Assessment of carbapenem resistance and carbapenemase genes in wastewater from cattle slaughterhouses: Implications for environmental antibiotic resistance surveillance

Serhat AL[1,a,✉], Adalet DIŞHAN[2,b], Mukaddes BAREL[1,c], Candan GÜNGÖR[1,d], Harun HIZLISOY[1,e], Fulden KARADAL[3,f], Nurhan ERTAŞ ONMAZ[1,g], Yeliz YILDIRIM[1,h], Zafer GÖNÜLALAN[1,i]

[1]Erciyes University, Veterinary Faculty, Department of Veterinary Public Health, Kayseri, Türkiye; [2]Yozgat Bozok University, Veterinary Faculty, Department of Food Hygiene and Technology, Yozgat, Türkiye; [3]Nigde Omer Halisdemir University, Bor Vocational School Milk and Dairy Products Technologies, Nigde, Türkiye

[a]ORCID: 0000-0003-2721-9275; [b]ORCID: 0000-0001-8097-1648; [c]ORCID: 0000-0002-1170-8632; [d]ORCID: 0000-0002-4850-7447; [e]ORCID: 0000-0003-3391-0185; [f]ORCID: 0000-0002-5113-5883; [g]ORCID: 0000-0002-4679-6548; [h]ORCID: 0000-0001-8783-3889; [i]ORCID: 0000-0002-3935-6296

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✉Corresponding author
serhatal@erciyes.edu.tr

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ABSTRACT

The objectives of the study were to determine the prevalence of carbapenem-resistant Gram-negative bacteria and assess the potential risks associated with cattle slaughterhouse wastewater. A total of 270 wastewater samples were collected from 10 different cattle slaughterhouses for microbiological analysis. Conventional culture methods were employed, followed by disc diffusion, the Modified Carbapenem Inactivation Method (mCIM), and the Modified Hodge Test (MHT) to identify carbapenem resistance. The Vitek® 2 compact system was used for species identification and antibiotic susceptibility profiling. Conventional and quantitative PCR (qPCR) were performed to detect specific carbapenemase genes (bla_{KPC}, bla_{NDM}, and bla_{OXA-48}), among the collected 17 (6.30%) carbapenem-resistant isolates, one *Pseudomonas fluorescens* (0.37%), one *Aeromonas hydrophila* (0.37%), and two *Aeromonas sobria* (0.74%) exhibited resistance to meropenem. Additionally, six *P. fluorescens* (2.22%) and two *A. hydrophila* (0.74%) isolates demonstrated intermediate resistance to meropenem. Furthermore, five carbapenem-resistant isolates were identified as *Stenotrophomonas maltophilia* (1.85%), known to be inherently resistant to most antibiotics. Ten different antibiotics were evaluated in the antibiotic resistance panel and all *Aeromonas* isolates were found to be resistant to cefazolin and one *A. hydrophila* was detected as multi-drug resistant. The revealed data indicates that slaughterhouse wastewater can serve as a reservoir for antibiotic-resistant opportunistic pathogens. However, it may not pose a substantial risk for the distribution of carbapenemases, thereby mitigating concerns related to potential public health and environmental hazards associated with this aspect of slaughterhouse operations. This study contributes to understanding of antibiotic resistance in livestock-related environments and underscores the importance of continued monitoring and surveillance.

Introduction

Antimicrobial resistance (AMR) is a critical global health concern with profound implications for public health and β-lactam class antibiotics are at the forefront of this concern. β-lactams, which include penicillins, cephalosporins, and carbapenems, have played important role in treatment of bacterial infections for decades (25). However, the overuse and misuse of these antibiotics have led to the development of resistance in many bacterial strains and preserving the effectiveness of β-lactams is

essential to ensure the success of modern medicine in bacterial diseases (8). Carbapenems are considered one of the last lines of defense against bacterial infections, especially those caused by multidrug-resistant Gram-negative bacteria (20). The emergence of carbapenem-resistant strains limit treatment options, making infections increasingly difficult to manage. One of the most common and epidemiologically significant mechanisms of carbapenem resistance (CR) in gram-negative bacteria is the production of carbapenemases that hydrolyze carbapenem antibiotics. These enzymes can be classified into different classes, including serine β-lactamases (KPC), metallo-β-lactamases (NDM, VIM), and oxacillinases (OXA-48) (19). They are often encoded by mobile genetic elements such as plasmids, which can be easily transferred between bacteria, facilitating the spread of resistance. CR represents serious healthcare challenges, as infections caused by pathogens difficult to treat and can lead to high mortality rates particularly in sensitive individuals. Ongoing surveillance and research are crucial strategies for preventing the spread of carbapenemases carried by mobile genetic elements. These elements have repeatedly been shown to be easily transmissible, and understanding their impact on human, animal, and environmental interactions is essential (21).

Slaughterhouse wastewater, if not managed properly, can serve as a significant source of environmental contamination. Within the environment, intestinal and environmental bacteria have the capacity to exchange AMR genes and other genetic elements through mobile genetic mechanisms (12). This exchange of genetic material raises concerns about the diversity of AMR genes, particularly in environments with rich microbiota such as slaughterhouse wastewater. The dispersion of multidrug-resistant microorganisms, even non-pathogenic strains, into the environment represents a substantial public health risk. To address these concerns, it is imperative to generate scientific data that clarify the risks associated with wastewater, thus advancing our understanding of the epidemiology of the contamination pathway. Thus, the primary objective of this study is to collect molecular data on the presence of CR in wastewater samples obtained from cattle slaughterhouses in Kayseri, Türkiye.

Materials and Methods

Bacterial strains: *E. coli* MSC228 (pMSC115) and *Klebsiella pneumoniae* ATCC BAA-1705 (for bla_{KPC}), *E. coli* MSC234 (pMSC122) (for bla_{OXA-48}), and *E. coli* MSC229 (pMSC116) (for bla_{NDM}) reference strains were used as positive controls. *E. coli* ATCC 25922 were also used as negative control for validation of all phenotypical and molecular analysis of the study.

Sampling: A total of 270 cattle slaughterhouse wastewater samples were aseptically collected after slaughtering before the sanitation process from the main drain of the plants to aseptic tubes from 10 different cattle slaughterhouses in Kayseri, Türkiye between June 2018 and March 2019. Sampling was performed each consecutive week covering just one sample on the same day at the same facility. The 50 mL of samples were carried to the laboratory under the cold chain and analyzed immediately.

Conventional culture technique: Carbapenem-resistant bacteria isolation from slaughterhouse wastewater was carried out following laboratory protocol proposed by the Centers for Disease Control and Prevention (CDC) with 100 µl wastewater samples (6). Suspicious isolates were also morphologically examined with streaked out directly onto Chromagar ™ KPC (Chromagar, France) and ChromID® Carba Smart (Biomerieux, France).

Determination of CR profiles of isolates: For this purpose, disc diffusion test, Modified Hodge Test (MHT) and Modified Carbapenemase Inactivation method (mCIM) were used in the study. Disc diffusion method for the phenotypic determination of CR was performed and the inhibition zones were evaluated as pointed out by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2, 28). The MHT and mCIM methods were also applied to verify the CR status by following the Clinical and Laboratory Standards Institute (CLSI) protocols (9, 10).

Species identification and antibiotic resistance profiles of carbapenem resistant isolates: Isolates found to be phenotypically CR were freshly cultured on blood agar at 37 °C for 24 h and were identified with using Vitek® 2 Compact system with GN ID enteric cards (Biomerieux, France). Besides, antibiotic resistance profiles and minimal inhibitory concentration (MIC) values of the isolates for piperacillin/tazobactam, cefazolin, cefoxitin, ceftazidime, cefepime, meropenem, amikacin, gentamicin, ciprofloxacin, colistin, trimethoprim/sulfamethoxazole were also determined by using the same system with using AST cards in according to the manufacturer's instructions (Biomerieux, France).

Determination of carbapenemase genes from carbapenem resistant isolates: Genomic DNA (gDNA) isolation was carried out using the Instagene DNA

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extraction kit (Bio-Rad, USA) and Qubit 3 Fluorometric quantitation device (Thermo, USA) was utilized to assess gDNA isolation efficiency and determine total gDNA quantities (ng/µl). All gDNA samples were stored at -20°C until further analysis. Both conventional and quantitative PCR (qPCR) analyses were performed to validate the presence of carbapenemase genes in carbapenem-resistant isolates. For conventional amplification of the *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ gen regions, gDNA isolates were subjected to multiplex PCR analysis with specific primers designed (24). DreamTaq Hot Start PCR (2x) Master Mix (Thermo Fisher, USA) was used for PCR analysis according to the manufacturer's instructions with Arktic™ Thermal Cycler (Thermo Fisher, USA). All obtained amplicons were loaded on 1.5% agarose gel with GelRed Nucleic Acid Gel Stain (Biotium, USA) and subjected to electrophoresis for 45 minutes at 120 V and visualized with Chemidoc XRS+ System (Bio-Rad, ABD). In the qPCR for same carbapenemase gene regions, primers and protocols designed by Subirats et al. (26) were employed using SYBR Green Master Mix (Bio-Rad, USA) on the CFX96 Real-Time PCR system (Bio-Rad, USA). The melting curve analyzes were also added to the protocol. Detection rates and quantitative values in the samples were determined according to amplification curves, melting curve analysis and Ct (dR) data. The primers and their properties used in the study are shown in Table 1.

Results

In the study, 102 different colonies were obtained as carbapenem-resistant (CR) suspicious Gram-negative bacteria from a total of 270 slaughterhouse wastewater samples analyzed by the conventional culture method. Of these, 72 were detected as CR due to their growth on two different chromogenic agars. Among these suspect isolates, 17 (6.30%) were found to be phenotypically resistant to meropenem and their CR condition was further validated through an automated system. However, it was noted that all the isolates tested negative in both the MHT and mCIM tests. Additionally, antibiotic susceptibility testing with the Vitek® 2 system revealed one *Pseudomonas fluorescens* (0.37%), one *Aeromonas hydrophila* (0.37%) and two *Aeromonas sobria* (0.74%) isolates to be meropenem resistant. The study also identified six *P. fluorescens* (2.22%) and two *A. hydrophila* (0.74%) isolates were found to be moderately resistant. Furthermore, it was revealed that five of the isolates (1.85%) were found as *Stenotrophomonas maltophilia* which was naturally resistant to carbapenem group antibiotics. Antibiotic susceptibility profiles and MIC values of isolates are shown in Table 2. None of three enzyme gene regions (*bla*$_{KPC}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$) were found in all 72 CR suspected isolates obtained in the study analyzed by conventional and qPCR analyses.

Table 1. Primers pairs used in the PCR and qPCR performed in the study.

Target Gene	Primer	Sequence (5'-3')	Product (bp)	Reference
bla$_{KPC}$	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	798	12
	KPC-Rm	CTTGTCATCCTTGTTAGGCG		
bla$_{NDM}$	NDM-F	GGTTTGGCGATCTGGTTTTC	621	
	NDM-R	CGGAATGGCTCATCACGATC		
bla$_{OXA-48}$	OXA-F	GCGTGGTTAAGGATGAACAC	438	
	OXA-R	CATCAAGTTCAACCCAACCG		
bla$_{KPC}$ alleles	Kpc-rtF	CAGCTCATTCAAGGGCTTTC	196	13
	Kpc-rtR	GGCGGCGTTATCACTGTATT		
bla$_{NDM}$ alleles	Ndm-rtF	GATTGCGACTTATGCCAATG	189	
	Ndm-rtR	TCGATCCCAACGGTGATATT		
bla$_{OXA-48}$ and related alleles	Oxa-rtF	AGGCACGTATGAGCAAGATG	189	
	Oxa-rtR	TGGCTTGTTTGACAATACGC		

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Table 2. CR Isolates and antibiotic resistance profiles obtained in the study.

Bacteria	**Antibiotics**																					
	Piperacillin/ tazobactam		Cefazolin		Cefoxitin		Ceftazidime		Cefepime		Meropenem		Amikacin		Gentamicin		Ciprofloxacin		Colistin		Trimethoprim /sulfamethoxazole	
	AS	MIC	AS	MIC	AS	MIC	AS	MIC	AS	MIC	AS	MIC	AS	MIC	AS	MIC	AS	MIC	AS	MIC	AS	MIC
P. fluorescens	S	8	-	-	-	-	S	2	S	≤0.5	I	8	S	≤2	S	≤1	S	≤0.25	S	≤0.5	-	-
P. fluorescens	S	8	-	-	-	-	S	2	S	2	I	8	S	≤2	S	≤1	S	≤0.25	R	≥16	-	-
P. fluorescens	S	8	-	-	-	-	S	2	S	2	I	8	S	≤2	S	≤1	S	≤0.25	R	4	-	-
P. fluorescens	S	16	-	-	-	-	S	2	S	2	I	4	S	≤2	S	≤1	R	2	S	≤0.5	-	-
P. fluorescens	R	32	-	-	-	-	S	8	S	8	I	4	S	≤2	S	≤1	S	≤0.25	R	≥16	-	-
P. fluorescens	R	≥64	-	-	-	-	R	≥64	R	16	I	8	S	4	S	≤1	S	≤0.25	S	≤0.5	-	-
P. fluorescens	R	32	-	-	-	-	R	16	S	8	R	≥16	S	4	S	≤1	S	≤0.25	S	≤0.5		
A. hydrophila	S	≤4	R	≥64	S	≤4	S	≤0.12	S	2	I	4	S	≤2	S	4	S	1	-	-	R	80
A. hydrophila	S	≤4	R	16	S	≤4	S	≤0.12	S	≤0.12	I	4	S	≤2	S	≤1	S	≤0.25	-	-	S	≤20
A. hydrophila	S	≤4	R	≥64	I	16	S	1	S	2	R	≥16	S	≤2	S	4	R	≥4	-	-	R	≥320
A. sobria	S	≤4	R	32	S	≤4	S	≤0.12	S	≤0.12	R	≥16	S	≤2	S	≤1	S	≤0.25	-	-	S	≤20
A. sobria	S	≤4	R	≥64	R	≥64	S	2	S	≤0.12	R	≥16	S	≤2	S	≤1	S	≤0.25	-	-	S	≤20
S. maltophilia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	80
S. maltophilia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	≤20
S. maltophilia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	≤20
S. maltophilia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	40
S. maltophilia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	≤20

AS: Antibiotic Susceptibility; **MIC:** Minimum Inhibition Concentration (mg/L); **S:** Sensetive; **I:** Intermediate; **R:** Resistant; **-:** Not Detected.

Discussion and Conclusion

The dissemination of AMR and its intricate interactions with the environment, food facility, and environmental microbiota represent a multifaceted concern. AMR genes, originating primarily from clinical and agricultural sources, can be released into the environment through various pathways, including the discharge of treated and untreated wastewater (13). Once in the environment, these genes can persist and accumulate, promoting the emergence of antibiotic-resistant bacteria in natural ecosystems. The environmental microbiota, comprising a vast array of microorganisms, serves as both a source and a reservoir of resistance genes. These genes can be mobilized and horizontally transferred between bacterial species, including potential pathogens. This genetic exchange not only threatens the effectiveness of antibiotics in healthcare but also raises concerns about the potential emergence of infectious diseases driven by multidrug-resistant pathogens (2). Consequently, comprehending the mechanisms underlying AMR spread in the environment is essential for developing strategies to mitigate its impact on human health and the ecological balance.

The main objective of the study is to assess the epidemiological significance of cattle slaughterhouse wastewater, known for its high bacterial community and abundance of mobile genetic elements, with respect to carbapenem resistance. Notably, insufficient data revealed regarding the presence of CR in slaughterhouse wastewater released into the environment in Türkiye. It is worth mentioning that epidemiological research on CR is limited in general, with many studies primarily focused on human infections and relying on conventional microbiology and phenotypic screening methodologies. The study is based on this background and the wastewater of cattle slaughterhouses was investigated to demonstrate the epidemiological importance in the spread of CR and some carbapenemase genes. In the study, 17 (6.30%) CR Gram-negative bacteria were isolated in 270 cattle slaughterhouse wastewater samples. In consequence of these findings, it is concluded that the analyzes based on the conventional method and selective agar separation need to be verified with molecular methods. All 72 suspected isolates obtained by conventional culture were examined by both PCR and qPCR for detection of bla_{KPC}, bla_{NDM}, and bla_{OXA-48} and none of these gene regions was found. In a study conducted in the same region of Türkiye, raw milk samples examined for same CR genes and these genes were also not found (1). It can be said that animal related environments have not been contributed carbapenemase gene distribution in Türkiye. Intrinsic resistance to carbapenems in environmental bacterial isolates can arise through mechanisms other than carbapenemase production. These mechanisms include alterations in the permeability of the bacterial cell wall, efflux pump overexpression, and mutation and transformation in antibiotic target structures. Changes in the outer membrane proteins can reduce drug entry, while active efflux pumps can expel the antibiotic before it reaches its target. Additionally, mutations in antibiotic target structures can decrease the binding affinity of carbapenems (3).

Carbapenem class antibiotics are classified as category A antibiotics and not authorized for use in veterinary fields and food-producing animals in the EU. This restriction can be seen as a main reason of these findings (11). CR screening of animal originated foods, animal environment, and food processing plants still crucial to protect public and animal health. Because carbapenem resistance have been reported in food animal niches in different part of the world. Carbapenem-resistant different *P. aeruginosa*, *Pseudomonas putida* and *Pseudomonas otitidis* were isolated from retail chicken and pork meats (30). Chabou et al. (7) reported poultry fecal samples were positive for bla_{OXA-58} carbapenemase. Also, in France carbapenemase-producing *Acinetobacter* spp. were isolated in rectal swabs were collected from cows and it is confirmed that nine isolates harbored bla_{OXA-23} β-lactamase gene (23). In addition to the carbapenemase gene regions we studied, several other carbapenemase gene variants have also emerged in the dissemination of carbapenem resistance among bacteria. Notably, genes like bla_{VIM}, bla_{IMP}, and bla_{GES} encode metallo-β-lactamases, which are enzymes capable of hydrolyzing carbapenems and other β-lactams. Understanding the diversity of carbapenemase gene regions beyond the well-known ones is essential for surveillance and control efforts aimed at limiting the spread of CR (14).

The ubiquitous presence of *Pseudomonas* species in various environmental niches has long been recognized (18). However, the emergence and spread of antibiotic-resistant strains within these environmental reservoirs present a critical concern for public health and environmental sustainability. The environmental distribution of antibiotic-resistant *Pseudomonas* species serves as a significant reservoir for the genetic elements conferring resistance. Antibiotic pollution in the environment leads ubiquitous bacteria resistant to promoting the development of resistance mechanisms. Antibiotics are spread into the environment via humans and domestic animals excretes (urine and feces) due to improper disposal or mishandling of unused drugs. Also, direct environmental contamination from animal production facilities contributes to antibiotic residues in the environment through waste streams (17). Investigating the prevalence and dynamics of resistance genes in these environments is crucial to better understanding of antimicrobial resistance diversity. The connection between environmental and clinical strains of *Pseudomonas* is an important aspect of antibiotic resistance dissemination.

Environmental reservoirs can act as sources of resistance genes, facilitating the transfer of these genes to clinically relevant strains. This horizontal gene transfer between food-borne, environmental and clinical cases emphasizes the importance of a comprehensive approach in understanding the epidemiology of antibiotic resistance (29). Also, the survival capabilities of *Pseudomonas* spp. conferred by resistance genes may influence the composition and diversity of microbial populations. Thus, ubiquitous bacteria have emerged as significant nosocomial infection agents, presenting clinical challenges that are difficult to overcome, such as carbapenem-resistant *P. aeruginosa* (CRPA) (16).

This ecological impact has broader implications for ecosystem functioning, emphasizing the need to assess the antibiotic resistance status in environmental microbiomes. The strains determined to be negative for the carbapenem genes investigated in the study are not significant in terms of antibiotic resistance distribution. However, studies in which other carbapenemase genes are investigated in detail need to be sustained. In a study, two *P. fluorescens* isolates were found to harbor IMP-22 isolated from wastewater collected from an urban sewage plant (22).

In our study, all phenotypically carbapenem-resistant *Aeromonas* isolates were detected as cefazolin resistant and similarly Hilt et al. (15) found carbapenem resistant *A. hydrophila* from two solid organ transplant patients were also resistant to cephazolin. In another study conducted in China, nine out of 58 *Aeromonas* isolates responsible for bacteremia were identified as multi-drug resistant (27). Likewise, one of our *A. hydrophila* isolates exhibited resistance to four different antibiotics can be classified as multi-drug resistant. This context emphasizes the significance of the environmental distribution of opportunistic pathogens, including *Pseudomonas* and *Aeromonas*, in shaping the patterns of antibiotic resistance distribution.

Recognizing the interaction of human, animal, and environmental health, the environmental distribution of antibiotic-resistant *Pseudomonas* species emphasizes the One Health approach (5, 29). Monitoring and mitigating resistance in environmental settings are essential components of a comprehensive strategy to combat antibiotic resistance across all domains. Comprehensive surveillance of environmental reservoirs is crucial for early detection of emerging resistance patterns. Understanding the prevalence and dynamics of antibiotic resistance in ubiquitous opportunistic pathogen species within diverse ecosystems allows for the implementation of timely interventions and mitigation strategies. Insights into the environmental distribution of antibiotic-resistant Gram-negatives provide critical information for designing effective antimicrobial control programs. Strategies aimed at preserving the efficacy of existing antibiotics must lead both clinical and environmental dimensions to address the complex interplay between human activities, microbial ecology, and antibiotic resistance. A multidisciplinary approach, integrating microbiology, ecology, and public health, is essential for unraveling the intricate dynamics of resistance dissemination and devising sustainable strategies to mitigate its impact on human and environmental health.

Our findings suggest that cattle slaughterhouse wastewater may not be a significant source for the distribution of CR and carbapenemase genes, reducing concerns about potential public health and environmental risks associated with this specific aspect of slaughterhouse operations in Kayseri, Türkiye. However, it is important to note that the prevalence of carbapenem-resistant bacteria in animal-related environments, including slaughterhouses, remains an important area of concern globally. The study contributes to our understanding of AMR in livestock-related environments, shedding light on the current status of carbapenem resistance in slaughterhouse wastewater. These results underscore the importance of continued monitoring and surveillance of AMR in environmental niches to assess potential risks in terms of One Health perspective. Future research should focus on expanding the scope of surveillance beyond known carbapenemase genes, as our study did not detect these specific genes in the isolates. Investigating the presence of other carbapenemase gene variants and exploring their dissemination dynamics in animal-related environments will provide valuable insights.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors have not reported any conflict of interest with this original research article.

Author Contributions

SA: Conceptualization, data curation, microbiological analysis; writing of original draft; AD: Microbiological analysis; MB: Microbiological analysis; CG: Microbiological analysis; HH: Microbiological analysis, validation; FK: Microbiological analysis, validation; NEO: Microbiological analysis, validation; YY: Validation, review & editing; ZG: Validation, review & editing.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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