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Investigation of the Resistance of Some Disinfectant Active Substances in ESBL-Producing *Enterobacteriaceae*

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Abstract

In this study, a total of 200 samples, including 100 neck skin and 100 cecum samples, were collected and analyzed from various poultry slaughterhouses on different sampling days. ESBL-producing *Enterobacteriaceae* were isolated and ESBL production was confirmed phenotypically by combined disk diffusion and E-test gradient strips. While ESBL production was confirmed in 10 (10%) of 100 neck skin samples, no significant ESBL production could be confirmed in 100 cecal samples. The broth microdilution method of Clinical and Laboratory Standards Institute (CLSI) was used to determine the resistance profiles against benzalkonium chloride (BC), cetylpyridinium chloride (CPC), N-alkyl dimethyl benzyl ammonium chloride (ADBAC) and potassium peroxymonosulfate (PPMS) disinfectants in 10 neck skin isolates with confirmed ESBL production. In the study, it has been determined that MIC_{50} and MIC_{90} values were respectively ADBAC (8 and 16 mg/L), BC (16 and 32 mg/L), CPC (16, and 32 mg/L), PPMS (≥ 1024 mg/L). The impacts of *Enterobacteriaceae* strains on food safety and public health are significant; Disinfectant resistance can lead to increased transmission of antibiotic-resistant bacteria, leading to serious infections in humans that are difficult to treat. For that reason, it is of great importance to develop effective control methods, including appropriate disinfectant use, hand hygiene and appropriate isolation measures, to prevent the spread of disinfectant resistant *Enterobacteriaceae* strains in food production systems.

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1. Introduction

Until the late 20th century, the safe use of empiric cephalosporins and fluoroquinolones by the clinician against major infections caused by *Enterobacteriaceae* strains has now been compromised by resistance conferred by extended-spectrum beta-lactamases (ESBLs) and AmpC enzymes. ESBLs are enzymes that can hydrolyze a wide variety of substrates, including most penicillins, cephalosporins (except cephamycins), and monobactams. ESBLs came to prominence in the early 1980s, first in Germany and shortly thereafter in France. These early ESBLs were reported to be structural mutants of TEM-1, TEM-2, and SHV-1, penicillinases common among *Escherichia coli* and *Klebsiella pneumoniae* (Denton, 2007).

Subsequently, in the 1990s, an increasing number of reports of TEM and SHV-induced ESBLs were published from many parts of the world, including the United Kingdom, particularly in association with nosocomial outbreaks. ESBL-producing *E. coli* (Palucha et al., 1999), *Salmonella enterica* serovar Typhimurium (Vahaboglu et al., 1996), *Citrobacter diversus* and *Klebsiella oxytoca* (El Harrif-Heraud et al., 1997) and *K. pneumoniae* have caused epidemics in various units of healthcare institutions (Venezia et al., 1995).

Although many epidemics are caused by the spread of microorganisms carrying certain resistance genes from patient to patient, Hibbert-Rogers et al. (1995) reported that the same microorganism carrying different resistance genes was transferred to different microorganisms through plasmid transfer between microorganisms.

Some studies have reported a significant increase in ESBL-producing *Enterobacteriaceae* from both community and patient origin. This increases the possibility that animals and food may play an important role in the epidemiology of ESBLs in community-acquired *Enterobacteriaceae* (Valverde et al., 2004; Liebana et al., 2006; Bertrand et al., 2006; Hasman et al., 2005; Riano et al., 2006).

Disinfectant resistance in ESBL-producing *Enterobacteriaceae* strains has become an issue of concern in the healthcare industry. Disinfectant resistance refers to the ability of bacteria to withstand the effects of disinfectants used to eliminate pathogen microorganisms from surfaces and equipment (Tong et al., 2021). ESBL-producing *Enterobacteriaceae* strains are a group of bacteria that produce extended-spectrum β-lactamases (ESBLs), enzymes that can break down certain types of antibiotics, making them resistant to treatment (Shaikh et al., 2015). These strains are often responsible for healthcare-associated infections and are a significant cause of morbidity and mortality worldwide (Navon-Venezia et al., 2017). Understanding the mechanisms of disinfectant resistance in these strains is crucial for developing effective infection control strategies and maintaining the effectiveness of disinfectants (Centers for Disease Control and Prevention [CDC], 2019).

Some different disinfectant resistance mechanisms have been reported in ESBL-producing *Enterobacteriaceae*. Efflux pumps are one of the main mechanisms of disinfectant resistance in ESBL-producing *Enterobacteriaceae* strains. Efflux pumps can facilitate the extrusion of a wide range of

substrates, conferring resistance to multiple antimicrobials (Anes et al., 2015). Many studies have reported efflux pumps as the primary mechanism of resistance to quaternary ammonium compounds (QACs), a common disinfectant used in healthcare settings (Nguyen et al., 2023; Hrovat et al., 2023). Efflux pumps expel the disinfectant from the bacterial cell, reducing its effectiveness and increasing the likelihood of bacterial survival (Tong et al., 2021).

Changes in cell membrane permeability may also contribute to disinfectant resistance in ESBL-producing *Enterobacteriaceae* strains. Resistance to disinfectants may result from changes in bacterial cell wall structure and function, which may reduce the ability of disinfectants to penetrate the bacterial cell (Boyce, 2023). Chlorine compounds commonly used as disinfectants can cause changes in membrane permeability, leading to resistance in bacterial strains (Rolbiecki et al., 2022). These changes may lead to reduced effectiveness of disinfectants and increased survival of bacteria, contributing to the spread of multidrug- resistant strains (Breijyeh et al., 2020; Chapuis et al., 2016).

Biofilm formation is another mechanism of disinfectant resistance in ESBL-producing *Enterobacteriaceae* strains. Biofilms are communities of bacteria that adhere to surfaces and can be difficult to eliminate with disinfectants (Dumaru et al., 2019). ESBL-producing *Enterobacteriaceae* strains have been shown to form biofilms that can protect themselves from disinfectants and antibiotics (Damiano et al., 2021; Laconi et al., 2023; Yılmaz & Güvensen, 2016). Murugesan et al. (2022) reported that ESBL-producing *E. coli* isolates had higher biofilm formation rates compared to non-ESBL-producing *E. coli* isolates (Murugesan et al., 2022). Biofilm formation may contribute to the persistence of antibiotic-resistant strains in healthcare settings, making them difficult to eradicate and increasing the risk of transmission to vulnerable patients.

The aim of the study was to determine the disinfectant resistance in phenotypically confirmed ESBL-producing *Enterobacteriaceae* isolates.

2. Materials and Methods

2.1. Isolation and Identification of ESBL-Producing *Enterobacteriaceae*:

Within the scope of this study, a total of 200 samples, including 100 neck skin and 100 cecum samples were collected from different poultry slaughterhouses in different time periods. Isolation of *Enterobacteriaceae* was done by classical culture technique using chromogenic ESBL (Extended spectrum beta-lactamase) medium (Himedia, M1829-500G). ESBL production in the obtained isolates was determined phenotypically by the combined disk diffusion method (Himedia, SD238-1KT) and E-test gradient strips (Himedia, EM132-30ST). Finally, the MIC values of the isolates that were found to produce ESBL phenotypically were determined with E-test gradient strips (Himedia, EM132-30ST) (The European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2017).

2.2. Detection of Disinfectant Resistance in Isolates:

The broth microdilution method recommended by CLSI was used to determine the resistance against BC (benzalkonium chloride, Sigma Aldrich 12060), CPC (cetylpyridinium chloride, Himedia GRM1526), ADBAC (alkyl (C12-16) dimethylbenzyl ammonium chloride, Sigma Aldrich) and PPMS (potassium peroxymonosulfate, Himedia RM2406) disinfectants (Clinical and Laboratory Standards Institute [CLSI], 2018) The disinfectant concentrations used to determine the disinfectant MIC values of the isolates were kept between 0.125- 1024 mg/L (Wu et al., 2015).

Enterobacteriaceae isolates, previously kept at -85 °C, were revived by incubating in Tryptic soy broth (Millipore, 43592) at 37 °C for 24 hours. The next day, they were planted on Tryptic soy agar (Millipore, 22091) and incubated at 37 °C for 24 hours. Colonies were taken from the agars and suspended in tubes containing 0,9 % sterile saline. The turbidity standard was verified using NanoDrop Spectrophotometer (NanoDrop ND-100, Delaware, USA). The value at which the culture in the broth was fixed at a wavelength of 0.08- 0.13 at 625 nm was considered optimum, and it was diluted at a ratio of 1:100 and inoculated into Mueller-Hinton broth (Millipore, 70192) (Wu et al., 2015). 96-well microtiter test plates containing double concentrations of disinfectant solution and MHB were added with 50 µl of suspended culture at 10^5 cfu/ ml bacteria per well. The plates were incubated at 37 °C for 24 hours. MICs were recorded as the lowest disinfectant concentrations that prevented visible growth of microorganisms (absence of turbidity) in the wells of microtiter plates (Wu et al., 2015). *Escherichia coli* ATCC 25922 and *Salmonella* Typhimurium ATCC 14028 were used as controls for disinfectant susceptibility tests.

2.3. Statistical Analysis

The data were evaluated using the Microsoft Excel application. Table 1 and Figure 1 were prepared with Microsoft Excel application.

3. Results

In the combined disk method applied for the phenotypic determination of ESBL production, ESBL production was detected in 66 (66%) of 100 neck skin isolates. In addition, ESBL production was detected in 50 (52.6%) of 95 cecum isolates. ESBL production was detected in 50 of 95 cecum isolates by the combined disk diffusion (Himedia, SD238-1KT) method. However, ESBL production could not be detected in the E-Test gradient strip (Himedia, EM132-30ST) application performed to confirm ESBL production and ESBL production was evaluated as negative. In the E-test gradient strip application performed to verify ESBL production and ESBL production was confirmed in 10 (10%) of the neck skin isolates.

E-test strips containing different cephalosporin group antibiotics were applied to these 10 confirmed isolates, based on the guideline determined by EUCAST (2017), to determine the MIC values.

Table 1. MIC values of ESBL-positive *Enterobacteriaceae* strains, respectively, against specific disinfectants.

Enterobacteriaceae **Isolates Number**	**Disinfectants and MIC's (mg/L)**			
	ADBAC*	**BC***	**CPC***	**PPMS***
1	8	16	16	>1024
2	16	16	16	>1024
3	2	8	16	>1024
4	8	16	16	>1024
5	4	8	8	>1024
6	8	4	16	>1024
7	4	8	16	>1024
8	32	64	32	>1024
9	16	32	32	1024
10	8	16	16	>1024

*ADBAC: N- alkyl dimethyl benzyl ammonium chloride
*BC: Benzalkonium chloride
*PPMS: Potassium peroxymonosulfate
*CPC: Cetylpyridinium chloride

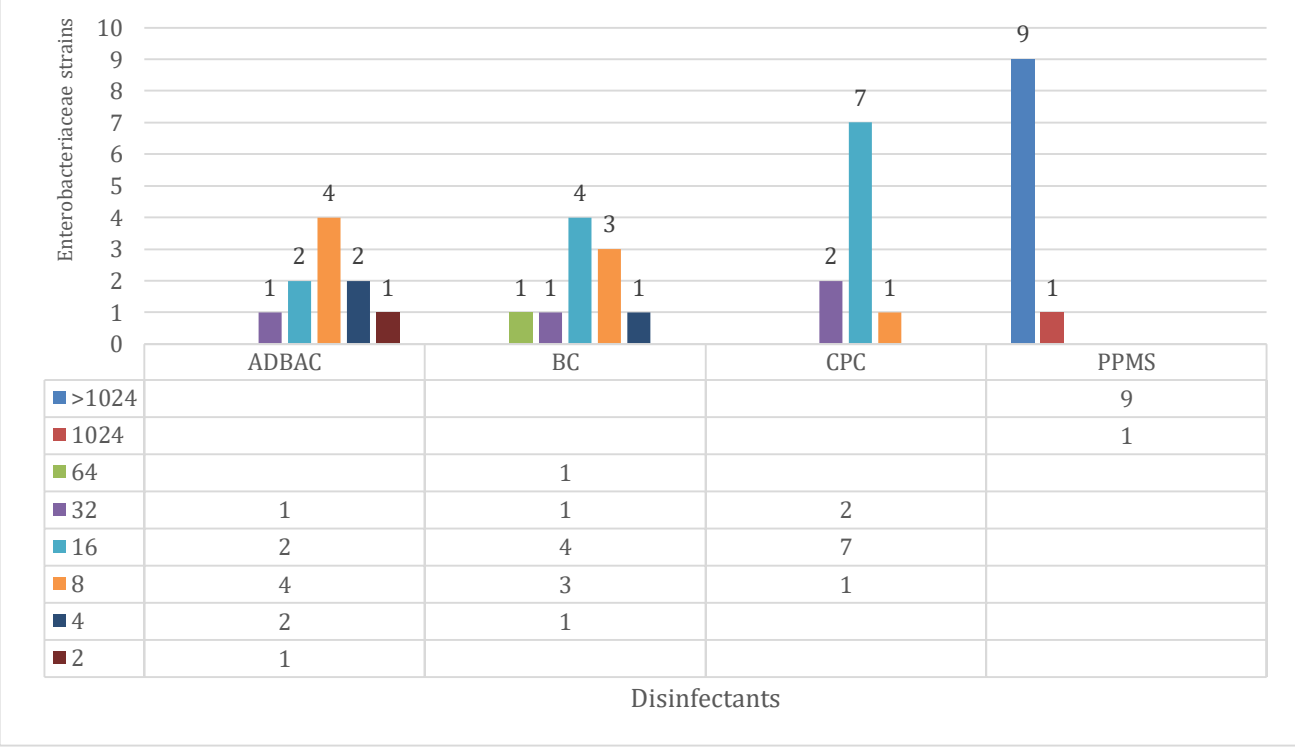

Figure 1. MIC values of ESBL-positive *Enterobacteriaceae* strains according to the microbroth dilution ratios developed against a certain disinfectant. (The numbers show how many strains in total have developed resistance to the relevant disinfectant at the relevant dilution rate, and the colors used symbolize the micro dilution rates.

As a result of the application, 6 (60%) of 10 chicken neck skin isolates were found to be highly resistant to ceftazidime and the other 4 (40%) were moderately resistant. Again, all 10 chicken neck skin isolates (100%) were found to be highly resistant to cefotaxime. Finally, 4 (40%) of 10 chicken neck skin isolates were found to be highly resistant to cefepime, the other 5 (50%) were moderately resistant and 1 (10%) was sensitive.

MICs of analyzed disinfectants were determined by CLSI's Broth microdilution method (2018) The highest MIC values were determined as >1024 mg/L (90%, n: 9) and 1024 mg/L (10%, n: 1) in PPMS. The other MIC values found 32 mg/L (10%, n: 1), 16 mg/L (20%, n: 2), 8 mg/L (40%, n: 4), 4 mg/L (20%, n: 2) and 2 mg/L (10%, n: 1) in ADBAC; 32 mg/L (20%, n: 2), 16 mg/L (70%, n: 7) and 8 mg/L (10%, n: 1) in CPC; 64 mg/L (10%, n: 1), 32 mg/L (10%, n: 1), 16 mg/L (40%, n: 4), 8 mg/L (30%, n: 3) and 4 mg/L (10%, n: 1) in BC.

In the study, it was determined that the samples had an ADBAC average of 10,6 mg/L and that it was the most sensitive active ingredient among the disinfectant active ingredients. It was found to have MIC values CPC 18,4 mg/L, BC 18,8 mg/L and PPMS >1024 mg/L, respectively.

It was found that MIC values varied between 32-2 mg/L for ADBAC, 64-4 mg/L for BC, 32-8 mg/L for CPC, and 1024 and above for PPMS. In the study, it has been determined MIC50 and MIC90 values were respectively ADBAC (8 and 16 mg/L), BC (16 and 32 mg/L), CPC (16 and 32 mg/L), PPMS (> 1024 mg/L).

4. Discussion

Studies have generally been carried out on the investigation of species and genus-specific disinfectant resistance in *Enterobacteriaceae*. For example, Boutarfi et al. (2019) conducted a study in Algeria with 77 *Enterobacter* spp. isolates, using hexachlorophene (CF) and benzalkonium chloride (BC) active ingredients. They found that their isolates tolerated disinfectants containing hexachlorophene (CF) and benzalkonium chloride (BC) at high rates such as 128 mg/L and 64 mg/L- 128 mg/L respectively. In another comprehensive study conducted by Morrissey et al. (2014), they collected 901 *Salmonella* spp., 368 *E. coli*, 60 *Klebsiella pneumonia*, 53 *Enterococcus faecium*, 56 *Enterococcus faecalis* and 54 *Enterobacter* spp. isolates obtained from certain university hospitals in Spain, the United Kingdom and Türkiye to determine the tolerance of benzalkonium chloride (BC). They found MIC_{50}- MIC_{90} rates 16- 16mg/L in *Salmonella* spp. isolates, 16- 32 mg/L in *E. coli* isolates, 8- 16 mg/L in *Klebsiella pneumonia* isolates, 16- 32 mg/L in *Enterobacter* spp isolates, 4- 8 mg/L in *Enterococcus faecium* isolates and 2 - 4 mg/L in *Enterococcus faecalis* isolates. In another comprehensive study conducted by Wu et al. (2015) collected 53 *Salmonella* spp. isolates, 33 *E. coli* isolates, 22 *Klebsiella pneumonia* isolates from retail raw meats, they found *Salmonella* MIC_{50}- MIC_{90} 128 mg/L in BC, 256- 256mg/L in CPC, 32-32 mg/L in DDAC (Didecyldimethylammonium chloride); for *E. coli* MIC_{50}- MIC_{90} 128- 128 mg/L in BC, 128- 128 mg/L in CPC, 16-32 mg/L in DDAC; for *Klebisella pneumonia* MIC_{50}- MIC_{90} 128- 128 mg/L in BC, 256- 256 mg/L in CPC, 32-32 mg/L in DDAC. Chapuis et

al. (2016) found that ADBAC and DDAC levels were between 64 and 512 mg/L in 43 environmental and clinical ESBL-positive *E. cloacae* isolates with microbroth dilution method in France. Zhang et al. (2016) found The MIC's of BC, CPC and DDAC in the 255 *E. coli* strains, from retail meat samples, were 16- 1024 mg/L, 8-512 mg/L, 4-1024 mg/L respectively. In another study conducted by Deus et al. (2017) in Germany, in 174 *E. coli* isolates were found to be ESBL-positive and the MIC_{50} - MIC_{90} values against BC were found to be 8 and 16 mg/L, respectively, according to the microbroth dilution method.

However, in this study, all strains belonging to the *Enterobacteriaceae* family, regardless of species and genus, were isolated from neck skin and cecum samples collected from poultry slaughterhouses, and the resistance of some disinfectant active substances from culture samples in this isolates was investigated. Since it is assumed that resistance transfer is easier among bacteria belonging to the *Enterobacteriaceae* family, studying disinfectant resistance as a whole with this bacterial family has made this study of high practical importance regarding the spread of antibiotic and disinfectant resistance in bacteria. Since this study contains important data for public health, it is thought to be a guide for future studies to be used in the food industry. In this study, although ADBAC and BC MIC values varied more among strains, these values were found to be more stable for CPC and PPMS. This suggests that resistant strains have emerged as a result of more frequent use of disinfectants containing BC and PPMS active ingredients in the field. Based on this, in order to inhibit all strains, PPMS (1024 mg/L <) should be used at the highest concentration and BC (64 mg/L) should be used at higher concentrations than ADBAC and CPC (32 mg/L).

Some studies have investigated whether there is a correlation between disinfectant resistance and biofilm formation. In this context. Sun et al. (2019) observed the susceptibility of 510 *E. coli* isolates isolated from retail poultry meat to 5 disinfectants and whether they formed biofilms. 194 isolates showed biofilm formation. Then, it was examined whether there was a correlation between disinfectant resistance and biofilm formation capacity of these isolates. They reported that while the biofilm formation capacity was directly correlated with BC resistance, no correlation was found with other disinfectants. In another study, Cai et al. (2018) investigated the response of biofilms formed by *Enterobacter cloacae*, *Klebsiella oxytoca* and *Citrobacter freundii* isolates, members of the *Enterobacteriaceae* family, to chloride-based disinfectants. For this purpose, chlorite-based SH (sodium hypochlorite), CD (chlorine dioxide), StAEW (strongly acidic electrolyzed water) and NEW (neutral electrolyzed water) were used. Biofilms formed by *E. cloacae* were more resistant to disinfectants than biofilms of the other two strains and SH (200 mg/L) was the most effective in the reduction of cell number in the biofilms of all strains.

Since biofilm formation varies depending on the surface, nutrient, pH, temperature, and other environmental conditions, conducting such studies by taking field conditions into account will significantly increase the applicability of the results to the field. (Chmielewski & Frank, 2003).

With the 2019 COVID epidemic, awareness of microorganisms in society increased and the use of

disinfectants increased approximately 3 times (Akyüz & Aytekin, 2022). There are 213 disinfectant and biocidal products licensed by the General Directorate of Public Health (Halk Sağlığı Genel Müdürlüğü, 2023) in Türkiye and allowed for use in personal areas, and the data obtained is expected to guide the use of disinfectants in the food and health sectors.

5. Conclusion

It is obvious that disinfectants will continue to exist in our lives as an indispensable element in the control of infectious diseases in abiotic environments by preventing or destroying the development of many pathogens, including bacteria and viruses. Understanding disinfectant resistance mechanisms in ESBL-producing *Enterobacteriaceae* strains; Using appropriate disinfection methods, selecting an effective disinfectant and using it in appropriate doses are of great importance in infection control and improving public health.

HACCP systems used in food processing plants do not directly mention bacterial biofilms, which can limit the effectiveness of disinfectants. Therefore, the creation of an updated HACCP system that foresees the assessment of biofilms in food environments and establishes an appropriate sanitisation plan, thus providing a much clearer contamination information and studies should be carried out to facilitate food production in biofilm-free processing systems of the food industry.

Among the disinfectant active ingredients used in this study, the most effective was found to be ADBAC, followed by CPC, BC and PPMS, respectively.

Frequent and high doses of the same disinfectant can cause damage to abiotic environments, chemical contamination of the environment, and the development of resistance. In order to prevent the use of high doses of disinfectants and to use appropriate disinfectants in effective doses, sero survey studies should be carried out to allow the development of resistance to be investigated at certain periods.

Future research directions should focus on identifying new disinfectants that can effectively control disinfectant-resistant bacteria. Additionally, research should adopt alternative and green approaches such as bacteriophages or probiotics to control the spread of disinfectant-resistant bacteria. In addition, more comprehensive studies should be conducted to understand the genetic basis of disinfectant resistance in ESBL-producing *Enterobacteriaceae* strains and to obtain information about the evolution and spread of these resistance mechanisms.

Ethical Statement

There is no need to obtain ethics committee permission for this study due to his article does not contain any studies with human or animal subjects. However, the study was conducted in accordance with ethical principles.

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Presentation Information

The findings of this study have not been presented at any conference or journal.

Conflicts of Interest

The authors declare no conflicts of interest regarding this study. Any institution or organization providing funding for this research did not have any role in the design, data collection, analysis, interpretation, or publication to influence or distort the findings.

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