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# Determination of the prevalence of *Salmonella* spp. and *S. aureus* in meat products by Real-Time PCR and testing their antibiotic susceptibility*⁾

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Summary

From a public health point of view meat products contain high pathogenic risk factors. This is because they can be contaminated with *Salmonella* spp. and *Staphylococcus aureus* microorganisms, and, more importantly, antibiotic resistance has been reported in these microorganisms at an increasing frequency. To examine the presence of *Salmonella* spp. and *Staphylococcus aureus* in samples of raw and semi-cooked (chicken doner, meat doner, chicken, beef, and lamb products) meat from markets of Izmir and Balikesir, Turkey were analysed. The presence of microorganisms in the samples was determined by Real-Time PCR method using *Salmonella* spp. and *S. aureus* specific primers. Following Real-Time PCR, microorganisms were isolated by selective culture methods and biochemical tests from the positive meat samples and tested for their antibiotic susceptibility using the Kirby-Bauer Disk Diffusion Method. The antibiotic disc diffusion method showed that *S. aureus* was resistant to penicillin G, oxytetracycline, sulfamethoxazole, tetracycline, erythromycin, and ampicillin, whereas *Salmonella* spp. was resistant to penicillin G, sulfamethoxazole, erythromycin, and ampicillin. As these products are consumed frequently, their contamination with *S. aureus* ($\geq 5 \times 10^3$ cfu/g) and *Salmonella* spp. can be a risk factor for food poisoning. The contamination of meat products' with *S. aureus* and *Salmonella* spp. can be a risk factor for public health and the antibiotics to be preferred in illness treatment are of critical importance.

Keywords: *Staphylococcus aureus*, *Salmonella* spp., Real-Time PCR, meat products, antibiotic sensitivity

Sufficient nutrition is essential to maintain a healthy life and enjoy protection from diseases. The reliability of the foods consumed is a major factor for tissue growth, regeneration, and the fruitful functioning of our bodies. A great majority of the nutrition quantity required for our body is provided by meat products. However, many food products, especially animal source foods, are convenient for contamination by pathogenic microorganisms. If the compulsory hygiene rules are not followed in the process extending from production to sale, undesirable microorganisms infect and spread in the food and the consumption of infected foods brings with it many health problems (40).

Food poisoning, which is caused by microorganisms transported through foods, is one of the most common global health problems, affecting many people around the world every year. Even though it only appears to affect people's health, it also has an economic impact on countries. Two of the most commonly identified species related to food infections are *Salmonella* spp. and *Staphylococcus aureus* (*S. aureus*) (23). *Salmonella* is one of the most common pathogenic microorganisms and causes foodborne outbreaks and infections that are known as salmonellosis in many countries. Cross-contamination, which is the transmission of microorganisms from a contaminated surface to a non-contaminated surface between human, environmental factors and foods, mostly leads to diseases such as salmonellosis (33). *Salmonella* is considered to be a risk factor for the consumer, even in low amounts. For this reason, it is not allowed to be present in food products. *S. aureus* is another pathogenic microorganism frequently encountered in food contaminations. The majority of food poisoning is caused by entero-

toxins produced by *S. aureus* strains as a by-product during growth, and is a significant global threat to human health (16).

The rapid and cost-effective diagnosis of foodborne pathogens throughout the food chain is an important issue for the industry and public health. Real-Time PCR (qPCR) is the preferred molecular technique as it can detect and quantify the amount of the targeted microorganisms with high specificity and sensitivity. It is also a reliable method as it can generate both qualitative and quantitative data about specific targeted microorganisms in a short period of time. Real-Time PCR analysis, which allows for rapid detection even in a small amount of bacterial DNA, has many advantages over other types of microorganism detection, regardless of the type and growth physiology of the microorganism (11, 43).

It is not always possible to identify the food types causing food poisoning, but unlike red meats, poultry meat contains a large percentage of reported foodborne pathogens such as *Campylobacter* spp. and *Salmonella* spp. (20). Day by day, the antimicrobial drug resistance of microorganisms is increasing because of the therapeutic applications in animal husbandry. The usage of antibiotics in inappropriate doses and time intervals as feed additives results in the development of resistant strains. Furthermore, animal-derived foods which contain antibiotic residues can lead to poisoning, allergies, and disruption in the human gut flora after consumption (6, 13, 24, 27). Importantly, it is more challenging to deal with pathogens that display multidrug resistance than with normal ones when treating infections. To improve food safety planning and infection-specific treatment strategies, it is necessary to observe the prevalence and antimicrobial resistance of pathogenic microorganisms in consumed food either raw or semi-cooked. The objective of this study is to assess the meat samples collected from the Izmir and Balikesir markets in terms of their contamination with *Salmonella* spp. and *S. aureus* strains and to evaluate antibiotic resistance profiles of these strains.

## Material and methods

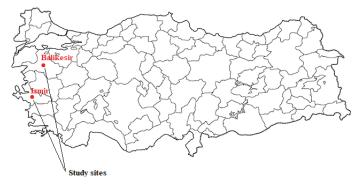**Sample collection.** The study sites are the Balikesir and Izmir provinces located in the west of Turkey (Fig. 1). In the present study, raw (chicken, beef, and lamb products) and semi-cooked (chicken and meat doner) samples were used. For semi-cooked products, generally a heat treatment below 100 degrees is applied in Turkey. Samples were collected from various bazaars (10), local markets (40), and large hypermarkets (50) to represent distinct production companies in the Izmir and Balikesir provinces randomly between April 2017 and September 2017. A total number of 100 samples that include 25 raw chicken meats, 25 raw red meats, 25 semi-cooked chicken meats, and 25 semi-cooked red meats, were collected in sterile bags. After being brought to the laboratory under cold chain conditions, the samples were marked according to date and area and processed 2 h after collection. For the prevention of cross-contamination throughout sampling, sterile gloves and equipment were used.

**Enrichment procedures.** First, samples were cut into small pieces (25 g) with a sterile scalpel. In pre-enrichment, 25 g of each of the meat samples were aseptically transferred into 225 mL of buffered peptone water (BPW) (Merck, Germany). Homogenised samples were incubated for 18 hours at 34-38°C. For isolation of both *Salmonella* and *S. aureus*, a part of BPW was then transferred to different enrichment media.

**Isolation of *Salmonella*.** The 0.1 mL and 1 mL of pre-enrichment culture were transferred into 10 mL of Rappaport Vassiliadis Medium (RVS) Broth (Merck, Germany) and Muller Kauffman Tetrathionate Novobiocin Broth (MKTTn) (Merck, Germany) respectively. Next, 24 h incubation was performed for RVS at 42°C, and for MKTTn at 37°C. Each selective enrichment culture was transferred to Xylose Lysine Desoxycholate (XLD) Agar (Merck, Germany) and Bismuth Sulphite Agar (BSA) media (Merck, Germany) on the following day. Plates were incubated at 37°C for 24 hours to isolate the visible colonies (22). Displayed pink-red colonies with a black centre in XLD agar and brown-grey-black colonies with a bright metallic centre in BSA media were considered to be *Salmonella*. For the confirmation, API 20E system (bioMérieux, Marcy-l'Étoile, France) was used according to the manufacturer's protocol.

**Isolation of *S. aureus*.** For *S. aureus* isolation, firstly $10^1$ dilutions were prepared from pre-enrichment culture. Next, Baird-Parker Agar (Merck, Germany), which is a selective medium due to its egg yolk emulsion and tellurite components, was utilised for 0.1 mL of dilution samples inoculation (15). Plates were incubated at 37°C for 24 hours to isolate the visible colonies. After 24 hours of incubation, the colonies that formed a black or grey transparent zone and had a bright and smooth appearance were accepted as *S. aureus*. Confirmation was performed by coagulase test and API Staph (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's protocol.

**DNA isolation.** Taking 1 ml of the pre-enrichment culture, DNA isolation was carried out using the High Pure PCR Template Preparation Kit (Roche Applied Science, Germany), a commercial DNA extraction kit, according to the manufacturer's protocol. The concentrations and purities of the DNAs obtained after isolation were measured by the Nanodrop and the DNAs were maintained at –20°C for the next step.



**Fig. 1. Areas of the study**

**Tab. 1. Primers and probes for Real-Time PCR amplification**

| Microorganism | Primer Name | Sequence (5'-3') | Probe |
|---|---|---|---|
| *Salmonella* spp. | Forward | CTCACCAGGAGATTACAACAT | 5' FAM-CACCGACGGCGAGACCGACTTT-TAMRA 3' |
| | Reverse | AGCTCAGACCAAAAGTGACCA | |
| *Staphylococcus aureus* | Forward | AATTAACGAAATGGGCAGAAACA | 5' FAM-AGAAATTAACTGGATGGTACGCGCGAAGA-TAMRA 3' |
| | Reverse | TGCGCAACACCCTGAACTT | |

**Real-time PCR analysis.** Real-time PCR and data analysis were performed in Roche LightCycler® 480 II real-time detection system (Roche Applied Science, Germany) using LightCycler® 480 Probes Master 2x (Roche Applied Science, Germany). First, from isolates of *S. aureus* (ATCC 25923) and *Salmonella* spp. (ATCC 700623), cloning was performed using the TOPO® TA Cloning Kit (Invitrogen, Carlsbad, CA). After the cloning, $10^9$ standards of plasmids were diluted until there were a total of $10^1$ copies which were prepared and the standard curve was drawn using these standards. PCR amplification was performed with the *Salmonella* spp. (30) and *S. aureus* (28) specific primer pairs and TaqMan probes (Tab. 1) using an in-house protocol. Primers were synthesised by Macrogen (Korea). The mixture contained 10 µL 2x LightCycler® 480 Probes Master; 1 µL of each primer; 0.4 µL of each TaqMan probe; 5 µL DNA template. To reach a total volume of 20 µL per well, distilled water was added. Thermal cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s. All runs included a negative control without target DNA and a standard.

The Roche LightCycler® 480 II Real-time PCR detection system and software were used for data analysis. The system records crossing point (Cp) number when fluorescence crosses a specific threshold value in the exponential phase of amplification. The Cp value is a measure of the quantity of transcript of interest. Thus, we have calculated unknown sample concentrations depend on Cp values and standard curve.

**Antimicrobial susceptibility testing.** Antibiotic susceptibilities were determined by Kirby-Bauer Disk Diffusion method (4) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (7). The typical colonies of both *Salmonella* spp. and *S. aureus* were diluted in sterile BPW and the McFarland turbidity was adjusted to a standard 0.5. Standardised suspension cultures were inoculated in Plate Count Agar (PCA) plates equally. Antibiotic discs were placed with at least a 15 mm gap to prevent overlapping of the zones on the plates and incubated at 37°C for 18 hours. Susceptibility to the following 10 antimicrobials were tested: chloramphenicol (30 µg), penicillin G (10 U), bacitracin (10 U), oxytetracycline (30 µg), sulfamethoxazole (25 µg), neomycin (30 µg), novobiocin (30 µg), tetracycline (30 µg), erythromycin (30 µg), and ampicillin (10 µg) (Oxoid, Basingstoke, UK). After incubation, the zone diameters around the antibiotic discs were measured with a ruler, and sensitivity and resistance profiles of microorganisms to antibiotics were determined as susceptible, intermediate, or resistant according to the CLSI guidelines (7).

**Statistical analysis.** The data were analysed using chi-square ($\chi^2$) tests, contingency tables. When interpreting the results, $p < 0.05$ was considered statistically significant.

## Results and discussion

Samples of raw and semi-cooked meat, either sourced locally or imported, were purchased from different locations in Balikesir and Izmir provinces. A hundred samples of meat were used in this study. Samples were examined using Real-Time PCR analysis in terms of the presence of *Salmonella* spp. and *S. aureus*, and microorganisms' resistance profiles were determined in 10 different antibiotics by disk diffusion method in samples with undesirable levels of bacteria according to the Turkish Food Codex.

**Examining the presence of *Salmonella* spp. and *S. aureus* in raw and semi-cooked meat samples by Real-Time PCR analysis.** Many methods are used to determine the presence of pathogenic microorganisms in food products. In addition to traditional methods, Real-Time PCR analysis method has become one of the most widely used analytical methods with its many benefits, such as being able to detect microorganisms in low concentrations and being fast and reliable (25). In our study, Real-Time PCR analysis was therefore the preferred method for detecting the presence of pathogenic microorganisms.

The numeric distribution of *Salmonella* and *S. aureus* found in each type of sample is displayed in Table 2. *S. aureus* was the most frequent contaminant of all types of samples. However, meat products containing $5.0 \times 10^3$ cfu/g or more microorganisms of *S. aureus* are considered to be inappropriate for consumption according to the Communique on Raw and Prepared Poultry Meat Mixture (26221) published

**Tab. 2. Proportional distribution of microorganisms in examined samples**

| Product type | The number of samples | The number of detected microorganisms by Real-Time PCR | |
|---|---|---|---|
| | | *Salmonella* spp. | *S. aureus* ($5.0 \times 10^3$-$3 \times 10^4$ cfu/g) |
| **Raw meat** | | | |
| Chicken meat | 25[a] | 6 | 5 |
| Red meat | 25[a] | – | 2 |
| **Semi-cooked** | | | |
| Chicken meat | 25[b] | – | 5 |
| Red meat | 25[b] | – | – |
| Total | 100 | 6 | 12 |

Explanations: [a] – Samples were collected as 5 from bazaars, 10 from local markets and 10 from hypermarkets; [b] – Samples were collected as 10 from local markets and 15 from hypermarkets

by the Turkish Food Codex. We observed *S. aureus* as more than $5.0 \times 10^3$ cfu/g bacteria in 5 of 25 raw chicken samples, 2 of the 25 raw red meat samples, and 5 of 25 semi-cooked chicken meat samples (Tab. 2). Therefore, according to these standards, a total of twelve samples were determined as unsuitable. In the present study, out of a total of fifty raw samples, six (27.3%) raw chicken meat samples were found to be contaminated with *Salmonella*. In contrast to raw chicken meat samples, no contamination was observed in red raw meat samples and semi-cooked meat samples. Even if a very low level of *Salmonella* is detected in food, the nutrient is considered to be in the risk group. According to the Communique on Raw and Prepared Poultry Meat Mixture (26221) and Regulation on Microbiological Criteria (28157) published by the Turkish Food Codex, the presence of *Salmonella* spp. in food groups should be zero (0/25 g-mL) so those samples were determined as inappropriate for consumption. Additionally, Regulation (EC) No 2160/2003 remarks that fresh poultry meat can be placed on the markets if *Salmonella* is absent in 25 grams of meat.

Many researchers have focused on the presence of pathogen microorganisms such as *Salmonella* spp. and *S. aureus* in different kinds of meat samples. Our data showed that in all of the samples, the rate of *Salmonella* spp. was 6% while *S. aureus* more than $5.0 \times 10^3$ cfu/g was 12%. In many studies conducted in Turkey or abroad, the reported contamination ratios with *Salmonella* spp. and *S. aureus* have varied widely (46, 48, 49). Greeson et al. (14) reported that 10.7% of various meat sources were contaminated with *Salmonella*. In another study by Akbar and Anal (2), 5.26% of poultry meat samples were shown to be contaminated with *Salmonella* while 18.18% were contaminated with *S. aureus*. Our results are similar to the values of those results. Moreover, in research conducted in Afyonkarahisar province in the west of Turkey, *Salmonella* presence was found to be 3% in chicken meat and giblets samples (1). Also, our *S. aureus* prevalence is similar to various studies. For instance, Heo et al. (19) reported that 11% of meat samples were contaminated with *S. aureus* while De Boer et al. (10) reported that 16% of chicken meat samples were contaminated with *S. aureus*.

*Staphylococcus*-induced food poisoning occurs due to the fact that these microorganisms produce heat-resistant enterotoxins. However, toxin production in the microorganism is present when the total number of cells in one gram of meat reaches 100,000. In the samples analysed, *S. aureus* could not be detected at the level that could produce enterotoxins, but in a study in which 225 meat samples were collected from Ankara, *S. aureus* with enterotoxigenic properties was found in 77.1% of samples (34).

The rate of both *Salmonella* (0/50) and *S. aureus* (5/50) contamination in semi-cooked meat samples was much lower than in raw meat samples. We interpret the presence of a lower number of microorganisms in semi-cooked meat products as a result of the heat treatment of these products during the fabrication phase. Taking into account all the samples used in our study, *Salmonella* was only observed in raw chicken meat samples. Unfortunately, raw chicken meat samples purchased from bazaars contained more *Salmonella* than purchased from local markets and hypermarkets. Similar to our results, Zwe et al. (50) found that the prevalence of *Salmonella* in chicken meat from wet markets was higher than in supermarkets. Another study conducted with meat samples collected at wet-markets in Cambodia showed that nearly half of the samples were contaminated with both *Salmonella* and *S. aureus* (39).

The results obtained were not suitable for public health in terms of pathogenic microorganisms. In this case, it is thought that contamination stems from some hygiene problems during the process of cutting, washing, freezing, or during storage between the production steps of the supplier and the sale of food products (36). Therefore, the conditions in both production and transfer for markets should be improved for raw meat products (45).

**Antibiotic resistance profile.** Our Real-Time PCR analysis results show that five samples of raw chicken meats, two of raw red meats, and five of semi-cooked chicken meat were contaminated with *S. aureus*, versus six samples of raw chicken meats contaminated by *Salmonella*. Following qPCR, microorganisms were isolated using selective culture methods and biochemical tests from the meat samples that were verified to be contaminated with *Salmonella* and *S. aureus* (more than $5.0 \times 10^3$ cfu/g) by Real-Time PCR analysis. Afterwards, antibiotic susceptibility of *Salmonella* spp. and *S. aureus* isolates was determined by Kirby-Bauer Disk Diffusion Method using 10 different antibiotics (4). Disk diffusion method is a fast and reliable method for detecting the resistance of bacteria to various antibiotics. For this reason, disc diffusion method was preferred for determining the antibiotics that can be used in the treatment of infections caused by isolated pathogenic microorganisms. The antibiotic resistance rates for the whole isolates are presented in Table 3. The response of *Salmonella* spp. and *S. aureus* microorganisms to tetracycline and oxytetracycline antibiotics was significantly different ($p < 0.05$).

To date, many studies have been performed in the area of *Salmonella* identification, antibiotic resistance and molecular characterisation. As shown in Table 3, *Salmonella* had a high level of resistance to penicillin G (100%), sulfamethoxazole (83.33%), erythromycin (66.66%), and ampicillin (66.66%). These results are in agreement with the findings of similar studies on meat products from Thailand (2), Thailand-Cambodia (44), China (48), Iraq (17), Argentina (36), Egypt (12, 41), and South Korea (21). Our findings are also

**Tab. 3. Antibiotic resistance profile**

| Type of antibiotic discs | Amount of microorganisms | | | | | | P value |
|---|---|---|---|---|---|---|---|
| | *Salmonella* spp. (total n = 6) | | | *S. aureus* (total n = 12) | | | |
| | Susceptible n (%) | Intermediate n (%) | Resistant n (%) | Susceptible n (%) | Intermediate n (%) | Resistant n (%) | |
| Chloramphenicol | 5 (83.33) | 1 (16.66) | 0 (–) | 12 (100) | 0 (–) | 0 (–) | 0.35 |
| Penicillin G | 0 (–) | 0 (–) | 6 (100) | 0 (–) | 2 (16.66) | 10 (83.33) | 0.58 |
| Bacitracin | 5 (83.33) | 0 (–) | 1 (16.66) | 10 (88.33) | 0 (–) | 2 (16.66) | 0.99 |
| Oxytetracycline | 6 (100) | 0 (–) | 0 (–) | 0 (–) | 3 (25) | 9 (75) | 0.0001 |
| Sulfamethoxazole | 0 (–) | 1 (16.66) | 5 (83.33) | 0 (–) | 2 (16.66) | 10 (83.33) | 0.99 |
| Neomycin | 4 (66.66) | 2 (33.33) | 0 (–) | 11 (91.66) | 1 (8.33) | 0 (–) | 0.41 |
| Novobiocin | 4 (66.66) | 2 (33.33) | 0 (–) | 10 (83.33) | 2 (16.66) | 0 (–) | 0.72 |
| Tetracycline | 6 (100) | 0 (–) | 0 (–) | 0 (–) | 3 (25) | 9 (75) | 0.0001 |
| Erythromycin | 0 (–) | 2 (33.33) | 4 (66.66) | 1 (8.33) | 1 (8.33) | 10 (83.33) | 0.35 |
| Ampicillin | 0 (–) | 2 (33.33) | 4 (66.66) | 0 (–) | 0 (–) | 12 (100) | 0.11 |

similar to those in another study performed on chicken meat samples in Anatolia in terms of a high susceptibility percentage for chloramphenicol antibiotic of *Salmonella* (49). In contrast, resistance of *Salmonella* isolates from retail chicken and beef in Iran of 4% to ampicillin were significantly lower than our result (8). Although our results for sulfamethoxazole are compatible with the *Salmonella* resistance profile obtained from frozen chicken samples in Bangladesh, the resistance percentages they observed for oxytetracycline and tetracycline do not support our results (35).

Pathogenic bacteria like *S. aureus* from different kinds of meat sources have been studied by many researchers all around the world. According to Table 3, *S. aureus* isolates show the highest resistance to ampicillin (100%). In addition, they were the most frequently resistant to penicillin G (83.33%), sulfamethoxazole (83.33%), erythromycin (83.33%), tetracycline (75%), and oxytetracycline (75%). Our findings are in compatible with the results of studies carried out in Turkey (5), Jordan (38), Saudi Arabia (14), Egypt (32), Bangladesh (9), Iran (18, 31), China (29, 47), and India (42). Our results reported that *S. aureus* isolates displayed a higher resistance percentage for tetracycline (44.73%) and ampicillin (55.26%) than the study in Thailand (2). Even though our resistance results are higher for penicillin, ampicillin, and erythromycin in *S. aureus* isolates, a study in Washington on meat products supports our findings for tetracycline and chloramphenicol (26). *S. aureus* isolates show the highest susceptibility for chloramphenicol. These findings are supported by studies in Louisiana (37) and Iran (3).

In this study, the presence and antibiotic resistance of food poisoning related pathogens *Salmonella* and *S. aureus* were researched among different meat products. The alteration of *Salmonella* and *S. aureus* contamination levels both in our study and others can be derived from the number of samples studied, detection methods utilised, and cross-contamination during fabrication, processing or transferring. In addition, by being more careful about storing raw and semi-cooked products in separate cabinets, possible contaminations can be reduced. The incidence of *S. aureus* and *Salmonella* in examined samples was observed to be higher in raw meat products than semi-cooked meat products. Due to the heat treatment for semi-cooked meat products, we supposed that food-related pathogen presence decreases during fabrication. Also, cooking procedures should be applied effectively at home for raw products. It can be concluded that prevention of foodborne diseases depends on hygienic conditions and cold storage during the production and marketing stages of meat products. This study also suggests the necessity for the application of the HACCP (Hazard Analysis and Critical Control Point) food safety system, which determines the hygiene principles for healthy food production and increases the quality by ensuring product safety.

Apart from the presence of pathogens in our samples, resistance to different antibiotic agents was accompanied to microorganisms. Increased antibiotic resistance of microorganisms is a cause of concern for public health. Antibiotic resistance enables bacteria which is why it decreases the treatment of infections. The difference in resistance to antibiotics among microorganisms may be due to the unconscious use of antibiotics and quick change in antibiotic susceptibility profiles of bacteria within a brief period. In this study, it is thought that the antibiotic resistances obtained in microorganisms occur because of the unconscious usage of antibiotics in both human and animal diseases. Especially, β-Lactams are widely used antibiotics to treat infections, but using those antibiotics may result in the formation of resistant strains. Our study highlights the importance of conscious antibiotic usage in animal farming to increase consumer safety. Furthermore, since pathogenic bacteria pose a higher

risk for young children, the elderly, and patients undergoing treatment, it is thought that a selective and conscious treatment may be more beneficial for them. Consequently, more restrictive politics should be taken into consideration and applied without discrimination to improve the current situation.

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