Determination of Carbapenem-Resistant Enterobacteriaceae from Chicken Meat by Advanced Modified ISO 21528-1:2017 Method

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RESEARCH ARTICLE

Abstract
Antibiotic resistance is critical today, and carbapenem-resistant Enterobacteriaceae (CRE) are the current reflection of this threat in terms of public health. Our study aimed to investigate the effectiveness of a known ISO method used to detect of CREs in chicken meat sold in the Aksaray Region. A total of 150 chicken samples (50 drumsticks, 50 breasts, and 50 wings) were analyzed in terms of CRE by modifying the ISO 21528-1:2017 method. For this purpose, meropenem and ertapenem powders were added into buffered peptone water and Violet Red Bile Glucose Agar at the resistance levels determined in EUCAST. At the same time, target DNA extraction was performed from all samples with a tissue isolation kit (Hybrigen) and stored for PCR to support and strengthen our results to compare the cultural method's results. According to the results of the cultural procedure, the existing flora of chicken meats was suppressed by antibiotic supplements, and no suspicious colonies were detected. Likewise, CRE was not detected in DNA samples obtained from 150 chicken meat samples. Carbapenem resistance, described as the last fortress, is today's significant public health problem. According to our results, CRE was not isolated in 150 chicken meat samples offered for sale in our region.

Keywords: Carbapenem-Resistance, Enterobacteriaceae, Chicken meat, ISO 21528, CRE

INTRODUCTION
Antibiotic resistance, which is developed by microorganisms, is a significant problem that has been on the agenda for many years and is still a critical problem for public health. Although newly developed antibiotics limit the growth of bacteria for a while, it has been reported that pathogens take on new resistance factors from other species in their environment, reducing our ability to prevent and treat infections (Larsson and Flach, 2022). Furthermore, structured environments such as biofilms promote the transfer of antibiotic resistance genes between bacteria (Alfonso et al. 2019). It has been reported that this annoying situation leads to treatment failures, economic losses, and some undesirable immunological reactions in susceptible individuals in practice. From this point of view, the success of a therapeutic agent is jeopardized by the potential development of tolerance from the first use, and the uncontrolled use of therapeutic agents poses a risk to public health (Davies and Davies, 2010; Frieri et al. 2017). In addition, it has been reported that antibiotic resistance reduces the success of treatments performed by disciplines such as organ transplantation, oncology, and surgery (Prabaker and Weinstein, 2011). Nowadays, it is known that many different groups and types of antibiotics are used for veterinary treatment. However, it has been reported that intensive and uncontrolled use of antibiotics causes the development of antimicrobial resistance (AMR). AMR can be enhanced by vertical point mutations or horizontally transferred to genes inserted into plasmids. The mobile blaNDM gene can
be an excellent example of a transferable AMR gene in this context. In addition, the blaNDM gene has been detected globally on plasmids from various gram-negative bacteria. Moreover, the β-lactamase enzyme encoded by this gene decreases the effectiveness of beta-lactam group antibiotics (Acman et al. 2022). Beta-lactam group antibiotics are among the most preferred antibiotic derivatives for medication. Chief members of this group have been identified as penicillin, cephalosporins, monobactams, beta-lactamase inhibitors, and carbapenems (Codjoe and Donkor, 2018; Essack, 2001). Thiencamycin, a molecule synthesized by Streptomyces cattleya, is the ancestor of carbapenems (Livermore & Woodford, 2000). Furthermore, it has been determined that carbapenems have the broadest spectrum among beta-lactam group antibiotics and are highly effective against many pathogens except mycobacteria (Bonfiglio et al. 2002). It has been reported that carbapenem group antibiotics imipenem, ertapenem, doripenem and meropenem occur. Imipenem and meropenem have the most comprehensive spectrum. They are effective against gram-negative and positive microorganisms. On the other hand, meropenem is resistant to all beta-lactamase except staphylococcal and some gram-negative bacterial carbapenemase (Birnbaum et al. 1985; Edwards, 1995).

One of the most recent results of uncontrolled use of antimicrobial agents has been shown as carbapenem-resistant Enterobacteriaceae (CRE) in recent years. It has been reported that treatment options for these infections have narrowed considerably due to the global spread of CREs and their threat to public health (Codjoe and Donkor, 2018; Gutiérrez-Gutiérrez et al. 2016). Today, alternative treatment protocols have been created by using polymyxin, aminoglycoside and tigecycline group antibiotics together in the treatment of CRE infections. However, the possibility of resistance to these combinations and the toxic side effects of multiple antibiotic use are important sources of concern (Daikos and Markogiannakis, 2011; van Duin et al. 2013). At the same time, long and costly treatment of CRE infections is another important problem. In the United States, it has been reported that the average cost of treatment for a CRE infection for hospitals is between $22,400 and $66,000 per patient. At the same time, the incidence of infection in the US was declared as 2.93 per 100,000 people. Centers for Disease Control and Prevention (CDC), Clinical and Laboratory Standards Institute (CLSI), and EUCAST have required that resistance to carbapenems be revealed either phenotypically or genotypically in order to prevent information pollution. (Bartsch et al. 2017).

In the Republic of Turkey, poultry meat consumption is a preferred protein source due to increasing awareness of healthy nutrition and being more readily available in economic conditions. In our country, annual poultry meat production was reported as 2137, 2157, 2138, and 2075 thousand tons in 2017, 2018, 2019, and 2020, respectively (TUIK, 2022). In addition, our country’s per capita annual poultry meat consumption has been reported as 22, 21, 21, and 21 kg in 2017, 2018, 2019, and 2020, respectively (Anon, 2022). Our study aimed to determine the presence of foodborne carbapenem-resistant Enterobacteriaceae (CRE) in the Aksaray region, to make genetic analyses of the isolated bacteria by molecular methods, to reveal the resistance profiles phenotypically. In addition, a method study was included in our project by modifying the known methods such as ISO 21528-1:2017 in order to detect CREs more easily.

**MATERIALS AND METHODS**

**Material**

In the present study, 150 chicken meat samples (50 drumsticks, 50 breasts, and 50 wings) were analyzed in Aksaray University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology laboratory between September 2020 – September 2021. Samples were purchased from markets and sale points in Aksaray/Turkey randomly. The samples were brought to the laboratory under a cold chain and analyzed immediately.

**Methods**

**Culture Technique**

Isolation and identification of carbapenem-resistant *Enterobacteriaceae* (CRE) were investigated using the ISO 21528-1:2017 method, which we modified (ISO 2017). For this purpose, two experimental groups were designed. In the first group Buffered Peptone Water (Merck 107228) and the second group, Violet Red Bile Glucose Agar (Merck 110275) was altered by adding meropenem and ertapenem powders (MERONEM and INVANZ) (Figure 1 and 2) in the frame of resistance levels determined in EUCAST v12.0 (0.5 mg/L Ertapenem and 8 mg/L Meropenem) (EUCAST 2022). All 150 samples were analyzed in parallel by both modified methods.

**Genomic DNA Analysis**

In order to confirm the results of classical culture analysis, genomic DNA was obtained from all chicken samples with a DNA extraction kit, and the presence of carbapenemase genes was investigated by PCR assay directly from specimens. In our study, the target gene regions *bla*<sub>KPC</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub> were selected for confirmation purposes.

**DNA Extraction**

In our study, Genomic DNA Isolation Kit (Hybrigen MG-GDNA-01-250) was used for DNA isolation from chicken meat samples. The DNA extraction procedure was carried out following the instructions suggested by the manufacturer, and the
obtained genomic DNAs were stored at -20 degrees until PCR analysis.

**Primer Pairs of Carbapenemase Genes Used in the Study**

For the determination of carbapenemase genes in the genomic DNAs of the chicken meat samples, the method and primer pairs recommended by Poirel et al. (2011) were used. Primer pairs are shown in Table 1.

**PCR and Amplification Conditions**

The PCR mix was prepared by adding 1 μM of each primer and 5 μL of target DNA into 2X Hybrigen Master Mix. PCR reaction was performed thermal cycler (Bio-Rad MJ Mini) with an initial denaturation of 95°C for 4 min followed by 36 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 5 min the final extension (Poirel et al. 2011). All amplification products were analyzed by agarose gel (1.5%) electrophoresis at 80 V and 1 h. Then the gels were stained with Hibrgen SYBR Safe gel stain.

**RESULTS AND DISCUSSIONS**

A total of 150 chicken meat samples (50 drumsticks, 50 breasts, and 50 wings) were analyzed both with classic culture technique and PCR in the present study. The classical culture technique recommended by ISO was applied by modifying two different steps then the samples studied in parallel with both methods. In the first group, only BPW, and the second group, only Violet Red Bile Glucose agar was altered with meropenem and ertapenem powders. Simultaneously, genomic DNA was extracted from all chicken meat samples to compare with culture technique results.

According to the classic culture technique, in all 150 samples, CREs have not been detected phenotypically by modified BPW and Violet Red Bile Glucose agar. Likewise, carbapenemase genes such as blaKPC, blaNDM, and blaOXA-48 did not establish in PCR analyses of genomic DNAs gained from chicken meat samples to confirm the modified classical culture technique.

Present-day, within the framework of “safe food from farm to fork”, it has become necessary to ensure that consumers reach healthy foods. At the same time, it has been determined that the uncontrolled use of antibiotics poses a significant risk to public health. It leaves residues in food and causes the emergence of bacteria resistant to antibiotics.

![Figure 1. The first experimental group, modification of ISO 21528-1:2017 method by adding antibiotics into BPW](image)

In our study, chicken meats purchased from Central Anatolia Region (Aksaray) were examined in terms of CRE content using the ISO 21528-1:2017 method modified by us. According to our results, CREs in purchased samples could not be determined either by conventional modified culture method or by PCR. However, in late research, chicken meat was reported positive for CRE in various countries. In a study handled in China, it was reported that 15% of E. coli isolates isolated from retail meat products in China carried the mcr-1 gene. However, they reported that only one isolate with carbapenem resistance genes was obtained in their study (Yao et al. 2016). In another study conducted in China, the prevalence of the blaNDM gene was investigated in meat products between 2016-2018. A total of 222 chicken meat was analyzed, and 42 (38 E. coli, 1 K. pneumoniae, 3 P. mirabilis) carbapenemase-producing isolates were identified (Zhang et al. 2019). In research carried out in Algeria and France, 833 poultry samples were collected between 2014-2015 for monitoring ESBL, and carbapenemase. According to their results, samples from France were negative for ESBL and carbapenemase encoding genes. However, a total of 503 samples were collected in Algeria, and 128 (25.4%), 83 (16.5%), 46 (9.1%), and 132 (26.2%) were found positive for blaTEM, blaSHV, blaCTX-M, and blaOXA-58, respectively (Chabou et al. 2018). A study conducted in Egypt investigated the prevalence of ESBL and carbapenemase-producing...
Enterobacteriaceae in chicken meat. A total of 106 isolates were obtained by the MALDI-TOF method. Twelve (11.32%) of them were phenotypically resistant to carbapenems (Abdallah et al. 2015).

The ISO 21528-1:2017 method, which we preferred in our study, was modified to gain selectivity in terms of CRE at different stages due to the intense presence of the Enterobacteriaceae family in raw chicken meats. With this design, antibiotic supplements were added to BPW and Violet Red Bile Glucose Agar at the MIC breakpoints recommended by EUCAST v12.0. It was observed that the antibiotics we added to both BPW and Violet Red Bile Glucose agar effectively suppressed all bacterial flora in both stages. In a similar methodological study conducted in Turkey, the effect of agar selection on the CRE isolation rate was investigated. According to the results of the study, it was determined that the chromogenic agars did not have sufficient sensitivity. At the same time, CREs were not detected in raw milk samples (Al et al. 2020).

CONCLUSIONS

Antibiotic resistance is accepted as one of today's critical public health problems. The cost and difficulty of treating infections, especially those caused by resistant bacteria, also bring significant disadvantages. At this point, the "farm to fork" perspective must be well understood by both producers and consumers.

According to our study findings, both phenotypic and genotypic CRE have not yet been detected in chicken meats in Aksaray region. In this case, raising the producers' awareness about the conscious use of antibiotics, effective implementation of environmental residue monitoring policies, and planning more comprehensive studies can be effective in dealing with the problem.

Author Contributions: Tahsin Onur KEVENK and Zeki ARAS were both collected samples, made all analysis together. Tahsin Onur KEVENK also wrote the article.

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Table 1. Primers were used in the study for confirmation

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>blaKPC</td>
<td>CGTCTAGTTCGTGCTGCTTG</td>
<td>798</td>
<td>(Poirel et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>CTTGTCATCCCTGTTAGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blANDM</td>
<td>GGTGTTGCGATCTGTTTTTC</td>
<td>621</td>
<td>(Poirel et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>CGGAAATGGCTCATCACGATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaOXA-48</td>
<td>GCCGTGTAAAGGATGAACAC</td>
<td>438</td>
<td>(Poirel et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>CATCAAGTTCACCCAACCAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
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Acknowledgments
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Conflicts of Interest
The authors declare that they do not have any conflict of interest.

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15. EUCAST. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. 2022; Version 12.0.