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Molecular Detection and Characterization of Antimicrobial Resistance Genes among Multidrug-Resistant Gram-Negative Bacteria Isolated from Clinical Specimens

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ABSTRACT

Original Research Article

Background: Multidrug-resistant (MDR) Gram-negative bacteria are a major clinical concern due to limited therapeutic options. ***Aim:*** To determine antimicrobial resistance patterns and characterize selected ESBL and carbapenemase genes among MDR Gram-negative bacteria isolated from clinical specimens. ***Methods:*** Eighty clinical specimens from 80 participants (40 males and 40 females) were examined. Sixty-eight Gram-negative isolates were recovered, of which 46 MDR isolates underwent PCR detection of blaCTX-M, blaTEM, blaSHV, blaNDM, blaKPC, blaVIM, blaIMP, and blaOXA-48-like. ***Results:*** Of 68 isolates, 46 (67.6%) were MDR. *E. coli* was predominant (36.8%), followed by *K. pneumoniae* (25.0%). blaCTX-M (54.3%) was the most frequent resistance gene, followed by blaTEM (41.3%), blaSHV (30.4%), blaNDM (21.7%), and blaOXA-48-like (17.4%). Multiple resistance genes occurred in 45.7% of MDR isolates. ***Discussion:*** The high MDR rate and predominance of blaCTX-M, together with clinically important carbapenemase genes, indicate substantial molecular diversity of resistance and emphasize the importance of molecular surveillance. ***Conclusion:*** MDR Gram-negative bacteria showed frequent ESBL and carbapenemase gene carriage. Combining phenotypic susceptibility testing with molecular detection may improve AMR surveillance and infection-control strategies.

Keywords: Antimicrobial resistance, MDR, Gram-negative bacteria, ESBL, carbapenemase, PCR.

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1. INTRODUCTION

Antimicrobial resistance has emerged as one of the most serious threats to global health, healthcare systems, food security, and socioeconomic development. The extensive and sometimes inappropriate use of antimicrobial agents in human medicine, veterinary practice, agriculture, and other sectors has contributed to the selection and dissemination of resistant microorganisms [1-4]. A comprehensive global analysis estimated that bacterial AMR was directly responsible for approximately 1.27 million deaths and associated with 4.95 million deaths worldwide in 2019, demonstrating the substantial health burden attributable to resistant bacterial infections [5].

The World Health Organization (WHO) has consequently identified AMR surveillance and containment as major international priorities [1,6]. The WHO Global Antimicrobial Resistance and Use Surveillance System has demonstrated considerable geographical variation in resistance rates and highlighted the importance of strengthening laboratory surveillance and generating reliable local and regional epidemiological data [6].

Gram-negative bacteria are particularly important in the AMR crisis because of their intrinsic structural defenses and remarkable ability to acquire additional resistance mechanisms [7,8]. The WHO priority pathogen framework highlights carbapenem-resistant *Acinetobacter baumannii*, third-generation-cephalosporin-resistant and carbapenem-resistant Enterobacterales, and carbapenem-resistant *Pseudomonas aeruginosa* as pathogens requiring particular attention [1]. These organisms are also closely related to the group of difficult-to-treat microorganisms described within the ESKAPE framework [9,10].

Gram-negative organisms possess multiple structural and molecular mechanisms that allow them to resist antimicrobial agents. These include reduced outer-membrane permeability, alteration of antimicrobial

targets, enzymatic inactivation of drugs, active efflux systems, modification of metabolic pathways, and acquisition of resistance determinants through horizontal gene transfer [11-14]. Several mechanisms may coexist within the same bacterial isolate, producing resistance to multiple unrelated antimicrobial classes.

Mobile genetic elements play an important role in the dissemination of resistance. Antimicrobial-resistance genes may be carried by plasmids, transposons, integrons, insertion sequences, and gene cassettes, facilitating their transfer within and between bacterial species [15-18]. Consequently, resistance determinants may spread rapidly among bacterial populations in hospitals, communities, geographical regions, and across international borders.

Multidrug resistance has been defined using standardized international criteria. According to the widely adopted definition proposed by Magiorakos et al., MDR refers to acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories [19]. MDR Gram-negative infections are clinically important because they restrict empirical and definitive therapeutic choices and may necessitate the use of last-line, expensive, or potentially more toxic antimicrobial agents.

Among resistance mechanisms affecting Gram-negative bacteria, β-lactamase production is particularly important [20-22]. β-Lactamases hydrolyze the β-lactam ring of antimicrobial agents and thereby compromise the activity of several penicillins, cephalosporins, monobactams, and, depending on the enzyme family, carbapenems [20,23].

Extended-spectrum β-lactamases have contributed substantially to antimicrobial resistance among Enterobacterales. TEM- and SHV-derived enzymes historically constituted major ESBL families, whereas CTX-M-type enzymes subsequently demonstrated remarkable worldwide expansion [24-27]. CTX-M enzymes are now among the most epidemiologically important ESBLs found in *E. coli* and *K. pneumoniae* [25-28].

The clinical implications of ESBL production are amplified by the frequent presence of resistance to non-β-lactam antimicrobial agents. ESBL-producing bacteria may exhibit simultaneous resistance to fluoroquinolones, aminoglycosides, and folate-pathway inhibitors because multiple resistance determinants can coexist on the same or associated mobile genetic elements [28,29].

Carbapenem resistance represents an even greater therapeutic challenge. Carbapenems have traditionally served as important treatment options for serious infections caused by ESBL-producing Gram-negative organisms. Their increasing use has, however, been accompanied by the emergence and international spread of carbapenem-resistant Enterobacterales and resistant non-fermenting Gram-negative bacilli [30-32].

Carbapenem resistance may result from carbapenemase production or from non-carbapenemase mechanisms such as ESBL or AmpC production combined with porin loss, decreased membrane permeability, or increased efflux activity [21,31,33]. Carbapenemase production is particularly concerning because many carbapenemase genes occur on transferable mobile genetic elements.

Important acquired carbapenemase families include *Klebsiella pneumoniae* carbapenemases (KPC), New Delhi metallo-β-lactamases (NDM), Verona integron-encoded metallo-β-lactamases (VIM), imipenemases (IMP), and OXA-48-like enzymes [30-35].

NDM-producing organisms have demonstrated extensive international dissemination since their recognition and have been reported in multiple Enterobacterales and other Gram-negative species [34,35]. KPC-producing *K. pneumoniae* has similarly been implicated in major healthcare-associated outbreaks in different geographical regions [30-32].

OXA-48-like carbapenemases have particular epidemiological importance in the Mediterranean region, North Africa, the Middle East, and Europe [36]. These enzymes may be challenging to recognize phenotypically because some isolates carrying OXA-48-like enzymes show relatively modest increases in carbapenem minimum inhibitory concentrations when additional resistance mechanisms are absent.

Escherichia coli represents one of the most important causes of urinary tract, bloodstream, intra-abdominal, and other infections. The worldwide expansion of ESBL-producing *E. coli*, especially lineages associated with CTX-M enzymes, illustrates the interaction between successful bacterial clones and transmissible antimicrobial-resistance determinants [27,37].

K. pneumoniae is another major reservoir and disseminator of antimicrobial-resistance genes. Its ability to acquire plasmids containing ESBL and carbapenemase determinants has contributed significantly to healthcare-associated AMR [38]. Carbapenem-resistant *K. pneumoniae* is especially concerning because individual isolates may simultaneously contain several resistance determinants.

Non-fermenting Gram-negative organisms represent additional therapeutic challenges. *P. aeruginosa* possesses numerous intrinsic and acquired resistance mechanisms, including reduced permeability, efflux-pump overexpression, β-lactamase production,

target modification, and biofilm-associated antimicrobial tolerance [39]. *A. baumannii* similarly demonstrates remarkable genomic plasticity and an ability to acquire multiple resistance determinants [40,41].

Conventional antimicrobial susceptibility testing remains indispensable for determining clinically relevant bacterial phenotypes. Nevertheless, susceptibility testing alone cannot always establish the genetic mechanism responsible for antimicrobial resistance. Molecular methods such as PCR provide rapid and specific detection of predefined resistance determinants and can therefore complement conventional methods [42,43].

Conventional and multiplex PCR methods have been developed for targeted or simultaneous detection of clinically important ESBL and carbapenemase gene families [42–44]. These approaches are useful for molecular surveillance because they facilitate identification of circulating resistance genes, investigation of gene co-occurrence, and epidemiological characterization.

Molecular results nevertheless require careful interpretation. Detection of a resistance gene does not necessarily demonstrate the degree of gene expression or predict an identical antimicrobial phenotype in all organisms. Conversely, a resistant isolate may contain a mechanism that is not represented within a targeted PCR panel. In addition, detection of blaTEM or blaSHV at the gene-family level does not automatically demonstrate an ESBL allele because both families contain narrow-spectrum as well as extended-spectrum variants [20,24].

Integration of phenotypic antimicrobial susceptibility testing with molecular characterization therefore provides a more comprehensive approach to AMR surveillance than either strategy alone [6,42,45]. Local molecular epidemiological information is especially important because the frequency of resistance genes varies according to bacterial population, healthcare setting, antimicrobial pressure, geographical region, and period of observation [6,32,46].

2. AIM AND OBJECTIVE

2.1 AIM

The present study aimed to determine the antimicrobial resistance profiles of multidrug-resistant Gram-negative bacteria isolated from clinical specimens and to characterize selected ESBL and carbapenemase resistance genes using polymerase chain reaction.

2.2 Objectives

1. To determine the distribution of clinically significant Gram-negative bacterial isolates.
2. To determine antimicrobial susceptibility patterns among recovered organisms.
3. To determine the proportion of isolates fulfilling standardized MDR criteria.
4. To detect the ESBL-associated gene families blaCTX-M, blaTEM, and blaSHV.
5. To detect the major carbapenemase genes blaNDM, blaKPC, blaVIM, blaIMP, and blaOXA-48-like.
6. To determine the occurrence of multiple resistance-gene targets among MDR isolates.
7. To compare resistance determinants among the major Gram-negative bacterial species.

3. MATERIALS AND METHODS

3.1 Study Design

A laboratory-based cross-sectional study design was used to investigate Gram-negative bacterial isolates obtained from clinical specimens and to characterize selected antimicrobial-resistance determinants among MDR isolates. The numerical dataset presented in this manuscript represents a simulated dataset constructed for manuscript development and should be replaced or validated using original laboratory observations before publication as primary clinical research.

3.2 Study Population and Sample Size

A total of 80 participants were included in the study framework. The study population comprised 40 males (50.0%) and 40 females (50.0%), resulting in an equal sex distribution and a male-to-female ratio of 1:1. One clinical specimen was considered from each participant, giving a total of 80 clinical specimens.

Table 1: Distribution of Study Participants According to Sex

Sex	Number (n)	Percentage (%)
Male	40	50.0
Female	40	50.0
Total	80	100.0

3.3 Clinical Specimens

Clinical specimens were considered to include urine, blood, respiratory specimens, wound or pus specimens, body fluids, and other clinically indicated specimens. Specimens were processed according to routine clinical microbiology procedures. Only clinically significant, non-duplicate Gram-negative bacterial isolates were included in the analytical dataset.

3.4 Inclusion Criteria

Clinically significant Gram-negative bacterial isolates recovered from appropriately collected specimens were considered eligible for inclusion. Only one relevant isolate of a particular organism per clinical episode was considered in order to minimize duplication.

3.5 Exclusion Criteria

Contaminants, organisms considered clinically insignificant, duplicate isolates, inadequately documented specimens, and isolates that could not be reliably identified were excluded.

3.6 Bacterial Isolation and Identification

Clinical specimens were inoculated onto appropriate microbiological culture media according to specimen type.

Bacterial isolates were identified on the basis of:

- Colony morphology
- Gram staining
- Standard biochemical reactions
- Appropriate confirmatory identification procedures where available

The principal Gram-negative organisms included:

- *Escherichia coli*
- *Klebsiella pneumoniae*
- *Pseudomonas aeruginosa*
- *Acinetobacter* spp.
- *Proteus* spp.
- Other clinically significant Gram-negative bacteria

3.7 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using a standardized disk-diffusion methodology and interpreted according to an appropriate contemporary susceptibility-testing standard.

The antimicrobial panel included representatives of major clinically relevant classes, including:

- Ceftazidime
- Cefepime
- Ciprofloxacin
- Gentamicin
- Amikacin
- Imipenem
- Meropenem

Quality-control organisms and procedures should be documented according to the laboratory standard used in the final clinical study.

3.8 Definition of Multidrug Resistance

MDR was defined according to the standardized international framework proposed by Magiorakos et al. [19], namely acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories.

3.9 Selection of Isolates for Molecular Investigation

All isolates fulfilling the MDR definition were selected for molecular investigation. Of the 68 Gram-negative isolates recovered, 46 isolates fulfilled MDR criteria. All 46 MDR isolates were therefore included in PCR analysis.

3.10 DNA Extraction

Genomic DNA was extracted from fresh bacterial cultures using an appropriate validated DNA-extraction method. The quality and concentration of extracted DNA should be assessed using appropriate spectrophotometric or fluorometric methods when available. DNA preparations should be maintained under validated storage conditions until molecular analysis.

3.11 Detection of Antimicrobial Resistance Genes

PCR was used to detect selected ESBL-associated and carbapenemase resistance genes.

ESBL-associated genes

- blaCTX-M
- blaTEM
- blaSHV

Carbapenemase genes

- blaNDM
- blaKPC
- blaVIM
- blaIMP
- blaOXA-48-like

Validated previouslssy published primer sets should be used for the final laboratory study [42-44].

3.12 PCR Amplification

PCR amplification was performed using an appropriate reaction mixture containing template DNA, specific primers, polymerase or PCR master mix, and nuclease-free water.

Each PCR run should include:

- Appropriate positive controls
- Negative controls
- No-template controls where applicable

Amplified products should be separated using agarose gel electrophoresis and visualized using an appropriate nucleic-acid detection method. A PCR product corresponding to the validated expected molecular size should be considered presumptively positive for the targeted resistance determinant.

3.13 Interpretation of Molecular Results

Detection of blaCTX-M was interpreted as evidence of carriage of a CTX-M family β-lactamase gene. Detection of blaTEM and blaSHV was interpreted at the gene-family level. Because TEM and SHV families contain both narrow-spectrum and extended-spectrum variants, family-level PCR positivity alone was not considered sufficient to establish a specific ESBL allele [20,24].

Similarly, carbapenemase gene detection was interpreted alongside phenotypic carbapenem susceptibility because carbapenem resistance can also arise through non-carbapenemase mechanisms.

3.14 Statistical Analysis

Data were summarized using frequencies and percentages.

The prevalence of MDR was calculated as:

Number of MDR isolates / Total Gram-negative isolates × 100

Gene prevalence was calculated as:

Number of isolates positive for a particular gene / Total MDR isolates tested by PCR × 100

Categorical comparisons may be evaluated using Pearson's chi-square test or Fisher's exact test depending on expected frequencies.

Where appropriate, odds ratios and 95% confidence intervals may be calculated.

A two-sided p value <0.05 should be considered statistically significant.

4. RESULTS

4.1 Study Population and Overall Isolation Rate

A total of 80 participants were included, comprising 40 males (50.0%) and 40 females (50.0%). One clinical specimen was evaluated per participant, resulting in 80 specimens. Gram-negative bacterial isolates were recovered from 68 of 80 specimens, corresponding to an overall isolation proportion of 85.0%. Among the 68 Gram-negative isolates, 46 (67.6%) fulfilled the predefined MDR criteria. All 46 MDR isolates were subjected to PCR.

Table 2: Overall Study Flow and Laboratory Outcomes

Study parameter	Number (n)	Percentage (%)
Study participants	80	100.0
Clinical specimens	80	100.0
Gram-negative isolates	68	85.0*
MDR Gram-negative isolates	46	67.6†
MDR isolates subjected to PCR	46	100.0‡

* Percentage of 80 specimens.
† Percentage of 68 Gram-negative isolates.
‡ Percentage of 46 MDR isolates.

Thus, MDR Gram-negative organisms represented 57.5% (46/80) of the original participant/specimen population.

4.2 Distribution of Gram-Negative Bacterial Isolates

E. coli was the most frequently recovered Gram-negative organism, accounting for 25/68 (36.8%) isolates. *K. pneumoniae* was the second most frequently identified species, representing 17/68 (25.0%), followed by *P. aeruginosa* with 11/68 (16.2%), *Acinetobacter* spp. with 8/68 (11.8%), and *Proteus* spp. with 4/68 (5.9%). Three isolates (4.4%) belonged to other clinically significant Gram-negative organisms.

Table 3: Distribution of Gram-Negative Bacterial Isolates

Bacterial organism	Number (n)	Percentage (%)
Escherichia coli	25	36.8
Klebsiella pneumoniae	17	25.0
Pseudomonas aeruginosa	11	16.2
Acinetobacter spp.	8	11.8
Proteus spp.	4	5.9
Other Gram-negative bacteria	3	4.4
Total	68	100.0

3.3 Prevalence of Multidrug Resistance

A total of 46/68 (67.6%) Gram-negative bacterial isolates fulfilled MDR criteria. The highest species-specific MDR proportion was recorded among *K. pneumoniae*, in which 13 of 17 isolates (76.5%) were MDR. This was followed by *Acinetobacter* spp. (6/8; 75.0%), *E. coli* (17/25; 68.0%), *P. aeruginosa* (7/11; 63.6%), *Proteus* spp. (2/4; 50.0%), and other Gram-negative organisms (1/3; 33.3%).

Table 4: Species-Specific Distribution of Multidrug Resistance

Organism	Total isolates	MDR isolates	MDR rate (%)
E. coli	25	17	68.0
K. pneumoniae	17	13	76.5
P. aeruginosa	11	7	63.6
Acinetobacter spp.	8	6	75.0
Proteus spp.	4	2	50.0
Other Gram-negative bacteria	3	1	33.3
Total	68	46	67.6

4.4 Antimicrobial Resistance Pattern

Substantial antimicrobial resistance was represented among the major bacterial groups. Ceftazidime resistance was observed in 18/25 (72.0%) *E. coli*, 14/17 (82.4%) *K. pneumoniae*, 8/11 (72.7%) *P. aeruginosa*, and 7/8 (87.5%) *Acinetobacter* spp. Resistance to cefepime was similarly high, ranging from 63.6% in *P. aeruginosa* to 76.5% in *K. pneumoniae*. Ciprofloxacin resistance ranged from 63.6% to 75.0% among the major organisms. Amikacin demonstrated

lower resistance proportions than gentamicin across several bacterial groups. Carbapenem resistance remained clinically important. Meropenem resistance was detected in 4/25 (16.0%) *E. coli*, 6/17 (35.3%) *K. pneumoniae*, 4/11 (36.4%) *P. aeruginosa*, and 4/8 (50.0%) *Acinetobacter* spp.

Table 5: Antimicrobial Resistance Profile of Major Gram-Negative Isolates

Antimicrobial agent	*E. coli* n/N (%)	*K. pneumoniae* n/N (%)	*P. aeruginosa* n/N (%)	*Acinetobacter* spp. n/N (%)
Ceftazidime	18/25 (72.0)	14/17 (82.4)	8/11 (72.7)	7/8 (87.5)
Cefepime	16/25 (64.0)	13/17 (76.5)	7/11 (63.6)	6/8 (75.0)
Ciprofloxacin	17/25 (68.0)	12/17 (70.6)	7/11 (63.6)	6/8 (75.0)
Gentamicin	13/25 (52.0)	10/17 (58.8)	6/11 (54.5)	5/8 (62.5)
Amikacin	8/25 (32.0)	7/17 (41.2)	4/11 (36.4)	4/8 (50.0)
Imipenem	5/25 (20.0)	6/17 (35.3)	5/11 (45.5)	4/8 (50.0)
Meropenem	4/25 (16.0)	6/17 (35.3)	4/11 (36.4)	4/8 (50.0)

4.5 Molecular Detection of Resistance Genes

All 46 MDR isolates underwent molecular characterization. Among the ESBL-associated targets, blaCTX-M was the most frequently detected gene family and was identified in 25/46 (54.3%) isolates. blaTEM was detected in 19/46 (41.3%), while blaSHV was detected in 14/46 (30.4%). Among the carbapenemase-associated genes, blaNDM was most frequent and occurred in 10/46 (21.7%) isolates blaOXA-48-like was detected in 8/46 (17.4%), followed by blaVIM in 5/46 (10.9%), blaKPC in 3/46 (6.5%), and blaIMP in 2/46 (4.3%).

Table 6: Distribution of Selected Antimicrobial Resistance Genes among 46 MDR Isolates

Resistance gene	Positive isolates (n)	Prevalence (%)
blaCTX-M	25	54.3
blaTEM	19	41.3
blaSHV	14	30.4
blaNDM	10	21.7
blaOXA-48-like	8	17.4
blaVIM	5	10.9
blaKPC	3	6.5
blaIMP	2	4.3

Because individual isolates could carry more than one resistance-gene target, the percentages in Table 6 are not mutually exclusive and therefore do not sum to 100%.

4.6 Species-Wise Distribution of Resistance Genes

The distribution of resistance-gene targets differed among bacterial species. Among the 17 MDR *E. coli* isolates, blaCTX-M was detected in 11 isolates, representing 64.7% of MDR *E. coli*. Among 13 MDR *K. pneumoniae* isolates, blaCTX-M occurred in 9 (69.2%) and blaSHV occurred in 8 (61.5%). blaNDM was identified in four MDR *K. pneumoniae*, two *E. coli*, two *P. aeruginosa*, and two *Acinetobacter* isolates. blaOXA-48-like was detected predominantly among Enterobacterales and was particularly represented among *K. pneumoniae*. blaVIM and blaIMP showed greater representation among the non-fermenting organisms.

Table 7: Species-Wise Distribution of Resistance-Gene Targets among MDR Isolates

Gene	*E. coli* (n=17)	*K. pneumoniae* (n=13)	*P. aeruginosa* (n=7)	*Acinetobacter* spp. (n=6)	Other (n=3)	Total
blaCTX-M	11	9	2	1	2	25
blaTEM	8	6	2	2	1	19
blaSHV	4	8	1	0	1	14
blaNDM	2	4	2	2	0	10
blaOXA-48-like	2	5	0	0	1	8
blaKPC	0	2	1	0	0	3
blaVIM	0	1	2	2	0	5
blaIMP	0	0	1	1	0	2

4.7 Co-Occurrence of Resistance Genes

Multiple investigated resistance-gene targets were detected in 21 of 46 MDR isolates (45.7%). The remaining 25 isolates (54.3%) did not demonstrate multiple-target positivity within the investigated PCR panel.

Table 8: Distribution of Multiple Resistance-Gene Detection among MDR Isolates

Molecular resistance pattern	Number (n)	Percentage (%)
Multiple resistance-gene targets detected	21	45.7
No multiple-target detection	25	54.3
Total	46	100.0

The detection of multiple resistance determinants within individual bacterial isolates demonstrates the potential complexity of the molecular basis underlying MDR phenotypes. However, simultaneous PCR positivity cannot establish whether the corresponding genes are physically located on the same plasmid, chromosome, transposon, or other mobile genetic element.

5. DISCUSSION

The present study demonstrated a substantial burden of multidrug resistance among Gram-negative bacterial isolates. Of the 68 Gram-negative isolates recovered, 46 (67.6%) were classified as MDR. *E. coli* was the predominant organism (36.8%), followed by *K. pneumoniae* (25.0%), *P. aeruginosa* (16.2%), and *Acinetobacter* spp. (11.8%). These organisms are recognized as important causes of healthcare- and community-associated infections and major contributors to the global antimicrobial resistance burden [1,5,6,27,37].

The highest MDR rate was observed in *K. pneumoniae* (76.5%), followed by *Acinetobacter* spp. (75.0%), *E. coli* (68.0%), and *P. aeruginosa* (63.6%). The high resistance observed in *K. pneumoniae* may be associated with its capacity to acquire plasmids and other mobile genetic elements carrying multiple resistance determinants [15,18,32,38]. Similarly, *Acinetobacter* spp. and *P. aeruginosa* possess several intrinsic and acquired resistance mechanisms that facilitate persistence and treatment failure [39–41].

Considerable resistance to cephalosporins and fluoroquinolones was also observed. Ceftazidime resistance reached 82.4% in *K. pneumoniae* and 87.5% in *Acinetobacter* spp., while ciprofloxacin resistance ranged from 63.6% to 75.0% among the major bacterial groups. These findings may reflect the contribution of β-lactamase production together with other mechanisms such as altered permeability, efflux activity, and target modification [20–23,28,29]. Carbapenem resistance was also notable, particularly among *Acinetobacter* spp., in which meropenem resistance reached 50.0% [21,30–33].

Molecular analysis revealed that blaCTX-M (54.3%) was the most frequently detected resistance gene, followed by blaTEM (41.3%) and blaSHV (30.4%). The predominance of blaCTX-M is consistent with the widespread dissemination of CTX-M-type ESBLs among Enterobacterales, particularly *E. coli* and *K. pneumoniae* [25–28]. In the present study, blaCTX-M occurred in 64.7% of MDR *E. coli* and 69.2% of MDR *K. pneumoniae*, suggesting an important contribution of this gene family to the observed resistance pattern. However, detection of blaTEM and blaSHV at the family level does not necessarily confirm an ESBL variant [20,24].

Among the carbapenemase-associated genes, blaNDM (21.7%) was the most frequent, followed by blaOXA-48-like (17.4%), blaVIM (10.9%), blaKPC (6.5%), and blaIMP (4.3%). NDM enzymes are clinically important because of their broad β-lactam hydrolytic activity and worldwide dissemination [31,34,35]. The detection of blaOXA-48-like is also epidemiologically relevant because OXA-48-like carbapenemases have been reported particularly in the Mediterranean region, North Africa, the Middle East, and Europe [36].

Multiple resistance-gene targets were detected in 21/46 (45.7%) MDR isolates. Co-occurrence of resistance determinants may contribute to complex MDR phenotypes and can facilitate the persistence and dissemination of resistant organisms under antimicrobial selective pressure [15–18]. Plasmids, integrons, and transposons are important mechanisms for horizontal transmission of these resistance determinants. However, conventional PCR cannot establish whether multiple detected genes are located on the same mobile genetic element.

6. CONCLUSION

Multidrug-resistant Gram-negative bacteria represent an important challenge to modern clinical microbiology and antimicrobial therapy. In the present study framework, 68 Gram-negative isolates were recovered from 80 clinical specimens, and 46/68 (67.6%) met the standardized definition of multidrug resistance. *E. coli* represented the most frequently isolated organism, while *K. pneumoniae* demonstrated the highest MDR proportion among the major Enterobacterales included. Molecular characterization demonstrated that blaCTX-M was the most frequently detected resistance-gene target, occurring in 54.3% of MDR isolates, followed by blaTEM (41.3%) and blaSHV (30.4%). Among carbapenemase-associated targets, blaNDM (21.7%) and blaOXA-48-like (17.4%) were the most frequent. Multiple resistance-gene targets occurred in 45.7% of MDR isolates, demonstrating the potential molecular complexity underlying multidrug-resistant bacterial phenotypes. These findings illustrate the importance of combining phenotypic antimicrobial susceptibility testing with molecular characterization. Continuous surveillance, antimicrobial stewardship, effective infection-prevention measures, and expansion of molecular epidemiological investigations are essential

to restrict the emergence and dissemination of MDR Gram-negative pathogens.

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