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Evaluation of *Escherichia coli* antibiotic resistance in brine fed to a closed-cycle graduation tower

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ABSTRACT

The objective of this study was to determine the drug resistance profile and the presence of genes that cause the ESBL mechanism in the *Escherichia coli* isolated from brine that circulates in a closed-cycle graduation tower. A disk diffusion method was employed to evaluate antibiotic resistance and a polymerase chain reaction (PCR) technique with specific primers was used to detect genes that cause the production of extended-spectrum β-lactamases (ESBLs). The total number of *E. coli* strains isolated was 40. The highest observed resistance levels were noted for ampicillin, amoxicillin/clavulanic acid, and ticarcillin. Most strains were resistant to 2 antibiotics, but one strain was found to be resistant to up to 8 antibiotics simultaneously. No MDR (multidrug-resistant) strains were observed. A total of 39 isolates were subjected to gene testing, and at least one gene conditioning the extended-spectrum β-lactamase (ESBL) mechanism was identified. The most prevalent genes responsible for β-lactamase production were *blaTEM* (87.5%) and *blaCTXM-9* (70%). The brine feeding the graduation tower was found to be a reservoir of drug-resistant *E. coli* strains, which represent a potential threat to public health. The presence of drug-resistant *E. coli* in the brine water may be a health risk to those inhaling in the graduation tower, which casts doubt on the health-promoting properties of such treatments. The results suggest the need for further research to observe the propagation of drug resistance in *E. coli*.

Keywords: closed-cycle brine graduation tower, drug-resistance, *Escherichia coli,* disk diffusion method, PCR.

INTRODUCTION

Poland has experienced a significant increase in the use of brine graduation towers in recent years. There are two primary reasons for this situation. Firstly, there is a growing awareness, particularly among residents of large urban areas, of the health benefits of brine. Secondly, there is frequently an excess of air quality standards in Poland, especially in the southern regions during the winter months, and smog is a recurring phenomenon. Krakow is among the Polish cities in which permissible air pollution levels are routinely exceeded. The pollutants responsible for this phenomenon include, but are not limited to, carbon oxides, nitrogen oxides, sulfur oxides, particulate matter, heavy metals, and dioxins (Wojtal, 2018; Rataj and Holewa-Rataj, 2020). Therefore, the authorities in Krakow, as well as in other large cities facing smog problems, are taking the measures to improve air quality. One of the concepts under discussion is the construction of municipal closed-cycle brine graduation towers, as they offer the possibility of taking health-promoting inhalations locally, without the need to visit a sanatorium.

Brine graduation towers, also known as thorn houses, are unique wooden structures made of spruce or oak wood. They are located in the open air and are used for inhalation of brine for therapeutic purposes. The structure of brine graduation towers consists in wooden logs filled with blackthorn branches. They have a complex brine distribution system, including valves, piping, and pumps (Figure 1) (Kostrzyn et al., 2018; Langer, 2012). The functioning of the graduation tower is based on the flow of brine from the top down. The brine droplets are broken up with the help of a blackthorn, followed by evaporation, creating a therapeutic aerosol. There are two types of graduation towers. Open-cycle graduation towers are built at the source of the brine, where its fresh supply is constantly provided. These types of graduation towers are often found in health resorts (sanatoriums) such as Ciechocinek or Inowrocław (Poland). On the other hand, the closed-cycle graduation towers are gaining popularity, especially in urban agglomerations, where brine is specially supplied (Faracik, 2020; Engelhardt and Von Borstel, 2015; d'Obyrn and Rajchel, 2015). The closed-cycle brine graduation tower operates on the principle of cyclic brine flow through the structure. In these towers, brine is used many times. Brine is drawn from a reservoir situated at the tower's base and pumped to the upper levels. From there, it is distributed through a system of trickles and gutters, traversing the tower branches and concentrating through the process of evaporation. Brine atomization is a phenomenon that occurs when it breaks into smaller droplets. The effect of evaporation is most noticeable on the days with abundant sunshine and strong winds. The final stage of the cycle involves the flow of brine down the gutter system into the tank. The collected brine is then directed back to the top of the graduation tower using pumps (Rogula-Kozłowska et al., 2022; Chudzińska and Dybczyńska-Bułyszko, 2019; Engelhardt and Von Borstel, 2015; d'Obyrn and Rajchel, 2015; Bodziacki and Wolny-Koładka, 2023).

As demonstrated by Burkowska-But et al. (2014), the quality of air surrounding the graduation tower is significantly superior to that of other areas of the city. As a result, the area near the graduation tower is considered an ideal location for rest and therapy for individuals with various health conditions. The microclimate of the area is comparable to that of a coastal climate, with similar therapeutic benefits (Kuchcik et al., 2013). Inhalation of brine, which is abundant in minerals such as sodium chloride, iodine, bromine, calcium, and iron compounds, can provide health benefits and support the treatment of respiratory and cardiovascular diseases, such as sinusitis, asthma, and rhinitis. It can also be helpful for people with immune system problems and iodine deficiencies, as well as those with hypertension or thyroid disorders (Pawalczyk et al., 2012; Kuchcik et al., 2013).

Currently, the scientific literature only provides information on the construction of graduation towers, their technological and functional aspects, and the benefits of brine inhalation for human health (Faracik, 2020; Tłoczek, 1958; Luścińska and Gadziemska, 2011; Chudzińska and Dybczyńska-Bułyszko, 2019; Engelhardt and Von Borstel, 2015; Affelt, 2003; Czubernat and Tomaszewska, 2021; Krzyżaniak-Sitarz, 2012). There is still a lack of information regarding potential microbial contamination of brine and the drug resistance of microorganisms isolated from it. However, our previous publications were the first in the world to assess the microbiological properties of brine and brine mist obtained in a

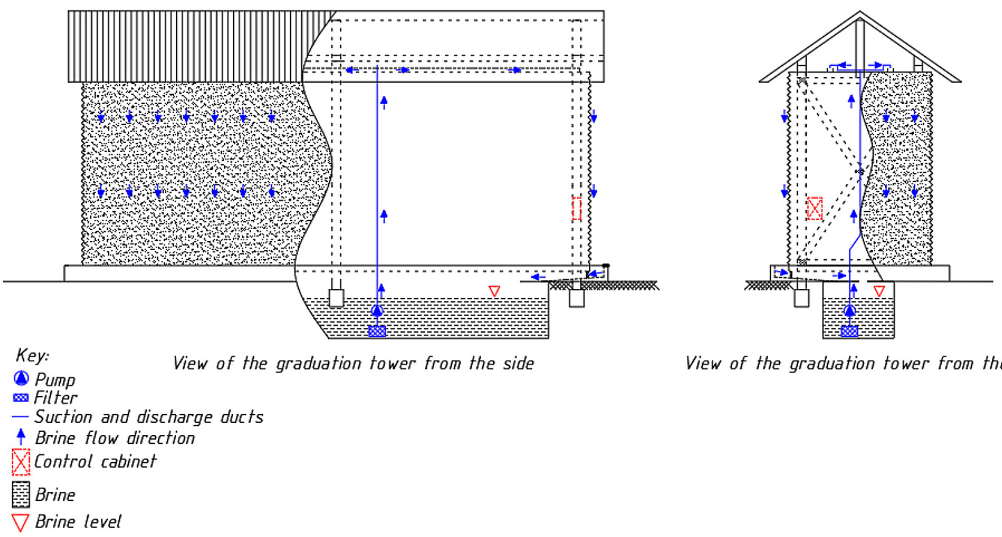

Figure 1. Schematic of a closed-cycle graduation tower structure (Bodziacki and Wolny-Koładka, 2023)

closed-cycle graduation tower (Bodziacki and Wolny-Koładka, 2023; Bodziacki et al., 2024). The study carried out by the authors' team indicates that the brine within the closed system, which supplies the brine graduation tower, is microbiologically contaminated with *Escherichia coli*. To further investigate this issue, a thorough analysis of the drug resistance profile of *E. coli* isolates from the graduation tower brine was conducted, as presented in this study.

E. coli is a bacterium that has a close association with humans and is frequently used to assess the microbial contamination of water. However, it can readily acquire resistance to antibiotics. As a conditionally pathogenic bacterium and commensal, it is constantly present in the digestive tract of mammals and in the environment, such as soil and water (Wolny-Koładka and Lenart-Boroń, 2016). There is a growing concern about the contribution of *E. coli* to the spread of drug resistance in aquatic environments (Wolny-Koładka, Lenart-Boroń, 2018; Wolny-Koładka, 2018; Bartoczewicz et al., 2014). Antibiotic resistance in bacteria is a significant and increasing problem worldwide. Therefore, it is important to continuously monitor this phenomenon, detect its sources, and fully understand its molecular mechanisms (Ahmed et al., 2010; Wolny-Koładka and Zdaniewicz, 2021). The production of extended-spectrum β-lactamases (ESBLs) has been identified as one of the most prevalent mechanisms of resistance observed in *E. coli*. The rapid transfer of these genes between different bacterial strains, including those from disparate species, is facilitated by their localization on conjugation plasmids. Consequently, the *E. coli* strains that produce ESBLs exhibit heightened pathogenicity and resistance to antimicrobials. (Rawat and Nair, 2010; Wolny-Koładka and Lenart-Boroń, 2018; Ibekwe et al., 2021; Shaikh et al., 2015; Wellington et al., 2013). It is important to note that these strains are highly virulent and difficult to treat with many antibiotics. Furthermore, these isolates have the capacity to transfer resistance genes to frequently utilized antibiotics. This phenomenon hinders the rational use of antibiotic therapy and results in the development of cross-resistance to multiple antibiotic classes (Picozzi et al., 2014).

Given the absence of literature on the drug resistance profile of *E. coli* isolates from the brine used for inhalation in a municipal graduation tower, a decision was made to bridge this knowledge gap.

Therefore, the objective of this study was to assess the drug resistance of *E. coli* isolated from brine circulating in a closed-cycle graduation tower. The analyses conducted in this study enabled the determination of whether the brine used for inhalation constitutes a source of antibiotic-resistant *E. coli* strains, posing a threat to public health.

MATERIALS AND METHODS

Sampling site characteristics

E. coli bacteria were isolated from the brine obtained from a closed-cycle graduation tower (Figure 2) located in Nowa Huta (Krakow, Poland; 50°04'52.7"N 20°03'05.7"E). The foundation of the graduation tower is triangular in shape, with a total area of 350 m^2. Its three arms, each measuring 18 m, are composed of larch wood and are filled with blackthorn branches. The closed-cycle graduation tower is equipped with a brine tank at its base, with a storage capacity of 60,000 dm^3. The brine used in the tower is sourced from a mine in Zablocie, in close proximity to Pszczyna in Upper Silesia, Poland. The graduation tower in Nowa Huta is accessible to both city residents and tourists from April to September. In accordance with the established regulations, the duration of inhalation sessions is strictly limited to 30 minutes, with a maximum permissible duration of 45 minutes (Zarząd Zieleni Miejskiej (Management of the Municipal Greenspace Authority); Bodziacki and Wolny-Koładka, 2022). Brine can replenish micronutrient deficiencies and strengthen immunity when it penetrates the body through the respiratory tract and skin (Gaweł and Kuczaj, 2012). The brine supplied to the graduation tower contains elevated concentrations of potassium, calcium, magnesium, sodium chloride, sulfates, strontium, iodides, and bicarbonates. The atomized mist exhibits antibacterial and anti-inflammatory properties and has the capacity to absorb impurities. The 28% brine is delivered to the graduation tower, where it is diluted to maintain a final concentration ranging from 10 to 18%. Consequently, sitting on benches, leisure strolls, or lounging around the graduation tower can elicit a therapeutic effect through inhalation of the minerals and trace elements contained in the brine (Kejna et al., 2022; Burkowska-But et al., 2014).

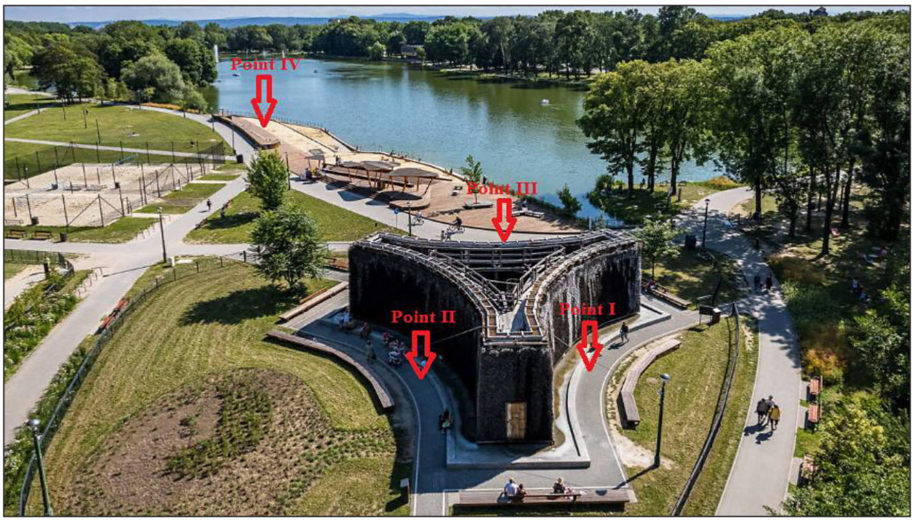

Figure 2. Bioaerosol sampling points (Bodziacki et al., 2024)

Isolation of *E. coli* strains and their identification

In the course of authors' prior investigation (Bodziacki and Wolny-Koładka, 2023), *E. coli* bacteria were isolated from the brine feeding the graduation tower. The collected isolates were inoculated onto chromogenic selective TBX medium (Tryptone Bile X-glucuronide agar, BTL, Poland) to facilitate the isolation and identification of *E. coli*. The bacterial cultures were then incubated for 24 hours at a temperature of 44 °C. The MALDI-TOF MS technique (Bruker Daltonik, Germany) was applied to confirm the species affiliation of green-blue colonies grown on TBX medium, following the manufacturer's recommendations and other studies (Bohme et al., 2010; Croxatto, 2012; Seng, 2010).

Phenotypic analysis of *E. coli* drug resistance profile

The disk diffusion method recommended by the European Committee on Drug Susceptibility Testing (EUCAST, 2023) was used to determine the drug resistance of 40 *E. coli* strains obtained from brine. For non-listed antibiotics (cephalothin, cefazolin, cefamandole, tetracycline), reference was made to publications (Wolny-Koładka and Lenart-Boroń, 2018; Wolny-Koładka, 2018). Twenty antibiotics, belonging to the basic and extended antibiogram, were used (Table 2). The ESBL mechanism was tested using the double-disk synergy method (Drieux et al., 2008). Following the incubation period of 18–24 h at a temperature of 37 °C, the growth inhibition diameters surrounding the antimicrobial disks were measured (in millimeters). The resulting data were then compared with the breakpoint values recommended by the EUCAST (2023). ATCC 25922, the reference strain of *E. coli*, was used as a quality control for the diffusion disk method employed.

Extraction of DNA and detection of ESBL-determining genes

In the course of the study, bacterial genomic DNA was extracted from the cultures collected and from the control *E. coli* strain, ATCC 25922. The Genomic Mini DNA extraction kit (A&A Biotechnology, Poland) was used according to the manufacturer's recommendations to ensure the effective extraction of DNA from the samples. PCR assays were conducted with the use of specific primers (Table 1) to identify the ESBL-determining genes: *blaCTXM-3* (Costa et al., 2006), *blaCTXM-9* (Simarro et al., 2000), *blaOXA*, *blaSHV*, and *blaTEM* (Sáenz et al., 2004). The reactions were carried out in a 25 µl reaction volume containing 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1 × PCR buffer, and 1 U Dream-Taq DNA polymerase (Thermo Scientific, US). The temperature profile applied for the reactions was as follows: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 94 °C for 45 seconds, annealing for 45 seconds at temperatures specific to individual primers, extension at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes, and then storage at 4 °C. The PCR amplifications were performed

Table 1. Primers used in the study with descriptions

Gene	5'-3' sequence	Annealing temperature (°C)	Product length (bp)	Reference
blaCTXM-3	F: GTTACAATGTGTGAGAAGCAG R: CCGTTTCCGCTATTACAAAC	60	800	Costa, 2006
blaCTXM-9	F: GTGACAAAGAGAGTGCAACGG R: ATGATTCTCGCCGCTGAAGCC	54	860	Simarro et al., 2000
blaOXA	F: ACACAATACATATCAACTTCGC R: AGTGTGTTTAGAATGGTGATC	61	813	Sáenz, 2004
blaSHV	F: CACTCAAGGATGTATTGTG R: TTAGCGTTGCCAGTGCTCG	52	885	Sáenz, 2004
blaTEM	F: ATTCTTGAAGACGAAAGGGC R: ACGCTCAGTGGAACGAAAAC	60	1150	Sáenz, 2004

using a T100 Thermal Cycler (Bio-Rad, USA). Subsequently, the PCR products were subjected to electrophoresis for 60 minutes in a 1% agarose gel with 1 x TBE buffer. The gel was then stained with Simply Safe (0.5 mg/ml; EurX, Poland), and its visualization was conducted under UV light. The documentation of the gel was facilitated by the Gel Doc system (Bio-Rad, US).

RESULTS AND DISCUSSION

The study enabled the isolation and identification of a total of 40 *E. coli* strains. Drug resistance was assessed phenotypically using disk diffusion tests, as illustrated in Table 2. The most prevalent resistances were observed to ampicillin (39 strains), amoxicillin/clavulanic acid (38 strains), and ticarcillin (19 strains). Conversely, there was no resistance to cefamandole, cephalothin, gentamicin, netilmicin, piperacillin/tazobactam, tobramycin, trimethoprim/sulfamethoxazole, and amikacin (Table 2). *E. coli* demonstrated resistance to a maximum of 8 antibiotics concurrently (1 strain). Of the *E. coli* strains that were collected, 21 exhibited resistance to 2 tested antibiotics, 16 to 3 antibiotics, and 2 to 4 antibiotics at the same time.

It is important to note that the entirety of the *E. coli* strains in the conducted study exhibited resistance to a single class of antibiotics (β-lactams). Consequently, the presence of multidrug-resistant (MDR) strains, defined as those resistant to a minimum of three antibiotic classes, was not observed. The MDR strains present a significant challenge for healthcare, as they can cause prolonged and difficult-to-treat infections, increasing the risk of complications and patient mortality. Effective therapy is severely hampered by the limited availability of antibiotics and the paucity of novel pharmaceutical agents approved for commercial distribution, resulting in a high mortality from infections (Bharadwaj et al., 2022; Ramos-Martín and D'Amelio, 2023).

No ESBL mechanism was detected in the analyzed strains using the disk diffusion method. However, the presence of an ESBL mechanism cannot be excluded based on the results of phenotypic analyses. Therefore, it is important to support the tests with genetic identification. Wolny-Koładka and Lenart-Boroń (2016) conducted a genetic and phenotypic analysis to identify the ESBL mechanism. Among the 94 tested *E. coli* strains, no ESBL mechanism was detected phenotypically. However, after PCR, the genes responsible for this type of resistance were detected in 36 strains. This is because the ESBL mechanism is multigene encoded. It is also noteworthy that while bacteria possess the genes that dictate the development of the ESBL mechanism, these genes are not always active, resulting in false negative outcomes (Wolny-Koładka and Lenart-Boroń 2016).

Given the absence of studies in the literature on drug resistance of *E. coli* isolated from the brine that circulates in a closed-cycle graduation tower, the publications describing the resistance of bacteria originating from water, particularly marine (saline) water, were used in the discussion.

Da Costa Andrade et al. (2015) analyzed the drug resistance of *E. coli* discovered in coastal waters collected near Ilha Porhat beach in Brazil. Approximately 25% of the *E. coli* strains isolated from seawater demonstrated resistance to ampicillin and amoxicillin/clavulanic acid. This type of resistance was also found in the present study. In contrast, the *E. coli* strains originating from brine exhibited nearly 100% resistance to these two antibiotics. This phenomenon may be caused by the continuous circulation of seawater in large bodies of water such as the sea (Da Costa Andrade

Table 2. Drug resistance profile of *E. coli*

Antibiotic (Symbol, µg)	Reference	Limit values (mm)		Number of *E. coli* strains		
		S≥	R<	S – sensitive	I – intermediate	R – resistant
Cefamandole (MA, 30)	Wolny-Koładka and Lenart-Boroń, 2018; Wolny-Koładka, 2018	18	14	40	0	0
Cefepime (FEP, 30)	EUCAST, 2023	27	24	36	2	2
Cefoxitin (FOX, 30)*	EUCAST, 2023	19	19	39	0	1
Cefalotin (KF, 30)	Wolny-Koładka and Lenart-Boroń, 2018; Wolny-Koładka, 2018	13	13	40	0	0
Cefazolin (KZ, 30)*	Wolny-Koładka and Lenart-Boroń, 2018; Wolny-Koładka, 2018	23	19	32	8	0
Ciprofloxacin (CIP, 5)	EUCAST, 2023	25	22	39	1	0
Gentamicin (CN, 10)	EUCAST, 2023	17	17	40	0	0
Netilmicin (NET, 30)	EUCAST, 2023	15	12	40	0	0
Piperacillin (PRL, 100)	EUCAST, 2023	20	20	38	0	2
Piperacillin/Tazobactam (TZP, 110)	EUCAST, 2023	20	20	40	0	0
Tetracycline (TE, 30)	Wolny-Koładka and Lenart-Boroń, 2018; Wolny-Koładka, 2018	15	11	39	1	0
Ticarcillin (TIC, 75)	EUCAST, 2023	23	20	3	18	19
Tobramycin (TOB, 10)	EUCAST, 2023	16	16	40	0	0
Trimethoprim/Sulfamethoxazole (SXT, 25)	EUCAST, 2023	14	11	40	0	0
Amoxicillin / Clavulanic acid (AMC, 30)*	EUCAST, 2023	19	19	2	0	38
Cefotaxime (CTX, 30)	EUCAST, 2023	20	17	39	0	1
Ceftazidime (CAZ, 30)	EUCAST, 2023	22	19	38	0	2
Amikacin (AK, 30)	EUCAST, 2023	18	18	40	0	0
Ampicillin (AMP, 10)	EUCAST, 2023	14	14	1	0	39
Aztreonam (ATM, 30)	EUCAST, 2023	26	21	38	0	2

Note: * Antibiotics used to detect the ESBL mechanism.

et al., 2015), resulting in a lower density of microorganisms compared to the closed cycle of brine graduation towers. Authors' previous study has shown that brine water is characterized by a high number of bacteria, including *E. coli* (Bodziacki and Wolny-Koładka, 2023). In addition, the brine tank's limited surface area and the installation's overall diminutive size promote the dissemination of antibiotic resistance genes among the brine microorganisms. Furthermore, da Costa Andrade et al. (2015) did not demonstrate any resistance of *E. coli* to tetracycline, which is consistent with the findings of the present study. Al-Sarawi et al. (2018) evaluated the drug resistance of *E. coli* found in marine waters off the coast of Kuwait near sewage discharge points. Their findings indicated that 95% of the strains exhibited resistance to ampicillin. This result closely aligns with the 95% resistance rate observed in authors' own study. Alves et al. (2014) isolated 166 *E. coli* strains from the seawater off the coast of Portugal. The strains showed resistance to streptomycin (100%), as well as to cephalothin (42%) and tetracycline (48%). In this study, all strains were found to be sensitive to both cephalothin and tetracycline. Analysis of the resistance of *E. coli* strains to ampicillin and amoxicillin/clavulanic acid once again confirmed that the bacteria isolated from seawater are less resistant to these antibiotics than those isolated from brine. Alves et al. (2014) detected drug resistance of *E. coli* in seagull droppings, suggesting that birds may be a source of seawater contamination with drug-resistant *E. coli* bacteria. This is relevant to the present study, because a similar phenomenon of contamination of the Krakow graduation tower with feces was observed when collecting samples for tests. The graduation tower is an open facility accessible to wild animals, especially birds, which build nests in the area and pollute the blackthorn with their feces. A study conducted on a freshwater reservoir, known as "Staw przy Kaczencowej" in Nowa Huta, Krakow, Poland, aimed to identify the presence of drug-resistant *E. coli* strains. The study revealed that the collected isolates were frequently resistant to ticarcillin, ampicillin, cefazolin, and cephalothin (Wolny-Koładka, 2018). In a previous study, Wolny-Koładka and Lenart-Boroń (2016) analyzed the drug resistance of the *E. coli* isolated from the Zalew Nowohucki Reservoir in Krakow, Poland. The isolated strains were frequently resistant to ampicillin, ticarcillin, cephalothin, amoxicillin with clavulanic acid, and cefazolin. However, no resistance was found to piperacillin-tazobactam and amikacin. Conversely, Lenart-Boroń et al. (2020) isolated *E. coli* strains from a river proximate to a wastewater treatment plant, which exhibited the highest resistance to amoxicillin with clavulanic acid (90%) and ampicillin (36%). Additionally, 40% of the strains exhibited multidrug resistance.

The presence of pesticides, herbicides, and heavy metals in surface waters has been demonstrated to facilitate the transmission of drug resistance genes among bacterial populations. This phenomenon can result in the dissemination of strains that are resistant to various classes of antibiotics (Vignesh et al., 2012; Vikesland et al., 2019). Apart from examining the drug resistance profile of bacteria, it is important to determine whether particular strains are resistant to multiple antibiotics at the same time. These strains impose limitations on the application of first-line antibiotics, thereby augmenting the probability of therapeutic failure. This phenomenon constitutes a grave challenge in the management of infectious diseases (Mazińska and Hryniewicz, 2020). Among the *E. coli* strains isolated from brine, simultaneous resistance to two, three, four, and even eight antibiotics was observed. The strains resistant to two antibiotics at the same time were the most frequent (52.5%), while those resistant to eight antibiotics were the least frequent (2.5%). Hence, the conducted study indicated a decline in the proportion of resistant strains with an increase in the number of antibiotics to which the strains are resistant. This relationship was also observed by da Costa Andrade et al. (2015) who reported that 78.4% of strains were resistant to a single antibiotic and 19.5% were resistant to two antibiotics. Only 2.1% of strains in their study were resistant to three antibiotics (da Costa Andrade et al., 2015). Wolny-Koładka (2018) demonstrated a similar relationship, finding a higher percentage of strains resistant to one antibiotic (21%) compared to those resistant to two (14%) or four antibiotics (8%) in ecological waters in Kraków. In their study, Wolny-Koładka and Lenart-Boroń (2016) reported that among the 94 strains examined, only 26% showed sensitivity to all the antibiotics tested, while the remaining 74% demonstrated resistance to one or more antibiotics. The study further revealed that 12% of the strains were resistant to a single antibiotic, while 9% were resistant to two or three different antibiotics. Additionally, 4% of the isolates exhibited resistance to up to twelve antibiotics simultaneously. Patola et al. (2010) found a similar relationship, with 33.34% of isolates being resistant to two antibiotics and only 3.70% being resistant to five antibiotics.

A genetic analysis was conducted on 40 isolated strains to ascertain the presence of genes responsible for the development of the ESBL mechanism. The results showed that the *blaTEM* gene was present in 35 isolates, making it the most common. The second most common gene was *blaCTXM-9* that was present in 28 strains (Table 3). However, none of the analyzed strains had the *blaCTXM-3*, *blaSHV*, or *blaOXA* genes. The co-occurrence of *blaTEM* and *blaCTXM-9* genes was observed in 60% of the strains

From an epidemiological perspective, the ESBL mechanism constitutes one of the most significant types of bacterial resistance (Rawat and Nair, 2010). Therefore, when testing *E. coli* strains, it is crucial to detect the presence of that resistance mechanism. In the present study, at least one gene determining the ESBL mechanism was detected in 39 *E. coli* isolates (98%) (Table 3). Wolny-Koładka and Lenart-Boroń (2016) found that 38% of the *E. coli* isolated from the Zalew Nowohucki Reservoir had the genes that determine the ESBL mechanism. Their study detected 4 genes: *blaTEM*, *blaSHV*, *blaCTXM-3*, and *blaOXA,* while in the present study, 2 genes were determined: *blaTEM* and *CTXM-9*. Lenart-Boroń et al. (2020) isolated the *E. coli* from the Szreniawa River near a wastewater treatment plant and detected three ESBL-determining genes: *blaTEM* (24% of strains), *blaOXA* (10% of strains), and *blaCTX-M* (8% of strains). The *blaTEM* gene was identified as the most prevalent gene in the present

Table 3. Genotypical and phenotypical profiles of *E. coli*, including antibiotic resistance and ESBL genes

Isolate No.	Antibiotic resistances	ESBL genes
1	TIC, AMC*, AMP	blaTEM
2	TIC, AMC*, AMP	blaTEM, blaCTXM-9
3	TIC, AMC*, CAZ, AMP	blaTEM, blaCTXM-9
4	TIC, AMC*, AMP	blaTEM, blaCTXM-9
5	AMC*, AMP	blaTEM, blaCTXM-9
6	TIC, AMC*, AMP	blaTEM
7	AMC*, AMP	blaTEM
8	TIC, AMC*, AMP	blaTEM
9	PRL, TIC, AMC*, AMP	
10	AMC*, AMP	blaCTXM-9
11	TIC, AMC*, AMP	blaTEM, blaCTXM-9
12	AMC*, AMP	blaTEM, blaCTXM-9
13	TIC, AMC*, AMP	blaTEM
14	TIC, AMC*, AMP	blaTEM
15	AMC*, AMP	blaTEM, blaCTXM-9
16	TIC, AMC*, AMP	blaTEM
17	FEP, ATM	blaTEM, blaCTXM-9
18	AMC*, AMP	blaTEM
19	TIC, AMC*, AMP	blaTEM
20	AMC*, AMP	blaTEM
21	FEP, FOX*, PRL, TIC, CTX, CAZ, AMP, ATM	blaCTXM-3
22	AMC*, AMP	blaTEM, blaCTXM-9
23	TIC, AMC*, AMP	blaTEM, blaCTXM-9
24	AMC*, AMP	blaTEM, blaCTXM-9
25	TIC, AMC*, AMP	blaTEM
26	AMC*, AMP	blaTEM, blaCTXM-9
27	AMC*, AMP	blaTEM, blaCTXM-9
28	TIC, AMC*, AMP	blaTEM, blaCTXM-9
29	AMC*, AMP	blaTEM, blaCTXM-9
30	AMC*, AMP	blaTEM, blaCTXM-9
31	TIC, AMC*, AMP	blaTEM, blaCTXM-9
32	AMC*, AMP	blaTEM, blaCTXM-9
33	AMC*, AMP	blaTEM, blaCTXM-9
34	TIC, AMC*, AMP	blaTEM, blaCTXM-9
35	AMC*, AMP	blaTEM, blaCTXM-9
36	AMC*, AMP	blaTEM, blaCTXM-9
37	AMC*, AMP	blaTEM, blaCTXM-9
38	AMC*, AMP	blaTEM, blaCTXM-9
39	AMC*, AMP	blaTEM
40	TIC, AMC*, AMP	blaTEM

Note: * Antibiotics used to detect the ESBL mechanism.

study and in the studies conducted by other authors (Lenart-Boroń (2017), Lenart-Boroń et al. (2020), Wolny-Koładka and Zdaniewicz (2021), Wolny-Koładka and Lenart-Boroń (2018)).

The obtained results are concerning from an epidemiological perspective, as they revealed the presence of drug-resistant *E. coli* in the brine utilized for inhalation in the municipal closed-cycle graduation tower. This study highlights the prevalence of drug-resistant bacteria in the environment and raises questions about the health benefits of saline inhalations. The obtained findings represent a novel and pioneering contribution to the scientific discourse on the microbiological safety of closed-cycle graduation towers. They may also encourage the measures to enhance the microbiological quality of the brine that circulates within the tower. It is important to note that the water used for inhalation must meet appropriate microbiological requirements, including the absence of *E. coli*. However, the Krakow graduation tower did not meet these requirements, which could potentially endanger the people who inhale the air, and expose them to infections with drug-resistant strains of *E. coli*. Management of the Municipal Greenspace Authority (Zarząd Zieleni Miejskiej, ZZM) in Krakow claims that the brine supplied to the graduation tower is thoroughly tested and free from microbiological contamination (ZZM 2022). However, the conducted study shows that after introducing it into the tank supplying the graduation tower, the water becomes contaminated with fecal bacteria (Bodziacki and Wolny-Koładka, 2023). The tank supplying the graduation tower is located outdoors and is therefore constantly exposed to contamination, including bird droppings, which may also be a source of drug-resistant *E. coli* (Alves et al., 2014). In addition, a significant issue in the operation of the graduation tower is non-compliance with the regulations established by ZZM by the individuals inhaling the brine mist. Despite the prohibitions, some individuals wash their unclean hands or feet in the brine water, introducing bacteria into the brine, which may also contain drug-resistant *E. coli* bacteria.

CONCLUSIONS

The present study enabled the isolation, identification, and assessment of the drug resistance profile of 40 strains of *E. coli* bacteria originating from brine that circulates in a closed-cycle graduation tower. A significant proportion of the strains exhibited resistance to the antibiotics evaluated in this study. The most common resistances were to ampicillin, amoxicillin/ clavulanic acid, and ticarcillin. The PCR technique was applied to ascertain the presence of the *blaTEM* and *blaCTXM-9* genes. The *blaTEM* gene was identified as the predominant gene responsible for the production of extended-spectrum β-lactamases. This study offers groundbreaking insights into the prevalence of multidrug-resistant strains of *E. coli* in public areas, such as municipal brine graduation towers. From an epidemiological perspective, the *E. coli* strains inhabiting the brine pose a risk to individuals who inhale it. The results suggest a need for further research to monitor drug-resistant bacteria in a closed-cycle brine graduation tower. In addition, procedures should be implemented to ensure constant microbiological control and reduce contamination of the brine with *E. coli*.

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