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The Silent Spread of Antibiotic-Resistant *E. coli* in Cemetery Soils of Sana'a City, Yemen: Implications for the One Health Approach

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Abstract:
Antibiotic-resistant *Escherichia coli* (*E. coli*) in environmental reservoirs represent an emerging One health concern, yet cemetery soils remain largely overlooked. This study aimed to determine *E. coli* prevalence and characterize resistance profiles in 115 soil samples collected from ten cemeteries (A–J) in Sana'a City, Yemen. *E. coli* was recovered from 44% (50/115) of samples, with prevalence varying markedly between sites, ranging from 0% (cemetery I) to 89% (cemetery F), reflecting spatial heterogeneity likely related to site-specific environmental factors. Antibiotic susceptibility testing of **50 selected isolates** against 15 clinically important antibiotics revealed high resistance to β-lactams [Ampicillin/Sulbactam (78%), Cefotaxime (89%), Amoxicillin/clavulanate (55%)], fluoroquinolones (Ciprofloxacin 69%, Levofloxacin 65%), Doxycycline (93%), sulfonamides, and phenicols. Meropenem retained high activity (92% susceptible); Gentamicin and Ceftolozane/Tazobactam showed moderate susceptibility (70–80%). Phenotypic analysis confirmed 82% ESBL producers (double-disc synergy test), 52% MDR (non-susceptibility to ≥3 classes), and 12% suspected pre-XDR strains. These findings confirm cemetery soils in Sana'a as significant reservoirs of ESBL/MDR *E. coli* within Yemen's One Health continuum. The data justify integrating cemetery environments into national AMR surveillance, with priorities for monitoring high-prevalence sites and protecting nearby groundwater resources.

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INTRODUCTION

The rapid global emergence of antibiotic-resistant bacteria (ARB) represents a critical public health threat by undermining the effectiveness of current antimicrobial therapies, thereby driving increased morbidity, mortality, and escalating healthcare costs worldwide (Ventola, 2015). Although clinical settings historically have been the main focus of antibiotic resistance surveillance, mounting evidence establishes environmental reservoirs, including soils, water bodies, and sediments, as crucial arenas for the persistence, evolution, and spread of antibiotic resistance genes (ARGs); In Sana'a, shallow wells, informal irrigation, and free-ranging livestock frequently occur near cemeteries, creating interfaces where resistant bacteria and ARGs can circulate between soil, water, animals, and humans within a One Health context (Wellington *et al.,* 2013).

Among environmental reservoirs, cemetery soils constitute a largely unexplored but potentially significant source of ARB (Figure 1). Repetitive burial activities introduce organic matter and diverse microbial communities into these soils, creating conditions favorable for microbial proliferation, enhanced horizontal gene transfer, and accumulation of ARGs in both endemic and invasive bacterial populations (Despotovic *et al.*, 2023). Recent comprehensive reviews highlight cemetery soils as silent yet critical reservoirs facilitating the persistence and environmental dissemination of antibiotic resistance into surrounding ecosystems and human communities (Tarnawska *et al.,* 2024). Despite their probable importance, cemetery soils remain an overlooked component in antimicrobial resistance research, particularly in low-resource and conflict-affected regions such as Yemen, where environmental health monitoring is scarce.

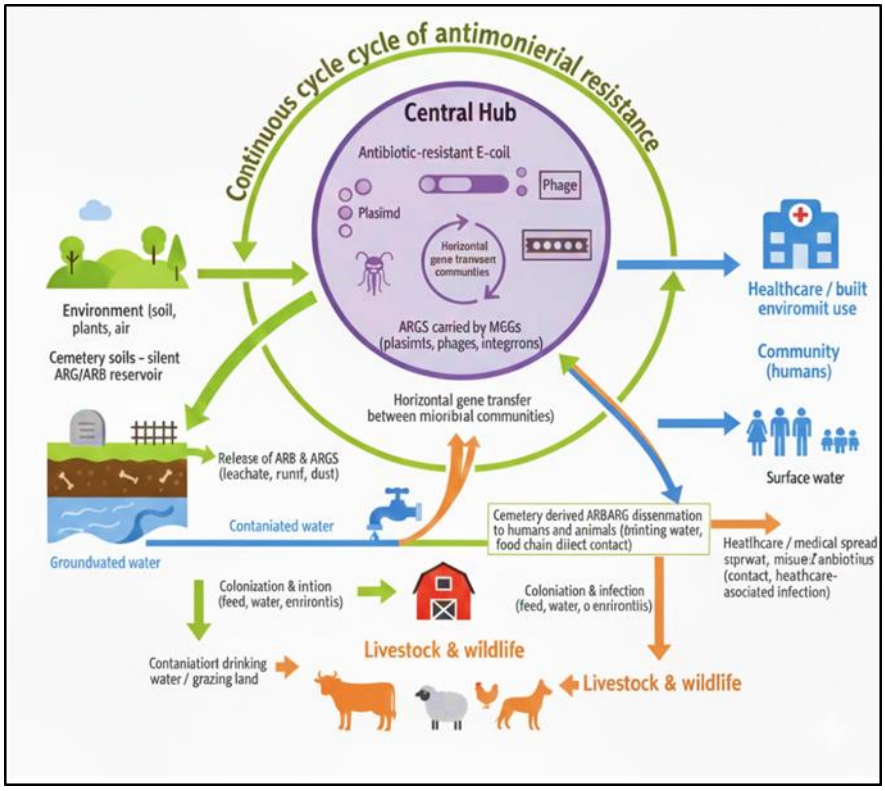

Fig. 1. One Health Dissemination of Antibiotic-Resistant *E. coli* and ARGs, Highlighting Cemetery Soils as a Silent Environmental Reservoir. Created with BioRender.com.

Understanding the hidden spread of resistant *E. coli* in cemetery soils is vital for developing effective local surveillance systems and guiding interventions to mitigate environmental reservoirs of antimicrobial resistance. This work contributes crucial new knowledge to the global antimicrobial resistance effort by emphasizing the need to integrate neglected environmental reservoirs in research and public health planning, particularly in regions facing complex infrastructural and humanitarian challenges (Kunhikannan *et al*., 2021).

This study presents the first investigation into the prevalence and diversity of antibiotic-resistant *E. coli* in cemetery soils in Yemen, focusing on Sana'a City; the country's densely populated capital and urban core. Specifically, this study aimed to (i) determine the prevalence of *E. coli* in soils from ten urban cemeteries in Sana'a City, Yemen, and (ii) characterize their antibiotic susceptibility patterns and the prevalence of ESBL, MDR, and pre-XDR phenotypes. By combining microbiological culturing with antibiotic susceptibility testing, this research generates essential baseline data on ARB within a unique environmental matrix in a vulnerable socio-environmental context. The findings illuminate previously unrecognized environmental reservoirs of antibiotic resistance that may pose significant public health and ecological risks, locally and potentially beyond.

MATERIALS AND METHODS

Research area

Samples were obtained from ten urban cemeteries in Sana'a city, Yemen: Khozayma cemetery (A); Al Khaneq cemetery (B); Al Dafei cemetery (C); Sam city cemetery (D); Rawdat Al Shohada'a cemetery (E); Al Hamra'a cemetery (F); Sawad Honish cemetery (G); Majel Al Demma cemetery (H); Al Jawmary cemetery (i) and Jame Al Ebada'a cemetery (J). All cemeteries in the study sample are located at an elevation of 2218.01 meters (7276.94 feet) above sea level, and under the same subtropical desert climate zone (Classification: BWh). The district's yearly temperature is 20.9ºC (69.62ºF) and it is -4.63% lower than Yemen's averages. Sana'a typically receives about 108.75 millimeters (4.28 inches) of precipitation and has 148.95 rainy days (40.81% of the time) annually.

Selection of samples

Ten operational cemeteries in Sana'a City were selected based on size, and burial density; a total of 115 within 10 cemeteries in Sana'a City (A, n = 11; B, n = 10; C, n = 10; D, n = 13; E, n = 15; F, n =9; G, n =10; H, n =13; I, n =14; J, n =10;) between Dec. 2024 and April 2025. Soil samples (50 g) were collected aseptically from the top 10-20 cm layer of cemetery sites using sterile spatulas, placed in clean bags, sieved (2 mm mesh) to remove stones and debris, and transported at 4°C for processing within 24 hours (Bhowmik and Ahsan, 2020).

Extraction of *E. coli* from soil

Ten grams of sieved soil were weighed into sterile 250 mL flasks containing 90 mL of 0.1% peptone water. Suspensions were vortexed vigorously for 10-15 min, followed by shaking at 200 rpm for 30 min at room temperature to detach bacteria. Mixtures settled for 10-30 min, and supernatants were collected as 10^{-1} dilutions. Tubes containing selective BGLB media were inoculated with test portion of supernatants using inoculum quantities of 1ml and incubated at 37°C for 24 hours. Each tube containing gas with yellow color was regarded as presumptive positive for coliforms. Subsequent confirmatory test with selective eosin methylene blue (EMB) and MacConkey agar media was performed.

Confirmative identification of *E. coli* on EMB and MacConkey agar media:

A loopful of culture from positive BGLB medium from each sample was streaked on EMB and MacConkey agar media for confirmative identification of the samples (Cheesbrough, 1985). The plates were incubated at 37°C for 24 hrs. Colonies with metallic green sheen on EMB and round, small, elevated colonies with pink pigmentation on MacConkey agar were thought to be *E. coli* and picked as positive isolates for further identification, repeatedly subcultured onto

EMB agar until the pure culture with homogenous colonies were obtained.

Biochemical identification

The laboratory biochemical tests such as Kligler's Iron Agar (KIA) test, indole production test, citrate utilization test, methyl red test and Voges-Proskauer test were used to confirm the identification of the selected colony from EMB and MacConkey agar media. Specific biochemical reactions such as fermentative metabolism, utilization of glucose, lactose, production of gases helped to identify *E. coli* (Bergey and Holt, 2000).

Antimicrobial susceptibility test (AST)

Fifty *E. coli* isolates were tested for AST using the Kirby-Bauer disc diffusion method on Mueller–Hinton (MH) agar plates (Bauer *et al.,* 1966; Iqbal *et al.,* 2016), following the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. The bacterial concentrations were standardized to a 0.5 McFarland standard and spread on the agar (*E. coli* ATCC 25922 was used for quality control). Isolates were tested against 15 clinically important antibiotics from multiple classes: Amoxicillin/Clavulanate (30 µg), Ampicilin/Sulbactam (20 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Cefuroxime (30 µg), Meropenem (10 µg), Piperacillin/Tazobactam (10 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Doxycycline (30 µg), Co-trimoxazole (25 µg), Chloramphenicol (30 µg), Gentamycin (10 µg), Streptomycin (10 µg), and Ceftolozane/Tazobactam (40 µg). The antibiotics were selected based on their frequent use in human medicine, and significance in antimicrobial resistance surveillance and were guided by CLSI guidelines and World Health Organization (WHO) medically important antimicrobials list (WHO, 2023). The results were interpreted as susceptible, intermediate, or resistant.

Phenotypic characterization of resistance phenotypes

Isolates that exhibited resistance to at least three antibiotic classes were considered multidrug resistant (MDR). MDR was defined as non-susceptibility to ≥1 agent in ≥3 antimicrobial categories (Magiorakos *et al.,* 2012). Antimicrobial categories considered were penicillins, cephalosporins, fluoroquinolones, tetracyclines, sulfonamides, phenicols, and aminoglycosides.

Pre-extensively drug-resistant (pre-XDR) isolates were operationally defined as those exhibiting non-susceptibility to ≥5 of the 7 categories above while retaining susceptibility to ≥1 last-line agent (Meropenem or Ceftolozane/Tazobactam), indicating strains approaching but not meeting full XDR criteria.

RESULTS

Isolation rates of *E. coli*

The study revealed variable prevalence of *E. coli* obtained from 44% of the 115 soil samples tested from ten cemeteries (A–J) in Sana'a City, Yemen, confirming widespread contamination in this novel environmental reservoir. The highest detection rate was observed in Cemetery F (89%), while Cemetery I yielded no *E. coli* isolates. Other cemeteries demonstrated intermediate isolation rates, with the lowest among them being 20% in Cemetery J and the highest at 70% in Cemetery C (Table 1).

AST patterns of all 50 *E. coli* isolates

Antibiotic susceptibility testing of 50 isolates further elucidated a concerning antimicrobial resistance pattern. The isolates exhibited pronounced resistance to multiple β-lactam antibiotics including Ampicillin/Sulbactam (78% resistant), Cefotaxime (89% resistant), and Amoxicillin/clavulanate (55% resistant). Fluoroquinolones, namely Ciprofloxacin and Levofloxacin, also showed critical resistance levels of up to 69%. Tetracycline class antibiotic Doxycycline was resisted by 93% of isolates, highlighting significant resistance challenges within this group. Phenicol and Sulfonamide resistance were similarly elevated. Conversely, carbapenem antibiotic Meropenem retained high potency with 92% susceptibility, underscoring its continued clinical utility. Other agents such as

Gentamicin and Ceftolozane/Tazobactam demonstrated moderate susceptibility rates ranging 70–80% (Figure 2). Collectively, these results define cemetery soils in Sana'a as important environmental reservoirs of antibiotic-resistant *E. coli*, exhibiting both substantial bacterial loads and multidrug resistance profiles.

Prevalence of MDR and ESBL-producing *E. coli*

Overall, of the 50 *E. coli* isolates recovered from cemetery soil, a very high proportion showed advanced resistance phenotypes. Specifically, 41 isolates (82%) were confirmed as extended-spectrum β-lactamase (ESBL) producers, indicating widespread ability to hydrolyze third-generation cephalosporins and related β-lactams. In parallel, 26 isolates (52%) met the definition of multidrug-resistant (MDR), being non-susceptible to at least one agent in three or more antibiotic classes. A small subset of isolates showed very advanced resistance phenotypes: six *E. coli* (12%) displayed non-susceptibility to multiple key antibiotic classes and were considered suspected pre-XDR strains. Although they did not fully meet formal XDR criteria are likely related to retaining susceptibility to at least one last-line agent.

DISCUSSION

This study investigated the AMR profiles of *E. coli* isolated from cemetery soils in Sana'a City, Yemen. The variable isolation frequencies of *E. coli*, ranging from complete absence to 89% among different cemeteries, reflected spatial heterogeneity likely influenced by micro environmental factors such as burial density, soil characteristics, organic matter, and moisture, as hypothesized based on patterns observed in similar anthropogenically impacted environments (Abia *et al.,* 2018; Alwash and Al-Rafyai*,* 2019). These reservoirs facilitate persistence and dissemination of *E. coli* in soils, groundwater, and adjacent ecosystems, posing potential public health risks.

The extensive multidrug resistance observed; with high resistance to β-lactams (Cefotaxime 89%, Ampicillin/Sulbactam 78%, Ceftriaxone 63%, Cefuroxime 62%, moxicillin/clavulanate 55%) and fluoroquinolones (Ciprofloxacin 69%, Levofloxacin 65%), and elevated tetracycline resistance (93%); parallels antimicrobial resistance patterns reported in clinical and environmental isolates from other regions such as Lusaka, Zambia, where similar resistance levels were attributed to widespread antibiotic misuse, incomplete treatment courses, and environmental contamination from human and animal waste (Kasanga *et al.,* 2024).

Table 1. The number and frequency (%) of isolations of *E. coli* in the cemetery soils, collected at the depths of 10-20cm in the ten cemeteries (A–J).

Cemetery	# of samples tested	# of Positive samples	%	# of Negative samples	%
A	11	6	55%	5	45%
B	10	5	50%	5	50%
C	10	7	70%	3	30%
D	13	5	38%	8	62%
E	15	7	47%	8	53%
F	9	8	89%	1	11%
G	10	4	40%	6	60%
H	13	6	46%	7	54%
I	14	0	0%	14	100%
J	10	2	20%	8	80%
Total	115	50	44%	65	56%

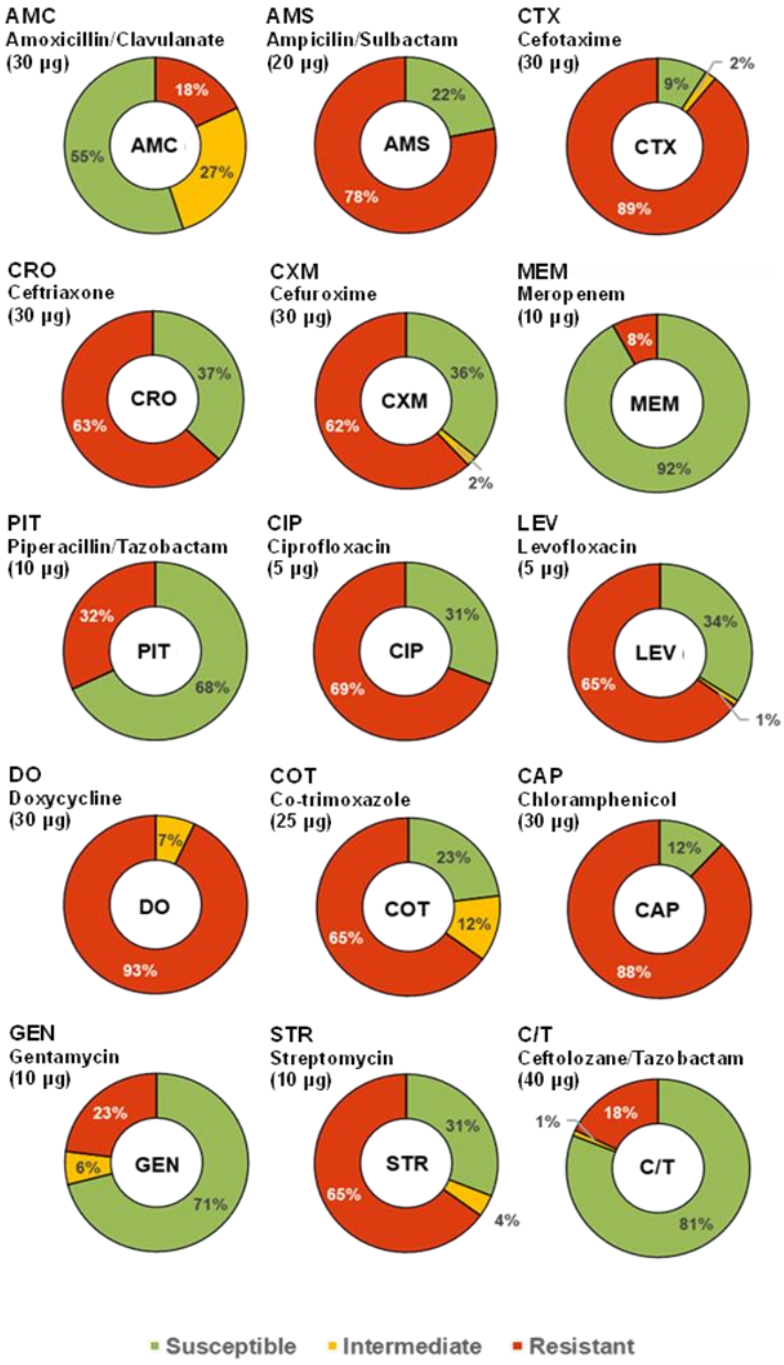

Fig. 2. Antibiotic susceptibility patterns of *E. coli* isolates.
AMC: Amoxicillin/Clavulanate; **AMS:** Ampicilin/Sulbactam; **CTX:** Cefotaxime; **CRO:** Ceftriaxone; **CXM:** Cefuroxime; **MEM:** Meropenem; **PIT:** Piperacillin/Tazobactam; **CIP:** Ciprofloxacin; **LEV:** Levofloxacin; **DO:** Doxycycline; **COT:** Co-trimoxazole; **CAP:** Chloramphenicol; **GEN:** Gentamycin; **STR:** Streptomycin; **C/T:** Ceftolozane/Tazobactam.

The resistance phenotypes of β-lactams been associated in previous studies with the production of extended-spectrum β-lactamases (ESBLs) and chromosomal or plasmid-mediated resistance mechanisms which enzymatically inactivate broad-spectrum penicillins and third-generation cephalosporins, thereby compromising effective clinical treatment as documented in prior studies (Mbanga *et al.,* 2023; Yuan *et al.,* 2023; Chekole *et al.,* 2025; Murugalakshmi *et al.*, 2025; Lin *et al.,* 2025; Lathakumari *et al.,* 2025; Ali *et al.,* 2025). High

resistance to ampicillin/sulbactam has been linked to the broad availability and common use of penicillins, often without medical supervision, which may contribute to selection for resistant strains (Ardillon *et al.,* 2023).

Similarly, resistance to Amoxicillin/clavulanate despite the β-lactamase inhibitor suggests the presence of potent enzymes such as CTX-M family β-lactamases that have been documented to confer resistance via multiple mechanisms on mobile genetic elements (Wang *et al.,* 2025). The absence of intermediate susceptibility in Ceftriaxone resistance suggests a population dominated by fully resistant ESBL producers, complicating clinical management (Assefa *et al.,* 2025).

Cefuroxime resistance, with a smaller intermediate fraction, points to emerging resistance mechanisms widening the resistance spectrum (Chekole *et al.,* 2025). Two studies conducted in Iraq reported high resistance of *E. coli* to ceftriaxone and cefotaxime (Naqid *et al.,* 2022; Jalil and Al Atbee, 2022), another one in India (Murugalakshmi *et al.,* 2025).

The marked resistance to fluoroquinolones Ciprofloxacin (69%) and Levofloxacin (65%), is consistent with global reports associating fluoroquinolone resistance in *E. coli* strains with point mutations in quinolone resistance-determining regions (QRDR) and plasmid-mediated mechanisms (Stapleton *et al.,* 2020; Jalil and Atbee, 2022; Sato *et al.,* 2023; Tewawong *et al.,* 2025; Chen *et al.,* 2025; Wan *et al.,* 2025).

The widespread misuse of fluoroquinolones for urinary and respiratory tract infections, exerting selective pressure that may promote resistant clones. Alarmingly, resistance can escalate even after reductions in fluoroquinolone prescriptions, suggesting persistent transmission and environmental reservoirs maintaining resistant strains (Tewawong *et al.,* 2025). Likewise, the high doxycycline resistance (93%) aligns with global tetracycline resistance trends driven by efflux pumps and ribosomal protection proteins on mobile genetic elements (Di Marcantonio *et al.,* 2025).

Resistance to co-trimoxazole at 65% reflects ongoing challenges with sulfonamide therapies, largely due to extensive and often inappropriate use that favors resistant strains (Alsheikh *et al.,* 2025).

The high chloramphenicol resistance (88%) despite reduced clinical use suggests environmental reservoirs sustain resistance genes such as cat determinants. This phenomenon has been supported by recent studies that demonstrate how antibiotic-resistant *E. coli* populations persist in environmental matrices like hospital sewage, polluted river water, and wastewater treatment plants, were long-standing use of chloramphenicol continues to drive ARG persistence and co-resistance in environmental *E. coli* populations (Alanazi *et al.,* 2018; Despotovic *et al.,* 2023; Kasanga *et al.,* 2024; Tufa and Birhanu, 2025).

Streptomycin resistance at 65% corresponds with global aminoglycoside resistance trends driven by modifying enzymes and target site mutations (Ali *et al.,* 2025). Intermediate susceptibility levels in certain drugs indicate evolving resistance mechanisms potentially leading to full resistance.

Encouragingly, high susceptibility to carbapenems such as Meropenem (92%) and the relatively favorable activity of Gentamicin and Ceftolozane/Tazobactam (70–80%) was observed in this study. The findings are consistent with global trends where carbapenems and select advanced agents retain efficacy against many ESBL-producing strains, although emerging carbapenemase producers warrant continued vigilance (Kasanga *et al.,* 2024; Lathakumari *et al.,* 2025). Similarly, other recent studies confirmed moderate to high susceptibility of *E. coli* to Gentamicin and newer β-lactam/β-lactamase inhibitor combinations like Ceftolozane/Tazobactam, which retain efficacy against many multidrug-resistant strains (Mousa *et al.,* 2025). These agents remain critical in treating severe infections caused by multidrug-resistant *E. coli,* especially in settings with widespread resistance to β-lactams, fluoroquinolones, and other commonly used

antibiotics (Lathakumari *et al.,* 2025). However, the emergence of carbapenemase-producing strains necessitates vigilant antimicrobial stewardship, routine resistance monitoring, and continued research into novel therapeutics to maintain these agents' clinical utility (Aljohni *et al.,* 2025). Taken together, these patterns indicate that *E. coli* in cemetery soils display resistance profiles comparable to other high-risk environmental reservoirs, supporting their role within the broader One Health continuum of ESBL- and MDR-*E. coli* circulation (Laspartriana *et al.,* 2023; Wan *et al.,* 2025; Murugalakshmi *et al.,* 2025).

The detection of substantial proportions of ESBL-producing (82%), MDR (52%), and suspected pre-XDR (12%) *E. coli* isolates confirm cemetery soils as highly relevant AMR reservoirs with limited therapeutic options and substantial dissemination potential. These findings justify including cemetery soils in national AMR surveillance programs, with initial priorities including environmental monitoring near high-prevalence sites (e.g., Cemetery F), urban planning restrictions on cemetery expansion near water sources, and community awareness campaigns about groundwater protection. This study establishes the first baseline dataset for Yemen and serves as a model for investigating cemetery soils as AMR hotspots in other low-resource, conflict-affected settings.

CONCLUSION

This study confirms cemetery soils in Sana'a City, Yemen, as significant environmental reservoirs of ESBL-producing (82%), MDR (52%), and suspected pre-XDR (12%) *E. coli*, exhibiting resistance patterns comparable to high-risk clinical and environmental sources. The marked spatial heterogeneity and advanced resistance phenotypes highlight these sites as neglected AMR hotspots within the One Health continuum. These baseline findings justify integrating cemetery soils into Yemen's national AMR surveillance framework and inform targeted priorities: environmental monitoring of high-prevalence cemeteries, groundwater protection measures, and urban planning safeguards. The study provides a replicable model for other resource-constrained settings facing similar environmental health challenges.

Limitations

This study relied exclusively on culture-based phenotypic methods for *E. coli* isolation, ESBL detection, and resistance profiling. No molecular analyses (e.g., PCR for blaCTX-M or other ARGs, whole-genome sequencing) were performed to confirm genotypic resistance mechanisms or phylogroups. Future studies should incorporate molecular typing to characterize the resistome and track ARG dissemination from cemetery soils.

CONFLICT OF INTEREST

Author hereby declares no conflict of interest.

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