic resistance rate of the isolates (%)

Antibiotic	R	I	S
Amicasin (30 µg)	4.7	-	95.3
Amoxicillin (20 µg)/Clavulanic acid (10 µg)	-	-	100.0
Ampicillin (10 µg)	23.3	-	76.7
Cefixime (5 µg)	-	-	100.0
Cephalothin (30 µg)	48.8	2.3	-
Cephazolin (30 µg)	4.7	-	95.3
Ciprofloksasin (5 µg)	4.7	4.7	90.7
Clindamycin (2 µg)	100.0	-	-
Cloramphenicol (30 µg)	11.6	-	88.4
Eritromycin (15 µg)	79.1	11.6	9.3
Gentamycin (10 µg)	4.7	-	95.3
Kanamycin (30 µg)	18.6	-	81.4
Nalidixic acid (30 µg)	65.1	-	34.9
Oksacillin (1 µg)	100.0	-	-
Penisillin G (10IU)	60.5	-	39.5
Streptomycine (10 µg)	-	4.7	95.3
Sulfamethoxazole (23.75 µg)–/Trimethoprim (1.25 µg)	37.2	-	62.8
Teicoplanin (30 µg)	100.0	-	-
Tetracycline (30 µg)	37.2	-	62.8
Vankomycine (30 µg)	79.1	4.7	16.3

R: Resistance, I: Intermediate Resistance S: Suscebtible

S. Enteritidis and S. Typhimurium. PCR mixes consisted of 1 U Taq DNA polymerase (Solis Biodyne, FIREPol®), 1 X Taq buffer without $MgCl_2$, 1.5 mM $MgCl_2$, 0.1 mM dNTPs (Solis Biodyne), 0.25 µl of *Inv-A* primers. For duplex PCR, *IE-1* and *Flic-C* primers were added as 0.4M per reaction. Total volume was adjusted to 20µl for both of the PCR reactions. Both PCR protocols consisted of an initial denaturation step for 5 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 58 °C, and 30 s at 72°C and by a final extension step for 7 min at 72°C (Paiao et al 2013).

Antibiotic susceptibility of Salmonella spp. *isolates*

Antibiotic susceptibility of the isolates to 20 antibiotics was carried out by the disk diffusion method. Briefly, the resuscitated isolates and the reference strain (*Escherichia coli* ATCC 25922) were cultured in Mueller Hinton Broth (Oxoid, CM0405) and the Optical density was adjusted to 0.5 Mc Farland with Mc Farland Optic Densitometer (DEN-1B McFarland Densitometer). The broth culture was streaked on to the Mueller Hinton Agar (Oxoid, CM0337) with sterile cotton swabs. The Antimicrobial Susceptibility Test Discs (Oxoid) were placed onto the surface of the plates which are 120 mm in diameter. The tested antibiotics were Amikacin (30 µg), Amoxicillin (20 µg)/Clavulanic acid (10 µg), Ampicillin (10 µg), Cefixime (5 µg), Cephalothin (30 µg), Cephazolin (30 µg), Ciprofloxacin (5 µg), Clindamycin (2 µg), Erythromycin (15 µg), Gentamycin (10 µg), Kanamycin (30 µg), Chloramphenicol (30 µg), Nalidixic acid (30 µg), Oxacillin (1 µg), Penicillin G (10IU), Streptomycin (10 µg), Sulfamethoxazole (23.75 µg)–/Trimethoprim (1.25 µg), Teicoplanin (30 µg), Tetracycline (30 µg), Vancomycin (30 µg). Following the incubation at 37°C for 18-24 hours, inhibition zones were measured. According to the measurement, the isolates were divided as resistant, susceptible and intermediate according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2014).

Results

Forty-three (43; 25.29 %) of the 170 samples were found to be positive for *Salmonella* spp. Distribution of the isolates according to the sample types are shown in Table 2.

All of the isolates were confirmed by *Inv-A* based PCR assay (Fig 1). According to the d-PCR assay for detection of S. Thyphimurium (*Flic-C*) and S. Enteritidis (*IE-1*) none of the samples were found positive (Fig 2).

Antibiotic resistance profile of the isolates

Distribution of isolates according to resistance profiling was as follows: 4.7 % amikacin, 23.3 % ampicillin, 48.8 % cephalothin, 4.7 % cephazoline, 4.7 % ciprofloxacin, all isolates clindamycin, 11.6 % chloramphenicol, 79.1 % erythromycin, 4.7 % gentamycin, 18.6 % kanamycin, 65.1 % nalidixic acid, all isolates to oxacillin, 60.5 % penicillin G, 37.2 % sulfamethoxazole-trimethoprim, all isolates to teicoplanin, 37.2 % tetracycline, and 79.1 % to vancomycin were resistant. The isolates were also resistant at intermediate levels to 2.3 % cephalothin, 4.7 % ciprofloxacin, 11.6 % erythromycin, 4.7 % streptomycin and 4.7 % vancomycin (Table 3). All isolates were resistant to three antibiotics (clindamycin, oxacillin, teicoplanin) and susceptible to two antibiotics (amoxicillin/clavulanic acid and cefixime) (Table 3).

Discussion

There are a number of studies with similar and different isolation rates than the current study. Arroyo and Arroyo (1995) found 83 of 264 (31.43 %) samples *Salmonella* spp. positive in a similar study from chicken and sheep internal organs that were sold in open and chilled conditions. Chang (2000), analyzed *Salmonella* spp. in broiler meat and eggs and found the contamination level of 25.9 %. In a similar study, Choi et al (2014) investigated the presence of *Salmonella* spp. in broiler breeder farm, truck, slaughterhouse and retail chicken meat samples and reported that *Salmonella* spp. was detected in 195 of the 1214 (16.06 %) samples. In a recent study by Naik et al (2015) detected 7 % of the 200 chicken meats as *Salmonella* spp. positive on the basis of cultural and biochemically confirmed isolates by targeting *Inv-A* gene with classical PCR assay.

Despite the high rate of *Salmonella* spp. contamination, none of the samples was detected to have S. Enteritidis and S. Typhimurium genes as the pathogenic species and this was regarded as satisfactory in terms of public health. Unlike the present study, S. Enteritidis and S. Typhimurium have been detected from a large number of subtype level studies (El-Aziz 2014; Zhao et al 2001; Yıldırım et al 2011; Abdellah et al 2009; Al et al 2017). El-Aziz (2014) performed in isolates identified as *Salmonella* spp. by the classical cultural method in the study of S. Typhimurium in chicken meat and internal organs. One of these studies were performed by El-Aziz (2014) with d-PCR of rfbJ and *Flic-C* genes in classical culturally confirmed *Salmonella* spp. isolates isolated from chicken meat and giblets. In *Salmonella* spp. isolates of chicken meat, liver and heart regions, S. Typhimurium rate was found at 44%, 40 % and, 48 %, respectively. The researcher stated not to detect *Salmonella* spp. in gizzard samples unlike to our study. Zhao et al (2001) investigated the prevalence of *Campylobacter* spp., *Salmonella* spp. and *E. coli* in retail chicken, pork, turkey and beef and detected that 25 (3 %) of the samples were contaminated with *Salmonella* spp. Yıldırım et al (2011) investigated the incidence of *Salmonella* spp. in 200 packaged fresh raw chicken carcasses in central Anatolia and found positive 34 % (68/200) of samples using cultural technique and PCR. The researchers stated the predominant serotypes included Typhimurium, Infantis and, Heidelberg among ten serovars identified. Abdellah et al (2009), analyzed a total of 576 samples and found 57 (9.90 %) of them positive for *Salmonella* spp, and they also detected S. Typhimurium (40.35 %) and S. Newport (26.31 %) as the most prevalent serotypes. In a recent study conducted by Al et al (2017) in Turkey, S. Typhimurium and S. Enteritidis were identified from 21 (8.3 %) and 2 (0.8 %) of the poultry products, respectively.

According to the sample groups, the obtained data demonstrated the highest contamination level of *Salmonella* spp. was in skin samples (19/30, 63.3 %). In a similar study (Capita et al 2003) in Spanish poultry products including chicken carcasses, giblets and, processed products showed that the highest contamination level with *Salmonella* spp. was detected in carcass skin samples.

Although the high prevalence of foodborne illnesses in the summer months was indicated by the CDC, Foodnet (2001), our results indicate that two important pathogenic subtypes have not been identified, despite the high level of contamination of *Salmonella* spp. In a seasonal study, researchers (Zhao et al 2001) stated that there was no significant difference when warm and cold months were compared in microbial contamination levels of *Salmonella* spp., *Campylobacter* spp. and *E. coli*.

In a similar study carried out by Chung et al (2003) between 1993-2001 in Korea 14.6 % of the isolates were susceptible to all of the tested antibiotics, 4.9 % were found to be resistant to one antibiotic, 14.6 % were resistant to two antibiotics, 22.0 % were resistant to three antimicrobial agents, 39.0 % were resistant to four antimicrobial agents, and 4.9 % were resistant to five antimicrobial agents. The researchers also stated that most of the isolates were resistant or intermediate resistant to streptomycin, ampicillin, carbenicillin, and/or tetracycline. Antunes et al (2003) found that *Salmonella* spp. isolates isolated from Portuguese poultry products were resistant to one or more antimicrobial agents. They stated to record eight different resistance profiles and 50% of the isolates were resistant to nalidixic acid and enrofloxacin. Researchers have also declared that all isolated samples were susceptible to amoxicillin/clavulanic acid, cephalothin, cefotaxime, ceftazidime, ciprofloxacin gentamicin, tobramycin, netilmicin and, ofloxacin.

In our study the multiresistance pattern of all the isolates

was Clindamycin+Oxacillin+Teicoplanin. Following this, the most frequent profile of multiresistant strains was Eritromycin+Nalidixic acid+Penicillin G+Vankomycine (65.1 %). In a similar study in Spain, Carraminana et al (2004) determined the antibiotic resistance profile to 19 antimicrobial agents of *Salmonella* spp. isolates obtained from a poultry slaughterhouse and found resistant the isolates to neomycin (53.4 %), streptomycin (11.3 %), sulfadiazine (96.2 %) and tetracycline (21.8 %). The researchers found the most frequent patterns as neomycin+sulfadiazine and neomycin+ tetracycline+sulfadiazine. They also declared to determine multiple resistance in 65.4 % of samples. Dallal et al (2010) determined the antibiotic resistance profiles in fresh chicken and beef meat in Tehran, Iran and found that *Salmonella* spp. isolates were resistant to nalidixic acid (82 %), tetracycline (69 %), trimethoprim (63 %) and streptomycin (52 %). They stated 68.5 % of the isolates were found to be multidrug resistant. In a recent study by Thung et al (2016) isolated the *Salmonella* spp. in retail raw chicken meat in Malaysia and detected multi-drug resistance in S. Enteritidis and S. Typhimurium isolates. All the isolates showed resistance to erythromycin, penicillin, and vancomycin. Besides it was also pointed out they were susceptible to Amoxicillin/Clavulanic acid, Gentamicin, Tetracycline and, Trimethoprim.

Conclusion

As a result, although *Salmonella* spp. were detected high prevalence, none of the isolates were responsible for human infections. Considering *Salmonella* spp. is common in the environment and other sources, it is suggested that food analysis made at the legislative level to prevent and control should focus on to determine pathogen serotypes for humans, should not be limited at the species level. Furthermore, appearance of antibiotic-resistant *Salmonella* spp. strains isolated from chicken meat and giblets would be a major concern in public health. In this context, a continuous surveillance system and prevention strategies would be implemented to take measures for more rational use of antibiotics.

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