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RESEARCH ARTICLE OPEN ACCESS

Antibiotic Resistance Profiles of Gram-negative *Enterobacteriaceae* Isolates from a Main Wastewater Pathway in Palestine

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Abstract

Multidrug-resistant (MDR) bacteria have been isolated from a major wastewater pathway. These bacteria harbor several antimicrobial resistance genes that confer resistance to several antibiotics simultaneously. The main aim of this study was to investigate the antibiotic resistance profiles of lactose-fermenting gram-negative bacteria isolated from the main wastewater pathway in the Nablus area of the West Bank, Palestine. A total of 162 lactose-fermenting isolates belonging to the *Enterobacteriaceae* family were isolated from a sample obtained from the main wastewater pathway. Most of the isolates obtained were identified to the species level using the API-20 E identification system. The proportions of MDR strains among the obtained *Escherichia coli* and *Citrobacter Koseri* isolates were 19.1% and 10%, respectively. Among all isolates, six were found to be extended-spectrum beta-lactamase (ESBL) producers. These included three *E. coli* isolates, one *Klebsiella pneumoniae* isolate, and two *C. Koseri* isolates. Approximately 12.3% of the total isolates were MDR and 3.7% were identified as ESBL producers. The prevalence of MDR isolates in our study was concerning, indicating that immediate and decisive measures are needed to halt its escalation and promote its reduction.

Keywords: Wastewater, Gram-negative, *Enterobacteriaceae*, Lactose-fermenter, Multidrug-resistant, Extended-spectrum beta-lactamases

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INTRODUCTION

The family *Enterobacteriaceae* includes approximately 250 species of Gram-negative, facultatively anaerobic, catalase-positive, non-spore-forming bacilli, most of which are motile. Although many members of this family inhabit the intestinal tracts of humans and animals, some are also free-living.[1,2]

Some members of this family are primary pathogens transmitted from infected humans or colonized animals.[3] These primary pathogens may cause gastroenteritis and other infections, some of which can be fatal.[3-6]

However, some members of this family are opportunistic pathogens, such as *Proteus*, *Escherichia coli*, and *Enterobacter,* which may cause various nosocomial and community-acquired infections, including urinary tract and wound infections, pneumonia, septicemia, and meningitis.[3,7]

Antibiotics are chemicals that target specific components of bacterial cells to either stop replication (bacteriostatic antibiotics) or cause death (bactericidal antibiotics).[8]

Beta-lactam and glycopeptide antibiotics interfere with bacterial cell wall biosynthesis, resulting in bacterial cell death via osmotic lysis.[9,10] Aminoglycoside, macrolide, tetracycline, and chloramphenicol antibiotics target ribosomes, inhibiting protein synthesis and preventing bacterial replication or causing bacterial death.[9,10] Sulfonamides and trimethoprim interfere with the biosynthetic pathway of purine nucleotides, halting bacterial replication.[11] Quinolones and fluoroquinolones kill bacterial cells by inhibiting DNA gyrase.[12,13] Finally, rifampin and rifampicin antibiotics kill bacterial cells by targeting their RNA polymerase.[14,15]

Antibiotics are used worldwide to treat bacterial infections in both humans and animals.[16] The widespread use of antibiotics has promoted the emergence of resistant bacterial strains of different species.[16,17] Interestingly, antibiotic resistance is now considered a global crisis.[18]

Various antibiotic resistance mechanisms have been developed by bacteria. These include decreased permeability, enzymatic inactivation, alteration of their target sites, and increased efflux.[19,20]

A multidrug-resistant (MDR) bacterium is defined as one that resists three or more antibiotics of different classes simultaneously.[21] This occurs because resistant strains can harbor several antibiotic resistance genes that confer resistance to different antibiotics. These genes may co-exist on transmissible genetic elements such as plasmids or transposons.[22,23]

Although infections caused by MDR Gram-negative bacterial strains were initially associated with nosocomial infections, recent studies have shown a notable increase in the prevalence of community-acquired infections caused by these strains.[24,25]

MDR bacteria of the family *Enterobacteriaceae* are increasingly becoming part of the gut microbiota in both humans and animals.[26-28] The passage of these MDR bacteria, along with fecal material from humans and animals, results in their environmental spread through both wastewater pathways and accumulation sites.[29-32]

Interestingly, it is estimated that 40%-90% of an antibiotic administered to humans or animals is excreted in their urine and feces in an active form.[33-37] Upon reaching wastewater pathways and accumulation sites, these excreted antibiotics may act as a driving force for the emergence of MDR bacteria.[29-32] Thus, wastewater pathways and accumulation sites can be important sources for the spread of MDR bacteria in the environment, increasing the risk of community-acquired infections with these highly resistant pathogens.[38-40]

Many studies in various countries have investigated the prevalence of MDR bacteria among isolates obtained from wastewater pathways and accumulation sites.[41-45] Therefore, the main goal of this study was to assess the prevalence of MDR Gram-negative bacteria of the family *Enterobacteriaceae* isolated from the main wastewater pathway in the Nablus area, West Bank, Palestine, a study that, to our knowledge, has never been conducted before in Palestine.

METHODOLOGY

Sample processing and identification of the obtained bacterial isolates

A single 300 mL wastewater sample was

obtained in June 2023 from the main wastewater pathway in the Nablus city, West Bank, Palestine. The sample was filtered through a sterile cotton cloth to remove solid waste material and collected in a sterile container. Approximately 10 mL of the drained sample was transferred into a sterile 50 mL screw-capped conical tube (obtained from Fisher Scientific (Loughborough, Leicestershire, UK). The tubes were then centrifuged at 1,958 × *g* for approximately 10 min. The supernatant was then removed, and the pellet was resuspended in 10 mL of sterile nutrient broth. The tubes were then incubated for 1 h in a shaker incubator at 37 °C under aerobic conditions. At the end of the incubation period, 10-fold serial dilutions were prepared from the cultures. The tube with the 10^4 dilution was used to inoculate 20 plates of MacConkey culture medium obtained from Oxoid (Basingstoke, Hampshire, UK). Each of the MacConkey agar plates was inoculated with 50 µL, which was spread all over the agar surface using a disposable sterile plastic spreader. The plates were incubated at 37 °C for 24 h under aerobic conditions. After incubation, 200 individual pink colonies (lactose fermenters) with smooth surfaces were randomly selected and then sub-cultured separately on MacConkey agar plates (Oxoid). The plates were incubated at 37 °C for approximately 24 h under aerobic conditions.

Subsequently, each bacterial isolate was identified using the API-20 E identification system obtained from BioMerieux (Marcy-l'Étoile, France) as described by the manufacturer.

Antibiotic susceptibility testing

Antibiotic susceptibility testing and interpretation of the obtained results were performed using the disc diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI).[46] Each isolate belonging to the family *Enterobacteriaceae* was examined for susceptibility to ampicillin, cefepime, cefotaxime, ceftazidime, trimethoprim-sulfamethoxazole, gentamicin, tetracycline, ciprofloxacin, meropenem, aztreonam, and chloramphenicol. The antibiotic discs were obtained from Oxoid (Basingstoke, Hampshire, UK), and the *E. coli* ATCC strain 25922 obtained from the American Type Culture Collection (Manassas, VA, USA), was used as a control.

Table 1. The number and the percentage of each of the obtained bacteria out of the obtained 162 isolates

Bacterial isolate	Out of the 162 isolates	% out of the 162 isolates
E. coli	94	58.0
Klebsiella pneumoniae	31	19.1
Klebsiella oxytoca	8	4.9
Citrobacter koseri	21	13.0
Ewingella americana	3	1.9
Enterobacter spp.	3	1.9
Providencia rettgeri	2	1.2
Total number	**162**	**100%**

Phenotypic characterization of ESBLs

Extended-spectrum beta-lactamase (ESBL) production was phenotypically characterized for each isolate that demonstrated resistance or intermediate resistance to cefotaxime and/or ceftazidime. This was conducted using the combination disc procedure with ceftazidime, ceftazidime-clavulanic acid, cefotaxime, and cefotaxime-clavulanic acid, as recommended by the CLSI.[46] An isolate was considered an ESBL producer if the inhibition zone diameter around ceftazidime-clavulanic acid or cefotaxime-clavulanic acid discs increased by more than 5 mm relative to the diameter around discs containing only cefotaxime and/or ceftazidime.

RESULTS

Identification of the isolated bacterial species

Out of 200 Gram-negative lactose-fermenting isolates, 162 were identified as members of the family *Enterobacteriaceae,* including the following species: *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter koseri*, *Ewingella americana*, *Providencia rettgeri*, and *Enterobacter cloacae*. The number and percentage of each species among the 162 isolates are shown in Table 1. Each of these species is an opportunistic pathogen that can both localized and potentially fatal systemic infections.[47-53]

Antibiotic susceptibility testing

Antibiotic susceptibility analysis was conducted for each of the obtained isolates of the family *Enterobacteriaceae* against eleven different antibiotics. The numbers and percentages of

Table 2. The numbers and the percentage rates of all the obtained isolates in terms of their susceptibility profiles to the tested antibiotics

	Out of obtained 162 isolates					
	S		IR		R	
Antibiotic	No. of isolates	%	No. of isolates	%	No. of isolates	%
Ampicillin	74	45.7	45	27.8	43	26.5
Cefepime	162	100	0	0	0	0
Cefotaxime	138	85.2	15	9.3	9	5.6
Ceftazidime	157	96.9	4	2.5	1	0.6
Meropenem	162	100	0	0	0	0
Aztreonam	158	97.5	3	1.9	1	0.6
Gentamicin	155	95.7	1	0.6	6	3.7
Tetracycline	120	74.1	2	1.2	40	24.7
Ciprofloxacin	81	50.0	70	43.2	11	6.8
Trimethoprim-Sulfamethoxazole	144	88.9	3	1.9	15	9.3
Chloramphenicol	142	87.7	0	0	20	12.3

(S: susceptible, IR: Intermediate-resistant or R: Resistant)

Table 3. The numbers and the percentage rates out of the 94 *E. coli* isolates

	E. coli					
	S		IR		R	
Antibiotic	No. of isolates	%	No. of isolates	%	No. of isolates	%
Ampicillin	49	52.1	20	21.3	25	26.6
Cefepime	94	100	0	0	0	0
Cefotaxime	80	85.1	8	8.5	6	6.4
Ceftazidime	91	96.8	2	2.1	1	1.1
Meropenem	94	100	0	0	0	0
Aztreonam	91	96.8	2	2.1	1	1.1
Gentamicin	87	92.6	1	1.1	6	6.4
Tetracycline	58	61.7	2	2.1	34	36.2
Ciprofloxacin	48	52.1	39	41.5	7	7.4
Trimethoprim-Sulfamethoxazole	77	81.9	3	3.2	14	14.9
Chloramphenicol	75	79.8	0	0	19	20.2

S: Susceptible, IR: Intermediate-Resistant or R: Resistant to each of the tested antibiotics

isolates that exhibited susceptibility, intermediate resistance, or resistance to each tested antibiotic are presented in Table 2. The highest rates of resistance were observed for ampicillin, tetracycline, chloramphenicol, and trimethoprim/sulfamethoxazole, at 26.5%, 24.7%, 12.3%, and 9.3%, respectively. Conversely, the lowest rates of resistance were observed for ciprofloxacin, gentamicin, and cefotaxime, at 6.8%, 3.7%, and 5.6%, respectively. Only 0.6% of the total isolates were resistant to ceftazidime and aztreonam, and none of the isolates were resistant to meropenem or cefepime (Table 2).

Table 4. The antibiotic resistant profiles of the multidrug-resistant *E. coli* isolates

Number of the *E. coli* isolates that were/was multidrug resistant	Resistant to
10	Ampicillin, Tetracycline and Chloramphenicol
1	Tetracycline, Trimethoprim/Sulfamethoxazole and Chloramphenicol
1	Ampicillin, Tetracycline, Trimethoprim/Sulfamethoxazole and Chloramphenicol
1	Gentamicin, Tetracycline, Ciprofloxacin and Chloramphenicol
1	Gentamicin, Tetracycline, Trimethoprim/Sulfamethoxazole, and Chloramphenicol
1	Ampicillin, Gentamicin, Tetracycline, and Trimethoprim/Sulfamethoxazole
1	Ampicillin, Gentamicin, Tetracycline and Ciprofloxacin
1	Gentamicin, Tetracycline and Ciprofloxacin
1	Ampicillin, Cefotaxime, Gentamicin, Tetracycline and Ciprofloxacin

Table 5. The numbers and the percentage rates out of the 31 *Klebsiella pneumoniae* isolates

	Klebsiella pneumoniae					
	S		IR		R	
Antibiotic	No. of isolates	%	No. of isolates	%	No. of isolates	%
Ampicillin	13	41.9	9	29.0	9	29.0
Cefepime	31	100	0	0	0	0.0
Cefotaxime	25	80.6	5	16.1	1	3.2
Ceftazidime	31	100	0	0	0	0
Meropenem	31	100	0	0	0	0
Aztreonam	30	96.8	1	3.2	0	0
Gentamicin	31	100	0	0	0	0
Tetracycline	31	100.	0	0	0	0
Ciprofloxacin	16	51.6	14	45.2	1	3.2
Trimethoprim-Sulfamethoxazole	31	100	0	0	0	0
Chloramphenicol	31	100	0	0	0	0

S**:** Susceptible, IR**:** Intermediate-resistant or R**:** Resistant to each of the tested antibiotics

Concerning the *E. coli* isolates, the highest rates of resistance were observed for tetracycline, ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole, at 36.2%, 26.6%, 20.2%, and 14.9% respectively. The resistance rate to ciprofloxacin was 7.4% and the resistance rates to Gentamicin and Cefotaxime were 6.4% for both. The lowest rate of resistance was observed for ceftazidime and aztreonam, at 1.1% for both (Table 3).

Interestingly, 18 (19.1%) of the obtained 94 *E. coli* isolates were MDR, meaning they were resistant to three or more antibiotics of different classes (21) (Table 3).

Of these 18 *E. coli* isolates, 10 were resistant to ampicillin, tetracycline, and Chloramphenicol, 1 was resistant to tetracycline, trimethoprim/sulfamethoxazole, and chloramphenicol, 1 was resistant to ampicillin, tetracycline, trimethoprim/sulfamethoxazole, and chloramphenicol, 1 was

Table 6. The numbers and the percentage rates out of the 8 *Klebsiella oxytoca* isolates

Antibiotic	*Klebsiella oxytoca*					
	S		IR		R	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Ampicillin	4	50	3	37.5	1	12.5
Cefepime	8	100	0	0	0	0
Cefotaxime	8	100	0	0	0	0
Ceftazidime	8	100	0	0	0	0
Meropenem	8	100	0	0	0	0
Aztreonam	8	100	0	0	0	0
Gentamicin	8	100	0	0	0	0
Tetracycline	7	87.5	0	0	1	12.5
Ciprofloxacin	2	25	6	75	0	0
Trimethoprim-Sulfamethoxazole	8	100	0	0	0	0
Chloramphenicol	8	100	0	0	0	0

S: Susceptible, IR: Intermediate-resistant or R: Resistant to each of the tested antibiotics

resistant to gentamicin, tetracycline, ciprofloxacin, and chloramphenicol, 1 was resistant to gentamicin, tetracycline, trimethoprim/sulfamethoxazole, and chloramphenicol, 1 was resistant to ampicillin, gentamicin, tetracycline, and trimethoprim/sulfamethoxazole, 1 was resistant to ampicillin, gentamicin, tetracycline, and ciprofloxacin, 1 was resistant to gentamicin, tetracycline, and ciprofloxacin, and 1 final isolate was resistant to ampicillin, cefotaxime, gentamicin, tetracycline and ciprofloxacin (Table 4).

Regarding the obtained 31 *K. pneumoniae* isolates, 29% of them were resistant to ampicillin and 3.2% to cefotaxime and ciprofloxacin (Table 5). Conversely, about 29.2%, 16.1%, 3.2%, and 45.2% of the *K. pneumoniae* isolates exhibited intermediate resistance to ciprofloxacin, ampicillin, cefotaxime, and aztreonam, respectively (Table 5). None of the obtained *K. pneumoniae* isolates showed resistance to chloramphenicol, trimethoprim/sulfamethoxazole, ceftazidime, meropenem tetracycline, or gentamicin (Table 5). None of the obtained *K. pneumoniae* isolates were MDR.

Regarding the eight *K. oxytoca* isolates, only one showed resistance to ampicillin and another one showed resistance to tetracycline. In contrast, three and six of these isolates showed intermediate resistance to ampicillin and ciprofloxacin, respectively. However, none of the isolates exhibited resistance or intermediate resistance to cefepime, cefotaxime, ceftazidime, meropenem, aztreonam, gentamicin, trimethoprim /sulfamethoxazole, or chloramphenicol (Table 6). None of the obtained *K. oxytoca* isolates were MDR.

Concerning the 21 *C. koseri* isolates obtained, the highest levels of resistance were observed against ampicillin, tetracycline, cefotaxime, and ciprofloxacin, at 38.1%, 19%, 9.2%, and 14.3%, respectively (Table 7). Only 4.9% of the isolates were resistant to either chloramphenicol or trimethoprim/sulfamethoxazole (Table 7).

Interestingly, two (12.9%) of the *C. koseri* isolates were MDR. One was resistant to ampicillin, tetracycline, and ciprofloxacin, while the other was resistant to ampicillin, cefotaxime, ciprofloxacin, and trimethoprim/sulfamethoxazole.

None of the three obtained *E. americana* or *E. cloacae* isolates showed resistance to any of the tested antibiotics. Only one of the two *P. rettgeri* isolates showed resistance to tetracycline. This can be explained by their small number among the total number of isolates obtained. The *E. coli* ATCC 25922 strain was sensitive to all antibiotics used.

Table 7. The numbers and the percentage rates out of the 21 *Citrobacter koseri* isolates

Antibiotic	*Citrobacter koseri*					
	S		IR		R	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Ampicillin	5	23.8	8	38.1	8	38.1
Cefepime	21	100	0	0	0	0
Cefotaxime	17	81	2	9.5	2	9.5
Ceftazidime	19	90.5	2	9.5	0	0
Meropenem	21	100	0	0	0	0
Aztreonam	20	95.2	1	4.8	0	0
Gentamicin	21	100	0	0	0	0
Tetracycline	17	81	0	0	4	19
Ciprofloxacin	11	52.4	7	33.3	3	14.3
Trimethoprim-Sulfamethoxazole	20	95.2	0	0	1	4.8
Chloramphenicol	20	95.2	0	0	1	4.8

S: Susceptible, IR: Intermediate-resistant and R: Resistant to each of the tested antibiotics

Identification of ESBL producers among the obtained isolates

Eighteen of the obtained isolates were either resistant or intermediately resistant to cefotaxime and/or ceftazidime. These isolates were examined for ESBL production, as described in the Methodology section. Our results confirmed that three *E. coli*, one *K. pneumoniae*, and two *C. koseri* isolates were confirmed to be ESBL producers. This implied that six (3.7%) of the total 162 isolates were ESBL producers.

DISCUSSION

Nablus City has several wastewater pathways that converge to form the main wastewater pathway from which the sample for this study was collected. Accordingly, the obtained single sample was representative of the city's wastewater.

In total, 162 lactose-fermenting isolates belonging to the family *Enterobacteriaceae* were obtained from this sample. *E. coli, K. pneumoniae*, and *C. koseri* represented approximately 91% of the total isolates, while *K. oxytoca*, *E. americana*, *Enterobacter* spp., and *P. rettgeri* represented approximately 8.9%.

An MDR bacterium is one that can resist to three or more different classes of antibiotics. This resistance may occur because of the simultaneous presence of several antibiotic resistance genes, such as those carried on a resistance plasmid.[54] Interestingly, a bacterium that shows intermediate resistance to an antibiotic *in vitro* may exhibit complete resistance *in vivo*.[55] Despite this, only isolates showing full resistance *in vitro* were considered MDR in this study.

Different mechanisms confer resistance to several antibiotics, such as an efflux pump that can export several antibiotics to the outside of the bacterial cell, thus preventing the antibiotics from reaching an effective concentration in the bacterial cytoplasm.[54]

The spread of MDR bacterial strains throughout the environment may increase the rate of community-acquired infections with strains that are clinically challenging to treat.[18]

Many studies from different countries have investigated the prevalence of MDR bacteria in sewage, wastewater pathways, collection areas, treatment plants, sewage sludge, and sewage-contaminated water. In Japan, several MDR bacteria were isolated from public wastewater, including carbapenem-resistant *Enterobacteria*, ESBL-

producing *Enterobacteria*, MDR *Acinetobacter*, MDR *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococci.*[56] In addition, antibiotic-resistant bacteria have been isolated from wastewater in different locations, such as *E. coli* in South Africa,[57] *Enterococcus* spp., *Staphylococcus* spp., *Pseudomonas* spp., and *Acinetobacter* spp. in Slovakia,[58] and *Pseudomonas*, *Aeromonas*, and *Bacillus* in Spain.[59] In an Ethiopian study, the prevalence rate of MDR *E. coli* isolates obtained from a sewage-contaminated river was 78%.[60] In an Austrian study, it was found that 16% of the *E. coli* isolates obtained from sewage sludge were MDR.[61] In addition, in a Saudi Arabian study, 7.7% of the *E. coli* isolates obtained from an urban sewage were MDR.[62] In our study, antibiotic-resistant bacteria including *E. coli*, *K. pneumoniae*, and *C. koseri* were isolated from the main wastewater pathway in the Nablus district of the West Bank, Palestine.

E. coli isolates from South Africa showed higher resistance levels to ampicillin (55.6%), gentamicin (0.5%), and tetracycline (60.1%) compared to the resistance level of *E. coli* in this study, with resistance levels of 26.6%, 6.4%, and 36.2%, respectively.[57]

In South India, two wastewater samples indicated more than 80% and more than 50% resistance among *E. coli* isolates to ampicillin, tetracycline, sulfamethoxazole–trimethoprim, and cefotaxime,[63] which is higher than resistance levels in this study for the same antibiotics.

The fact that none of the isolates were resistant to meropenem or cefepime can be explained by the fact that these antibiotics are not commonly used in Palestine.[64,65]

E. coli is an opportunistic pathogen that causes various types of infections. Although it is the most common cause of urinary tract infections, it may also cause wound infections, pneumonia, septicemia, septic shock, and meningitis.[66]

E. americana was first described by Grimont et al. in 1983.[67] Although it may cause plant infections, it mainly affects immunocompromised patients and neonates, and causes various life-threatening infections.[68] In our study, only three *E. americana* isolates were obtained, one of which was susceptible to all antibiotics used, and the other two exhibited intermediate resistance to one of the tested antibiotics (data not shown).

A previous study conducted in Saudi Arabia described an MDR strain of *E. americana* that caused severe pneumonia in a young patient.[51,69]

Fecal colonization with MDR Gram-negative bacteria of the *Enterobacteriaceae* family has been found to occur in both humans and animals. Accordingly, it is not surprising that these MDR Gram-negative bacteria are found in wastewater pathways, collection areas, or treatment plants.

The ability of organisms to share and transfer genetic material not connected to a parental relationship is known as horizontal gene transfer (HGT). HGT has been widely investigated as the cause of adaptation mechanisms that facilitate the transfer of antimicrobial resistance and virulence factors, enhancing the ability of the bacterium to overcome challenging environments.

Wastewater contains a large collection of organisms that interact with one another. The evolution of bacteria relies on HGT between organisms, which results in the spread of resistance genes. Gene transfer between bacteria results in the simultaneous development of resistance to different types of antibiotics. Monitoring these resistances is critical for public health, and appropriate policies must be applied to minimize their impact.

CONCLUSION

In our study, 12.3% of the total isolates and 19.1% of the *E. coli* isolates were MDR. Although the prevalence rate in our study was less than that in that mentioned in the Ethiopian study, it is similar to that reported in the Austrian study and it is clearly higher than that reported by the Saudi study mentioned earlier.

This requires prompt measures from the people in charge of the Palestinian Ministry of Health to prevent further progression of the prevalence of MDR bacteria in our environment.

Limitation of the study

Although this study was the first of its kind in Palestine, the number of isolates obtained was relatively small, and this study focused on some lactose-fermenting species of the family *Enterobacteriaceae*. A more comprehensive

study is needed to shed further light on the environmental prevalence of MDR Gram-negative bacteria as well as the prevalence of ESBL producers. In addition, antibiotic resistance genes were not characterized or detected in this study; however, these will be investigated in a future study.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

MMA and ODA conceptualized the study. MMA, ADA, and NND applied methodology. MMA performed data curation. ZHAD, ARB and FKO performed formal analysis. MA and ARQ performed data investigation. AOA and FKO supervised the study. FKO and ZHAD performed visualization. AOA and MMA wrote the manuscript. MA reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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