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Evaluation of a selective colistin susceptibility testing practice and identification of antimicrobial resistance indicators associated with the need for colistin testing.

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Evaluation of a selective colistin susceptibility testing practice and identification of antimicrobial resistance indicators associated with the need for colistin testing.

Abstract

The development of multidrug-resistant (MDR) pathogens has renewed the clinical significance of colistin as a last-line antibacterial drug. However, colistin susceptibility testing (CST) is often selectively caried out, and the antimicrobial resistance features associated with test selection remain unsuccessfully defined. This retrospective case study evaluated selective CST practices in routine clinical microbiology at a governmental medical city. Associations between colistin-test selection and bacterial groups, MDR status, and individual antimicrobial resistance patterns were assessed using exact statistical tests, odds ratios, relative risks, and Phi coefficients. Hierarchical clustering and biochemical profiling were additionally performed for colistin-tested resistant isolates. Only 19 isolates underwent CST, all of which were MDR and resistant to colistin. Enterobacterales were meaningfully more likely to be selected for testing than other bacterial groups. Test selection was strongly associated with MDR status and resistance to several clinically important antimicrobial agents, particularly carbapenems, as well as aminoglycosides, fluoroquinolones, and piperacillin/tazobactam, whereas cephalosporin resistance showed no significant association. Resistance clustering revealed substantial phenotypic heterogeneity, while

biochemical profiling identified conserved characteristics despite taxonomic diversity. These findings suggest that MDR and carbapenem resistance may serve as practical indicators for considering CST. However, because colistin susceptibility was unknown for non-tested isolates, these factors should not be interpreted as predictors of colistin resistance itself. Multicenter validation is warranted to support the development of standardized, risk-based colistin-testing strategies. The present study nevertheless provides important practice-based evidence and an exploratory framework for designing larger multicenter studies.

Keywords: Multidrug-resistant (MDR), odds ratios (ORs), relative risks (RRs), Colistin, Heterogeneous resistance

1. Introduction

The global emergence and spread of MDR Gram-negative bacteria are one of the most serious challenges for modern healthcare systems. Members of the Enterobacterales family and non-fermenting Gram-negative pathogens, including *Pseudomonas aeruginosa* and *Acinetobacter* spp., exhibit increasingly resistance to multiple classes of antimicrobial agents, including β-lactams, fluoroquinolones, aminoglycosides, and carbapenems (Jean et al., 2022). The rapid increase in AMR has significantly limited the therapeutic choices and has been linked to increased hospitalization, costs of health care, treatment failure and mortality rates (Willemsen et al., 2022).

Colistin has re-emerged as a critical therapeutic option among limited antimicrobial agents active against MDR Gram-negative pathogens (Zeb et al., 2026). Colistin remains an important treatment option for difficult-to-treat infections, particularly in the absence of or resistance to other antimicrobial agents, despite concerns about nephrotoxicity and neurotoxicity. Thus, the accurate determination of colistin susceptibility has become increasingly important in clinical microbiology laboratories (Lim et al., 2010).

Unlike numerous commonly tested antimicrobial agents, colistin susceptibility testing (CST) presents unique technical and operational challenges (Singh et al., 2025). Some conventional susceptibility testing methods have been shown to give unreliable results with colistin, and some international bodies have recommended particular testing methods for this agent. Routine CST is not systematically performed across all clinical isolates due to laboratory

workload, resource constraints, and selective institutional testing policies (Chauhan et al., 2022). Thus, susceptibility testing for colistin is often performed only on isolates with extensive AMR or on isolates deemed to be clinically important by the laboratory and treating physicians (Hindler & Humphries 2013).

Colistin resistance prevalence, molecular mechanisms and epidemiology have been extensively studied, but the characteristics of bacterial isolates selected for CST in routine clinical practice have been largely overlooked (Azzam et al., 2025; Xu et al., 2025). The decision to perform colistin testing is often based on complex microbiological and clinical considerations such as bacterial species identification, AMR, and multidrug resistance. But these factors have seldom been systematically evaluated with laboratory surveillance data. Determining the bacterial groups and resistance phenotypes most closely linked with colistin-test selection might offer useful insights for laboratory testing practices and AMR surveillance strategies (Uwizeyimana et al., 2020; Mondal et al., 2024).

Biochemical characterization is essential for bacterial identification in clinical microbiology. Biochemical reaction profiles are a useful phenotypic framework for describing bacterial diversity and can be used to identify species (Aslanzadeh, 2006). Although many studies have been published on the molecular and phenotypic mechanisms of colistin resistance, empirical research regarding the biochemical characteristics of colistin-resistant isolates remains limited. For example, Attalla et al. (2023) characterized colistin-resistant *Klebsiella pneumoniae* isolates using conventional biochemical tests and automated identification systems before genomic analysis, while Furqan et al., (2022) used biochemical identification methods as part of the phenotypic characterization of colistin-resistant Enterobacterales. However, the use of biochemical testing in both studies was mostly for isolate identification, rather than comparative phenotypic profiling of colistin-tested resistant populations.

AMR among Gram-negative bacteria remains a major public health concern in Saudi Arabia (Thabit et al., 2023). However, the factors influencing laboratory selection for CST, along with the comprehensive phenotypic profiles of such isolates, remain ambiguous. Generating local evidence regarding these issues may help to improve antimicrobial stewardship programs, laboratory testing strategies and resistance surveillance efforts. Consequently, the present study aimed to characterize Gram-negative clinical isolates selected for CST in a governmental medical

city. Specifically, the study was designed to (i) describe the distribution of bacterial species and analytical bacterial groups subjected to CST, (ii) identify bacterial and AMR characteristics associated with selection for colistin testing using exact statistical methods, odds ratio, and relative risk analyses, (iii) evaluate the relationship between multidrug resistance and selection for CST, and (iv) characterize the antimicrobial susceptibility, intrinsic resistance status, and biochemical profiles of colistin-tested resistant isolates using phenotypic and clustering-based approaches.

2. Methodology

2.1 Study design and setting

An analytical retrospective study was performed using routine microbiology laboratory data from a governmental medical city. The name of the medical city was blinded at the review stage and will be revealed after the peer review process is completed. The institutional research ethics committee approved the study. All data were analyzed in an anonymized form and did not include any patient-identifiable information. CST was not routinely performed for all Gram-negative isolates and was not triggered by a predefined study protocol or by MDR classification as a formal eligibility criterion. Instead, isolates were selected for colistin testing on a case-by-case basis following direct clinical requests when determination of colistin susceptibility was considered necessary for patient management. Consequently, the 19 colistin-tested isolates represented a clinically selected subgroup rather than a systematically screened population.

2.2 Bacterial isolate collection and data preparation

A retrospective collection of Gram-negative bacteria isolates was retrieved from routine clinical specimens received at the microbiology laboratory of a governmental medical city. Sampling and analysis occurred from January 1, 2025, to July 31, 2025. All isolates were previously identified by the VITEK® automated identification system (bioMérieux, Marcy-l'Étoile, France). The bacterial identification and the corresponding BioNumber profiles were interpreted according to the manufacturer's recommendations and the standard laboratory operating procedures. The susceptibility to antimicrobial agents was determined by the VITEK® automated antimicrobial susceptibility testing system. Susceptibility results were interpreted

according to the clinical breakpoints and laboratory guidelines applicable at the time of testing by the microbiology laboratory.

Routine testing for colistin susceptibility was not performed for all isolates. Rather, 19 isolates were chosen from the population for colistin testing based on laboratory and clinical considerations. The broth microdilution (BMD) method was used to determine the colistin susceptibility of these isolates in appropriate testing plates following standardized laboratory procedures (Chandankhede et al., 2025). MIC values were interpreted according to the internationally accepted recommendations for CST.

Following the data cleaning, records with no valid bacterial identification were excluded and 168 Gram-negative bacterial isolates were included in the final analysis. The bacterial species were examined separately at first and then combined into broader analytical groups for statistical comparisons, including Enterobacterales, *Pseudomonas* spp., *Acinetobacter* spp. and other non-fermenting Gram-negative bacteria.

CST was not routinely performed on all isolates. Conversely, testing was performed selectively on a subset of isolates, guided by institutional laboratory practices and precise clinical considerations. Therefore, a colistin value of 0 was interpreted as "colistin not tested" rather than "colistin susceptible" for this reason. Thus, the overall prevalence of colistin resistance in all bacterial isolates was not calculated in this study. Instead, colistin-related analyses were restricted to isolates selected for CST and factors associated with colistin-test selection. Isolates with a valid colistin result were classified as colistin tested and those with a 0 code as not tested. All colistin testing isolates in the dataset were reported to be colistin-resistant.

2.3 Antimicrobial susceptibility data coding

Antimicrobial susceptibility results were coded as follows: 1 = sensitive, 2 = intermediate, 3 = resistant, and 0 = not tested, not conducted, or not applicable which means that isolates not tested for a specific agent were excluded from its respective analysis. A value of 0 was not interpreted as antimicrobial susceptibility. Therefore, values coded as 0 were excluded from antibiotic-specific susceptibility percentage calculations and from the corresponding association analyses. For each antimicrobial agent, the numbers and percentages of sensitive, intermediate, resistant, and non-tested isolates were calculated. Susceptibility percentages were calculated using

the number of tested isolates for each antibiotic as the denominator, whereas the percentage of non-tested isolates was calculated relative to the total number of isolates.

2.4 Definition of multidrug resistance

The study definition for MDR phenotype was resistance to one or more antimicrobial agents in three or more antimicrobial classes (Magiorakos et al., 2012). The antimicrobial classes were aminoglycosides, beta-lactam/beta-lactamase inhibitor combinations, penicillins, cephalosporins, carbapenems, fluoroquinolones, nitrofurans, glycylcyclines and folate pathway inhibitors. Antibiotics with code 0 in the MDR classification were not considered for the respective antimicrobial class. The guideline used in this paper followed the latest clinical breakpoints standard by CLSI 100 35^{th}/36^{th} Edition (CLSI, 2026).

2.5 Biochemical profile analysis

Biochemical test results were analyzed for the 19 isolates that underwent CST and were reported as resistant. For each biochemical reaction, the frequency and percentage of positive results were calculated. Biochemical reactions were subsequently ranked according to their frequency of occurrence, and the most prevalent reactions were visualized using a horizontal bar chart (Eisen et al., 1998; Buffalo, 2015).

To explore phenotypic similarities and differences among isolates, a biochemical heatmap was generated using Python-based data analysis and visualization workflows. Analyses were implemented using custom Python scripts executed within the Google Colab environment and OriginPro 2018 (OriginLab Corporation, USA). In the heatmap, rows represented bacterial isolates and columns represented biochemical reactions. The resulting visualization was used to identify conserved and variable biochemical characteristics and to provide an overview of phenotypic heterogeneity among the analyzed isolates. This approach is consistent with commonly applied exploratory data analysis and microbial phenotypic profiling methodologies used for pattern recognition and visualization of complex biological datasets.

2.6 Hierarchical clustering analysis

Hierarchical cluster was analyzed for the 19 colistin-tested resistant isolates to explore similarity patterns based on antimicrobial susceptibility profiles and biochemical test profiles.

Dendrograms were generated to visualize the clustering patterns among isolates using OriginPro 2018 (OriginLab Corporation, USA) (Chen et al., 2024). The analysis was considered exploratory and was used to describe similarity and heterogeneity among the colistin-tested resistant isolates.

2.7 Statistical analysis

Categorical variables were presented as counts and percentages. Descriptive statistics were used to describe the distribution of bacterial species and analytical bacterial groups. A binary outcome variable was created to identify factors associated with selection for CST. Isolates with valid colistin susceptibility results were classified as colistin-tested, and isolates coded as 0 for colistin were classified as not tested. For analyses of antibiotic-specific associations, isolates with the code resistant (3) were considered resistant and isolates with the code sensitive (1) or intermediate (2) were considered non-resistant. Isolates with a code of 0 for the antibiotic being studied were not included in that analysis.

Two-by-two contingency tables were built to compare resistance status for each antimicrobial agent to colistin-test selection status. Fisher's exact test was used for small, expected cell frequencies. The magnitude and direction of associations were assessed using odds ratios (ORs), relative risks (RRs), 95% confidence intervals (95% CIs), and Phi coefficients. Where there were sparse or zero cells a continuity correction was used.

Additional analyses were conducted to assess the association between colistin-test selection and broader resistance phenotypes, i.e., resistance to at least one cephalosporin and MDR status. All 19 isolates were tested for colistin susceptibility and multivariable logistic regression was not performed due to complete separation and model overfitting. All statistical analyses were carried out using Python and OriginPro 2018. A two-sided P value < 0.05 was considered statistically significant.

3. Results

3.1 Distribution of bacterial species

The study included a total of 168 Gram-negative bacterial isolates. The most frequent isolated species was *Escherichia coli* (51 isolates, 30.4%), followed by *K. pneumoniae* subsp. *pneumoniae* (47 isolates, 28.0%) and *P. aeruginosa* (34 isolates, 20.2%). These three species accounted for most of the isolates recovered during the study period. The other species were found

at much lower frequencies. When the isolates were classified into analytical bacterial groups (Table 2), Enterobacterales was the largest group, with 116 isolates (69.0%), followed by *Pseudomonas* spp. with 36 isolates (21.4%) and *Acinetobacter* spp. with 15 isolates (8.9%). Only one isolate (0.6 %) belonged to the other group of non-fermenting Gram-negative bacteria.

Table 1. Distribution of bacterial species among Gram-negative clinical isolates included in the study (n = 168)

Bacterial species	Total isolates (n)	Percentage (%)
E. coli	51	30.4
K. pneumoniae ssp. *pneumoniae*	47	28
P. aeruginosa	34	20.2
A. baumannii	8	4.8
A. baumannii/*calcoaceticus* complex	6	3.6
K. pneumoniae ssp. *ozaenae*	4	2.4
Proteus mirabilis	4	2.4
K. aerogenes	2	1.2
Enterobacter cloacae	2	1.2
K. oxytoca	2	1.2
P. putida	2	1.2
A. lwoffii/*haemolyticus*	1	0.6
Citrobacter koseri	1	0.6
Providencia stuartii	1	0.6
Salmonella species	1	0.6
Serratia marcescens	1	0.6
Stenotrophomonas maltophilia	1	0.6
Total	**168**	**100**

Table 2. Distribution of analytical bacterial groups among Gram-negative clinical isolates included in the study (n = 168)

Analytical group	Total isolates (n)	Percentage (%)
Enterobacterales	116	69.0
Pseudomonas spp.	36	21.4
Acinetobacter spp.	15	8.9
Other non-fermenters	1	0.6
Total	168	100

Table 3: Distribution of isolates selected for colistin susceptibility testing according to analytical bacterial groups

Analytical group*	Total isolates	Colistin tested	Not tested
Enterobacterales	116	18	98
***Pseudomonas* spp.**	36	1	35
***Acinetobacter* spp.**	15	0	15
Other non-fermenters	1	0	1

*Colistin susceptibility testing was not routinely performed for all isolates. Isolates with recorded colistin results were considered to have undergone colistin susceptibility testing, whereas isolates not tested for a specific agent were excluded from its respective analysis (see Section 2.3).

Table 4. Odds ratio, relative risk, and Fisher's exact test analysis of Enterobacterales association with colistin-test selection

Analytical Group	**Colistin tested***		**Colistin not tested**
Enterobacterales	18		98
Non- Enterobacterales	1		51
Odds-Ratio	OR-95CI-Lower		OR-95CI-Upper
9.367	1.216		72.181
Relative-Risk	RR-95CI-Lower	RR-95CI-Upper	Fisher exact (p-value)
8.069	1.106	58.85	0.0081

*Colistin testing status was defined as "tested" when a valid colistin susceptibility result was available and "not tested" when the colistin value was recorded as 0. Non-Enterobacterales included *Pseudomonas* spp., *Acinetobacter* spp., and other non-fermenting Gram-negative bacteria. Odds ratios (ORs), relative risks (RRs), and their corresponding 95% confidence intervals (95% CIs) were calculated using a 2×2 contingency table. Statistical significance was assessed using Fisher's exact test. No continuity correction was required because all contingency table cells contained non-zero values.

3.2 Colistin susceptibility

A total of 19 isolates underwent CST. *K. pneumoniae* subsp. *pneumoniae* was the most frequently represented species, accounting for 8 isolates (42.1%), followed by *P. mirabilis* with 4 isolates (21.1%). *E. coli* contributed 2 isolates (10.5%), whereas *S. marcescens*, *E. cloacae*, *P. stuartii*, *P. aeruginosa*, and *K. pneumoniae* subsp. *ozaenae* were each represented by a single isolate (5.3%). Collectively, Enterobacterales accounted for 18 of the 19 isolates (94.7%) subjected to colistin susceptibility testing, while only one isolate (5.3%) belonged to the non-Enterobacterales group (Table 5).

Table 5: Species distribution of isolates subjected to colistin susceptibility testing (n = 19)

Species*	n	%
K. pneumoniae subsp. *pneumoniae*	8	42.1
P. mirabilis	4	21.1
E. coli	2	10.5
P. aeruginosa	1	5.3
E. cloacae	1	5.3
P. stuartii	1	5.3
S. marcescens	1	5.3
K. pneumoniae subsp. *ozaenae*	1	5.3

* Colistin susceptibility testing was performed for a subset of isolates based on laboratory testing practices and clinical considerations. All isolates included in this table underwent colistin susceptibility testing and were reported as colistin-resistant. Percentages were calculated using the total number of colistin-tested isolates (n = 19) as the denominator.

3.4 Antimicrobial resistance predictors of colistin-test selection

Table 6 summarizes the association between resistance to individual antimicrobial agents and selection for CST. For each antibiotic, only isolates with valid susceptibility results for that specific antimicrobial agent were included in the corresponding analysis; therefore, the number of isolates analyzed (Valid n) varies among antibiotics. Importantly, the Valid n value does not represent the number of isolates tested for colistin. Instead, it represents the number of isolates with available susceptibility results for the antibiotic under investigation after excluding isolates coded as “0” (not tested or not applicable).

The contingency table counts are presented as follows: RCT represents antibiotic-resistant isolates that underwent CST, RCnT represents antibiotic-resistant isolates that did not undergo colistin testing, nRCT represents antibiotic-non-resistant isolates that underwent colistin testing, and nRCnT represents antibiotic-non-resistant isolates that did not undergo colistin testing. Thus, for each antibiotic, the sum of RCT, RCnT, nRCT, and nRCnT equals the corresponding Valid n.

Table 6. Association between resistance to other antimicrobial agents and selection for colistin susceptibility testing.

Antibiotics*	Valid n**	RCT	RCnT	nRCT	nRCnT	OR*	RR	Fep	Phi	Cor.
IPM	162	13	28	2	119	27.625	19.183	0	0.451	No
NIT	154	19	63	0	72	44.528	34.301	0	0.352	Yes
TZP	167	11	24	8	124	7.104	5.186	0.0002	0.325	No
AMK	167	10	20	9	128	7.111	5.074	0.0003	0.324	No
C/T	150	10	18	9	113	6.975	4.841	0.0003	0.332	No
GEN	133	11	25	7	90	5.657	4.234	0.0011	0.303	No
MEM	166	10	29	9	118	4.521	3.618	0.0032	0.247	No
CIP	104	14	54	1	35	9.074	7.412	0.017	0.241	No
LVX	103	14	55	1	33	8.4	6.899	0.0186	0.231	No
ETP	155	13	57	6	79	3.003	2.631	0.0469	0.175	No
AMC	155	14	64	5	72	3.15	2.764	0.0479	0.175	No
SXT	157	14	68	5	70	2.882	2.561	0.0527	0.159	No
TGC	136	8	25	11	92	2.676	2.27	0.0795	0.168	No
AMP	155	15	125	4	11	0.33	0.402	0.0914	-0.144	No
FEP	164	9	53	8	94	1.995	1.851	0.1939	0.106	No
CAZ	166	9	53	10	94	1.596	1.51	0.4501	0.074	No
CXM	154	14	91	5	44	1.354	1.307	0.7932	0.044	No
CRO	157	11	79	8	59	1.027	1.024	1	0.004	No
CZO	95	11	84	0	0	0.136	0.24	1		Yes

*OR, odds ratio; RR, relative risk; Phi, Phi coefficient; Cor., continuity correction; RCT, antibiotic-resistant and colistin-tested isolates; RCnT, antibiotic-resistant and colistin-not-tested isolates; nRCT, antibiotic-non-resistant and colistin-tested isolates; nRCnT, antibiotic-non-resistant and colistin-not-tested isolates. For each antibiotic, isolates coded as resistant (3) were classified as resistant, whereas isolates coded as sensitive (1) or intermediate (2) were classified as non-resistant.

**Valid n refers to the number of isolates with available susceptibility results for the corresponding antimicrobial agent and should not be interpreted as the number of isolates subjected to colistin susceptibility testing. Only 19 isolates underwent colistin testing in the entire dataset. Isolates coded as 0 for the antibiotic under investigation were excluded from the corresponding antibiotic-specific analysis

Among all evaluated antibiotics, resistance to imipenem showed the strongest association with colistin-test selection. Of the 162 isolates with valid imipenem susceptibility results, 13 imipenem-resistant isolates and 2 imipenem-non-resistant isolates underwent colistin testing, yielding an odds ratio (OR) of 27.625, a relative risk (RR) of 19.183, and a Fisher's exact test p-value of <0.0001. Significant associations were also observed for nitrofurantoin (OR = 44.528, p < 0.0001), piperacillin/tazobactam (OR = 7.104, p = 0.0002), amikacin (OR = 7.111, p = 0.0003),

ceftolozane/tazobactam (OR = 6.975, p = 0.0003), gentamicin (OR = 5.657, p = 0.0011), meropenem (OR = 4.521, p = 0.0032), ciprofloxacin (OR = 9.074, p = 0.0170), levofloxacin (OR = 8.400, p = 0.0186), ertapenem (OR = 3.003, p = 0.0469), and amoxicillin/clavulanate (OR = 3.150, p = 0.0479). No statistically significant associations were observed for the remaining antimicrobial agents (p > 0.05).

The contingency table counts are presented as follows: RCT represents antibiotic-resistant isolates that underwent CST, RCnT represents antibiotic-resistant isolates that did not undergo colistin testing, nRCT represents antibiotic-non-resistant isolates that underwent colistin testing, and nRCnT represents antibiotic-non-resistant isolates that did not undergo colistin testing. Thus, for each antibiotic, the sum of RCT, RCnT, nRCT, and nRCnT equals the corresponding Valid n.

Among all evaluated antibiotics, resistance to imipenem showed the strongest association with colistin-test selection. Of the 162 isolates with valid imipenem susceptibility results, 13 imipenem-resistant isolates and 2 imipenem-non-resistant isolates underwent colistin testing, yielding an odds ratio (OR) of 27.625, a relative risk (RR) of 19.183, and a Fisher's exact test p-value of <0.0001. Significant associations were also observed for nitrofurantoin (OR = 44.528, p < 0.0001), piperacillin/tazobactam (OR = 7.104, p = 0.0002), amikacin (OR = 7.111, p = 0.0003), ceftolozane/tazobactam (OR = 6.975, p = 0.0003), gentamicin (OR = 5.657, p = 0.0011), meropenem (OR = 4.521, p = 0.0032), ciprofloxacin (OR = 9.074, p = 0.0170), levofloxacin (OR = 8.400, p = 0.0186), ertapenem (OR = 3.003, p = 0.0469), and amoxicillin/clavulanate (OR = 3.150, p = 0.0479). No statistically significant associations were observed for the remaining antimicrobial agents (p > 0.05).

Figure 1 shows the odds ratios (and 95% confidence intervals) of AMR factors significantly associated with selection for CST. Resistance to nitrofurantoin was the most strongly associated with an odds ratio of 44.53 (95% CI: 2.63–752.51; p < 0.0001) followed by resistance to imipenem with an odds ratio of 27.62 (95% CI: 5.89–129.45; p < 0.0001).

Significant associations were also found for ciprofloxacin (OR = 9.07; 95% CI: 1.14–72.12; p = 0.0170), levofloxacin (OR = 8.40; 95% CI: 1.06–66.85; p = 0.0186), amikacin (OR = 7.11; 95% CI: 2.57–19.65; p = 0.0003), piperacillin/tazobactam (OR = 7.10; 95% CI: 2.59–19.51; p = 0.0002), ceftolozane/tazobactam (OR = 6.97; 95% CI: 2.49–19.52; p = 0.0003), gentamicin (OR = 5.66; 95% CI: 1.99–16.10; p = 0.0011), and meropenem (OR = 4.52; 95% CI: 1.68–12.14;

p = 0.0032). Statistically significant, albeit weak, positive associations were observed for amoxicillin/clavulanate (OR = 3.15; 95% CI, 1.07–9.23; p = 0.0479) and ertapenem (OR = 3.00; 95% CI, 1.08–8.37; p = 0.0469).

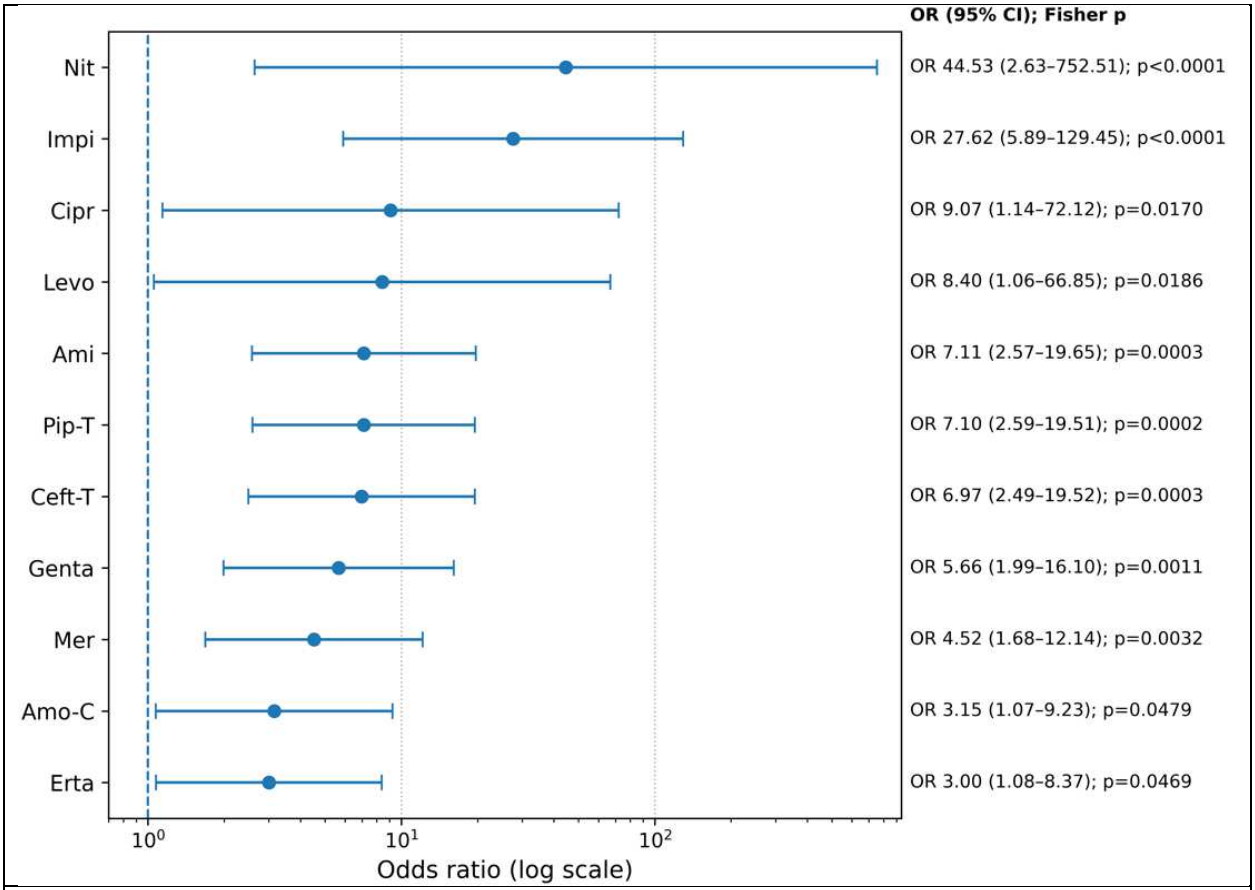


Figure 1: Forest plot of odds ratios for AMR factors associated with selection for colistin susceptibility testing.
Note: Odds ratios (ORs) and their corresponding 95% confidence intervals (95% CIs) were calculated using antibiotic-specific 2 × 2 contingency tables. The outcome variable was selection for colistin susceptibility testing rather than colistin resistance. Only antimicrobial agents showing statistically significant associations (Fisher's exact test, $p < 0.05$) are presented. Isolates with unavailable susceptibility results (coded as 0) for the corresponding antibiotic were excluded from that antibiotic-specific analysis. The dashed vertical line represents the null value (OR = 1), indicating no association with colistin-test selection.

The confidence intervals shown were mostly to the right of the null reference line (OR = 1), suggesting positive associations between resistance to these antimicrobial agents and the probability of an isolate being selected for CST. The strength of the association was different between antibiotics with the strongest effects for nitrofurantoin and imipenem resistance (Table 7).

There was no significant association between colistin-test selection and resistance to at least 1 cephalosporin (OR = 1.838, 95% CI: 0.579–5.830; p = 0.4326). In contrast, the MDR phenotype was strongly and statistically associated with colistin-test selection (OR = 22.257, 95% CI: 1.318–375.951; p = 0.0005). Likewise, the estimated relative risk for MDR isolates was 18.652 (95% CI: 1.147-303.288). The Phi coefficient indicated a moderate positive association between the MDR status and colistin-test selection (Phi = 0.246).

Table 7: Association of cephalosporin resistance and MDR phenotype with selection for colistin susceptibility testing.

Predictor*	**OR**	**RR**	**Fisher exact *p*-value**	**Phi coefficient**	**Continuity correction used**
Resistance to at least one cephalosporin	1.838(0.579-5.83)	1.728(0.602-4.958)	0.4326	0.081	No
MDR phenotype	22.257(1.318-375.951)	18.652(1.147-303.288)	0.0005	0.246	Yes

*MDR phenotype was defined as non-susceptibility (resistant or intermediate) to at least one antimicrobial agent in three or more antimicrobial categories, according to the standardized international definitions proposed by Magiorakos et al. (2012). Odds ratios (ORs), relative risks (RRs), 95% confidence intervals (95% CIs), Fisher's exact p-values, and Phi coefficients were calculated to assess associations with colistin-test selection. A continuity correction was applied, when necessary, because of sparse contingency table cells.

All 19 bacterial isolates that investigated CST were classified as MDR, representing 100% of the colistin-tested strain. Importantly, selection of these isolates for colistin testing was not based on a predefined systematic laboratory protocol or MDR status as an eligibility criterion; rather, testing was performed on a case-by-case basis following direct clinical requests when colistin susceptibility information was considered necessary for patient management. Among the 149 isolates that were not tested for colistin, 95 (63.8%) were classified as MDR, whereas 54 (36.2%) were non-MDR. Overall, MDR isolates accounted for 114 of the 168 isolates (67.9%)

included in the study. Remarkably, none of the non-MDR isolates underwent CST (Table 8). This distribution exhibited a strong association between MDR status and selection for CST. The isolates that underwent CST were classified as MDR, representing 100% of the colistin-tested group. In contrast, among isolates that did not undergo colistin testing, 95 of 149 isolates (63.8%) were classified as MDR, whereas 54 isolates (36.2%) were non-MDR. In general, MDR bacterial strains accounted for 114 of 168 isolates (67.9%) included in the study. No non-MDR isolate was subjected to CST.

Table 8: Distribution of MDR status according to colistin-test selection

MDR status	Colistin tested n (%)	Colistin did not test n (%)	Total n (%)
MDR	19 (100.0)	95 (63.8)	114 (67.9)
Non-MDR	0 (0.0)	54 (36.2)	54 (32.1)
Total	19 (100.0)	149 (100.0)	168 (100.0)

3.5 Antimicrobial susceptibility and biochemical profile clustering

Hierarchical clustering based on antimicrobial susceptibility profiles divided the 19 colistin tested resistant isolates into two major clusters at a distance of approximately 4.5 (Figure 2). Abbreviated isolate identifiers were created for graphical representation, using the first letter of the genus name followed by the first letter(s) of the species or subspecies designation, with numerical suffixes reflecting the order of appearance of isolates in the dataset. These identifiers were used only as analytical labels and are not genetically verified strains.

The first major cluster was represented by *K. pneumoniae* isolates, including *K. pneumoniae* subsp. *pneumoniae* and *K. pneumoniae* subsp. *ozaenae*. Within this group several closely related subclusters were observed, indicating that some *Klebsiella* isolates are very similar in antimicrobial susceptibility patterns. However, isolates from the same bacterial species were not always grouped together in the same subcluster, suggesting heterogeneity in resistance profiles despite taxonomic similarity.

Hierarchical clustering based on the biochemical test profiles also segregated the 19 colistin tested resistant isolates into two major clusters (Figure 3). In terms of antimicrobial

susceptibility analysis, one major cluster was formed by *K. pneumoniae* isolates and several closely related subclusters were characterized by high biochemical similarity. The second cluster was made up of isolates that were *P. mirabilis*, *E. coli*, *P. stuartii*, *P. aeruginosa* and *S. marcescens*.

The biochemical clustering pattern was more similar to phenotypic characteristics at species level than the antimicrobial susceptibility clustering. Isolates of the same bacterial species showed generally greater similarity and tended to cluster together. However, several subclusters were identified within species, indicating that there is a phenotypic variability among isolates belonging to the same taxonomic group.

The second major cluster comprised isolates of several bacterial species, including *P. mirabilis*, *E. coli*, *P. stuartii*, *P. aeruginosa* and *S. marcescens*. Within this cluster several closely related branches were observed among *P. mirabilis* isolates, which is consistent with shared antimicrobial susceptibility characteristics. In general, the dendrogram showed that isolates of the same species often clustered together, but they were not restricted to a single branch, indicating that AMR phenotypes vary within species.

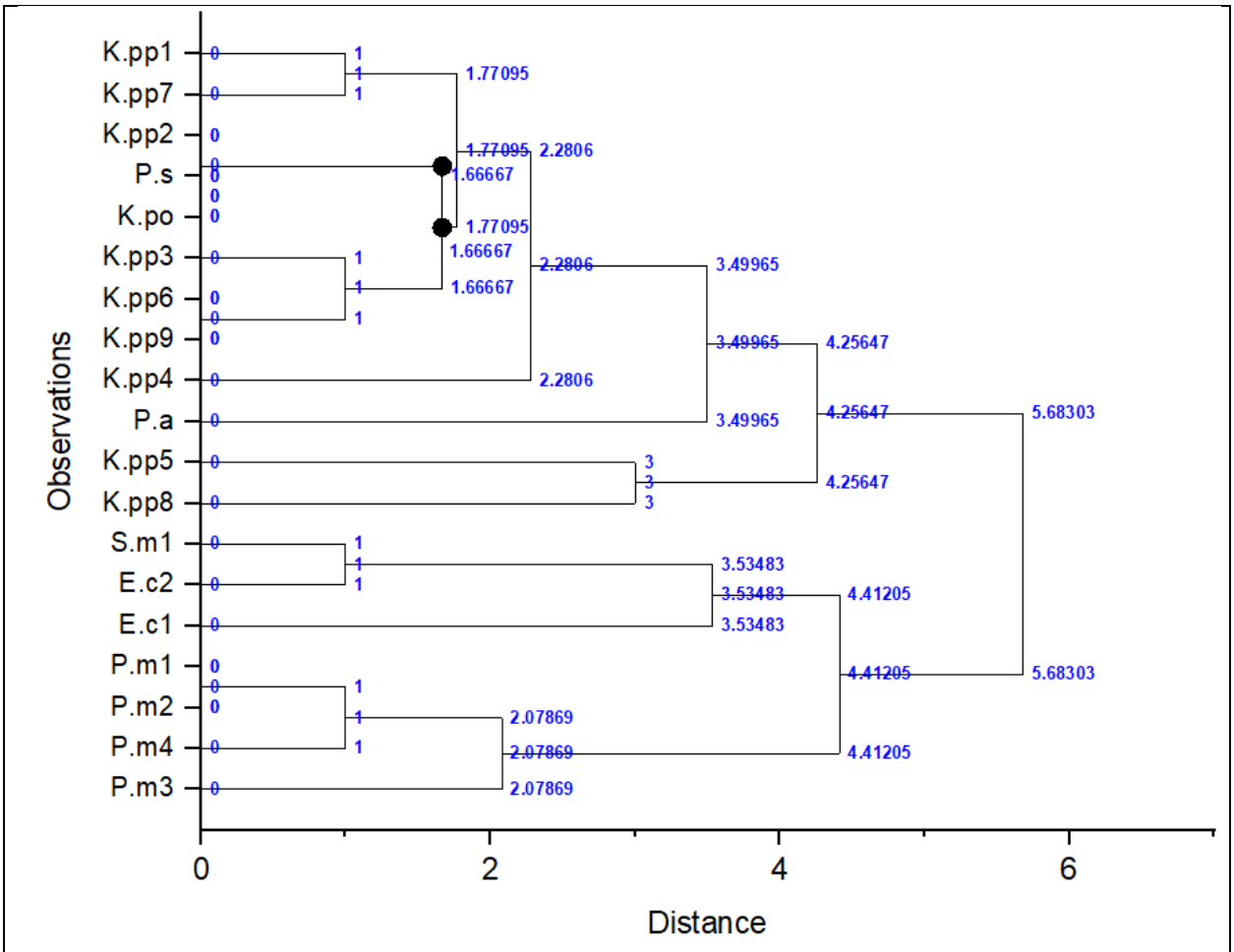


Figure 2: Hierarchical clustering reveals distinct AMR profile groups among colistin-tested resistant Gram-negative isolates.
K.pp= *K. pneumoniae* ssp *pneumoniae*; S.m= *S. marcescens*; P.m= *Proteus mirabilis*; P.a= *P. aeruginosa* ; P.S= *P. stuartii*; and K.po=*K. pneumoniae* ssp *ozaenae*

Note: Hierarchical cluster analysis was performed using antimicrobial susceptibility results obtained for the 19 colistin-tested resistant isolates. Susceptibility data were coded according to routine laboratory interpretation (susceptible, intermediate, and resistant categories), while unavailable test results were excluded from the analysis. Branch lengths represent the degree of dissimilarity among isolates based on their overall antimicrobial susceptibility patterns. Isolates clustering at shorter linkage distances exhibited more similar resistance profiles than those connected at larger distances.

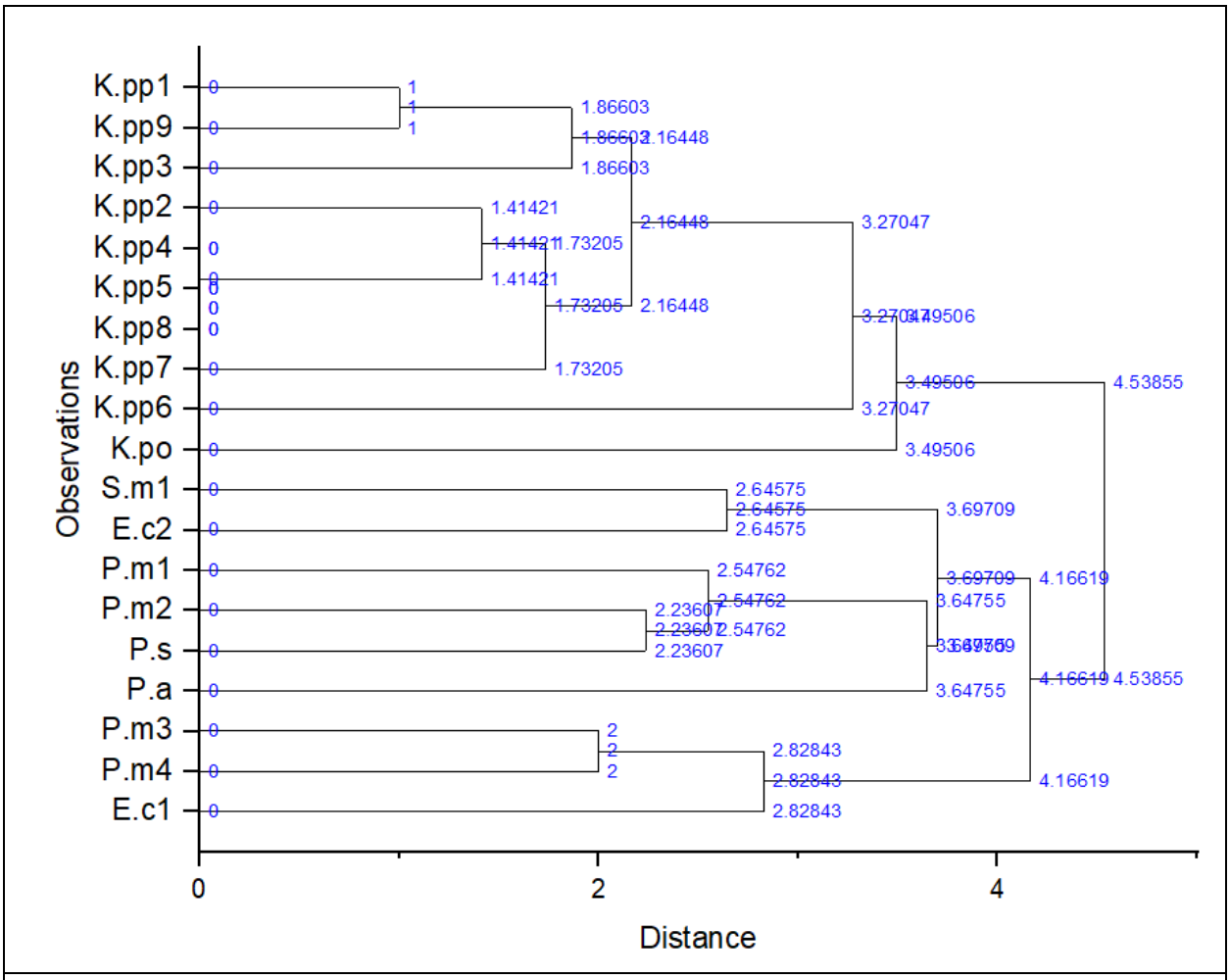


Figure 3: Hierarchical clustering of colistin-tested resistant isolates based on biochemical test profiles. K.pp= *K. pneumoniae* ssp *pneumoniae*; S.m= *S. marcescens*; P.m= *P. mirabilis*; P.a= *P. aeruginosa* ; P.S= *P. stuartii*; and K.po=*K. pneumoniae* ssp *ozaenae*.

Note: Hierarchical clustering was performed using biochemical test results obtained for the 19 colistin-tested resistant isolates. Branch lengths represent the degree of dissimilarity between isolates based on their biochemical characteristics. Isolates sharing similar biochemical profiles clustered together, whereas increasing linkage distance indicates greater phenotypic divergence.

3.6 Conserved biochemical signatures and phenotypic heterogeneity among colistin-tested resistant isolates

The most frequent positive biochemical reactions observed among the 19 colistin tested resistant isolates are summarized in Figure 4. Three biochemical reactions (A_GLPRB, N_LPROT and A_LTRY) were positive for all isolates (100%). Two other reactions, A_LPHET and M_NAG, were positive in 94.7% of isolates. Reactions R_BALL, A_LPYR, A_GLYB and A_LPROB were

seen in 89.5% isolates while A_GUGAH was positive in 84.2% isolates. These reactions were the most conserved biochemical features despite the taxonomic diversity of the analyzed isolates.

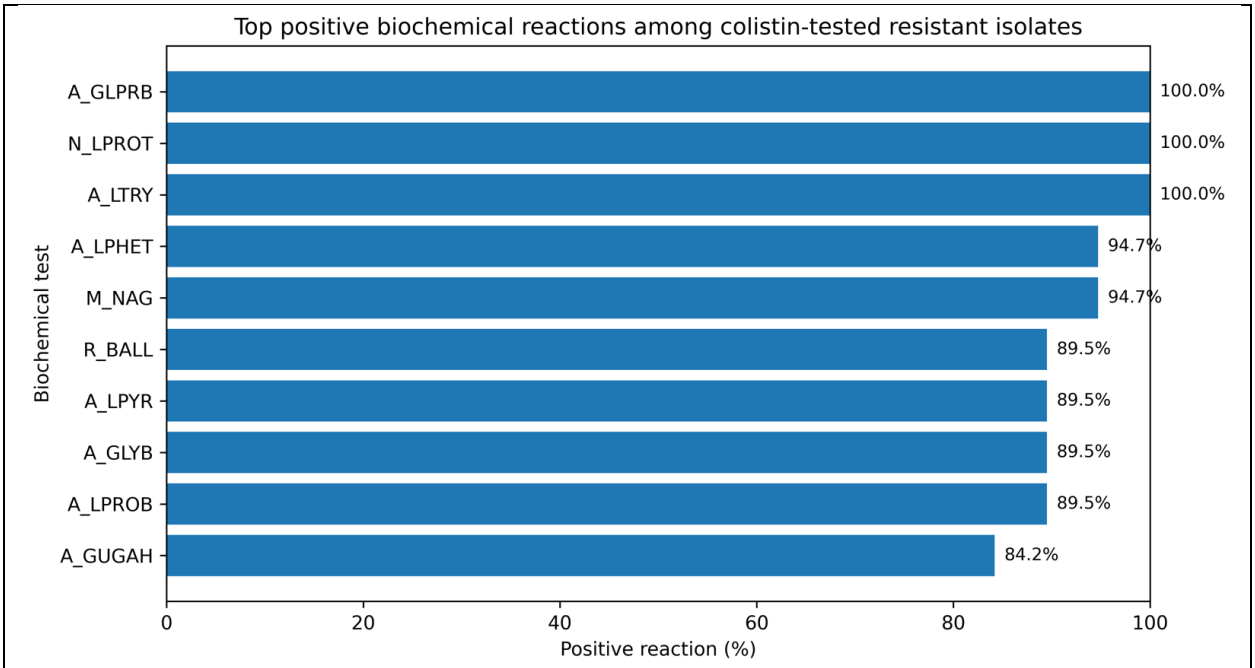


Figure 4. Most frequent positive biochemical reactions among colistin-tested resistant isolates (n = 19).
Bars represent the percentage of isolates exhibiting positive reactions for the indicated biochemical tests. Only the ten most frequently positive biochemical reactions are shown. Percentages were calculated based on the 19 colistin-tested resistant isolates included in the study.

Figure 5 presents the biochemical reaction profiles of the 19 colistin tested resistant isolates in all the biochemical tests performed. Several biochemical reactions showed highly conserved patterns, being consistently positive or negative in most isolates, whereas other reactions showed considerable variation between isolates. Although the isolates belonged to different bacterial species, several biochemical characteristics were found in common and species-specific differences were noted in the different reaction patterns of several tests. The heatmap also shows a significant phenotypic heterogeneity of the isolates studied, with clusters of similar biochemical profiles found among isolates belonging to related bacterial taxa.

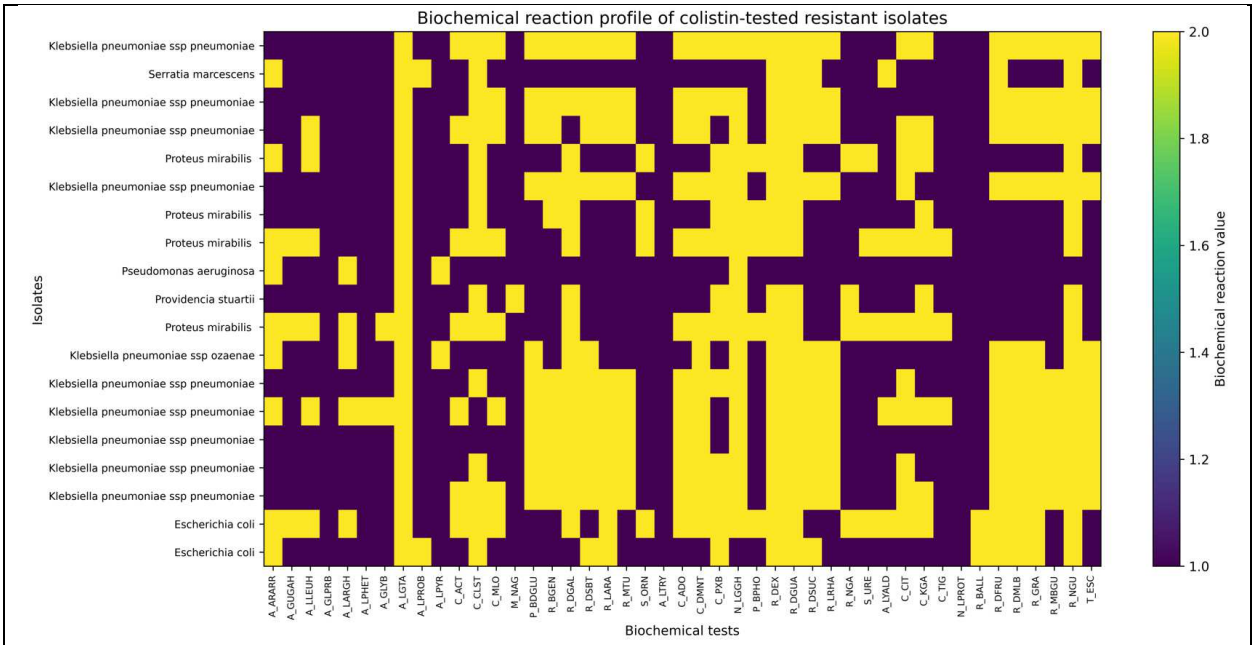


Figure 5: Heatmap of biochemical reaction profiles among colistin-tested resistant isolates (n = 19). Rows represent individual bacterial isolates and columns represent biochemical tests. Colored cells indicate the recorded biochemical reaction outcome for each isolate-test combination. The heatmap provides a visual overview of similarities and differences in biochemical profiles among the analyzed isolates and highlights both conserved and variable phenotypic characteristics.

3.7 Distribution of Intrinsically and Non-Intrinsically Colistin-Resistant Species Among Colistin-Tested Resistant Isolates

Table 9 shows the distribution of bacterial species categorized as intrinsically and non-intrinsically resistant among the 19 colistin resistant isolates tested for colistin. Of the total isolates analyzed, six isolates (31.6%) were of bacterial species with known intrinsic resistance to polymyxins. These were *P. mirabilis* (n = 4, 21.1%), *P. stuartii* (n = 1, 5.3%) and *S. marcescens* (n = 1, 5.3%). All these intrinsically resistant species together represented nearly a third of all resistant isolates tested for colistin.

The remaining 13 isolates (68.4%) were of bacterial species not considered to be intrinsically resistant to colistin. Most of this group was represented by *K. pneumoniae* (including *K. pneumoniae* subsp. *pneumoniae* and *K. pneumoniae* subsp. *ozaenae*, 9 isolates, 47.4%). Additional non-intrinsically resistant species included *E. coli* (n = 2, 10.5%), *E. cloacae* (n = 1, 5.3%), and *P. aeruginosa* (n = 1, 5.3%).

In general, more than two-thirds of the total number of bacterial species with no known intrinsic resistance to colistin were colistin-tested resistant isolates, while intrinsically resistant species were less than one-third of the studied population.

Table 9. Distribution of intrinsically and non-intrinsically colistin-resistant bacterial species among colistin-tested resistant isolates (n = 19)

Resistance category*	Species	No. of isolates	Percentage (%)
Intrinsic colistin resistance	*P. mirabilis*	4	21.1
	P. stuartii	1	5.3
	S. marcescens	1	5.3
Subtotal		**6**	**31.6**
Non-intrinsic colistin resistance	*K. pneumoniae* subsp. *pneumoniae*	8	42.1
	K. pneumoniae subsp. *ozaenae*	1	5.3
	E. coli	2	10.5
	E. cloacae	1	5.3
	P. aeruginosa	1	5.3
Subtotal		**13**	**68.4**
Total		**19**	**100.0**

* Classification of bacterial species as intrinsically or non-intrinsically resistant to colistin was based on published reviews describing natural polymyxin resistance patterns (Olaitan et al., 2014; Gogry et al., 2021). *P. mirabilis*, *P. stuartii*, and *S. marcescens* were categorized as intrinsically resistant species, whereas *K. pneumoniae*, *E. coli*, *E. cloacae*, and *P. aeruginosa* were classified as non-intrinsically resistant organisms in which colistin resistance is generally considered acquired or adaptive.

4. Discussion

In this study, we analyzed the Gram-negative clinical isolates from a governmental medical city and focused on the microbiological and statistical characteristics of isolates selected for CST. A major methodological issue in this dataset was that colistin testing was not routinely done for all isolates. This distinction is essential to prevent misinterpreting routine laboratory triage as true epidemiological prevalence.

The overall isolate distribution showed that Enterobacterales were the most prevalent bacterial group followed by *Pseudomonas* spp. and *Acinetobacter* spp. This distribution is in line with the clinical relevance of Gram-negative pathogens in healthcare-associated infections, especially Enterobacterales and non-fermenting Gram-negative bacilli (Brauncajs et al., 2022; Apetroaei et al., 2025; Dihmane et al., 2025). The dominance of Enterobacterales in the present dataset is also of interest because this group includes important clinical organisms such as *K. pneumoniae*, *E. coli*, *E. cloacae*, *P. mirabilis*, *P. stuartii*, and *S. marcescens*, several of which were represented among the isolates selected for colistin testing.

The study found that Enterobacterales were significantly more likely to be selected for CST than non-Enterobacterales isolates. This observation does not mean that Enterobacterales had a higher prevalence of colistin resistance in the total dataset, as most isolates were not tested for colistin. Rather, it suggests that the laboratory selection for colistin testing was mainly focused on Enterobacterales. This observation is consistent with clinical laboratory practice, where colistin testing is more likely to be requested or performed for isolates with complex or extensive resistance patterns, especially among Enterobacterales with limited therapeutic options.

This interpretation was also supported by the species distribution among the 19 isolates tested for colistin. Most of the colistin-tested isolates were Enterobacterales, where *K. pneumoniae* subsp. *pneumoniae* was the predominant species, followed by *P. mirabilis*, *E. coli*, *E. cloacae*, *P. stuartii*, *S. marcescens*, and *K. pneumoniae* subsp. *ozaenae*. One isolate was *P. aeruginosa*. This pattern suggests that colistin testing was not consistently performed across all bacterial groups but was targeted at organisms with clinically relevant resistance patterns or inherent polymyxin-related issues. It should be noted here that the polymyxins (Colistin, Polymyxin B) have been shown in scientific research to be critical, last-resort agents for MDR Gram-negative bacteria. However, significant emerging patterns of resistance and severe intrinsic toxicities greatly limit their clinical utility (Silva et al., 2022; Ray et al., 2022).

The presence of *P. mirabilis*, *P. stuartii* and *S. marcescens* among the tested isolates was an important interpretive issue. These organisms have been reported in the literature as being intrinsically resistant to polymyxins including colistin (Olaitan et al., 2014; Chakkour et al., 2024). Therefore, their presence in isolates tested for colistin resistance should not be interpreted in the same way as acquired colistin resistance in organisms such as *K. pneumoniae* or *E. coli*. Rather

than representing a deliberate diagnostic choice by clinicians to evaluate colistin efficacy, testing these intrinsically resistant species is a recognized artifact of automated systems such as VITEK cards; where colistin is frequently included as part of a standard, non-customizable panel. However, it should be noted that while automated systems exist for testing and reporting colistin results, there is currently a global consensus among standardization organizations (EUCAST and CLSI) that automated systems are inaccurate and entirely unacceptable for colistin testing. This is because colistin molecules are large and tend to adhere to the plastic used in the test cards employed by these systems, thus reducing the actual antibody concentration in the solution and leading to false susceptibility/major errors. It has been scientifically confirmed that the only internationally recognized method for testing colistin resistance is broth microdilution (BMD) using untreated plates to minimize polymer adhesion.

This is important because it differentiates intrinsic resistance patterns from acquired resistance and prevents overinterpretation of resistance mechanisms that are not directly investigated in our present study. In developing these procedures, priority must be granted to MDR Enterobacterales, particularly those exhibiting carbapenem resistance, rather than considering CST as a universal screening measure. Future AMR surveillance efforts must rigorously arrange data to isolate developed resistance mechanisms from intrinsic ones, thus avoiding the misinterpretation of regional epidemiological trends.

The association analyses revealed that several AMR phenotypes were significantly associated with selection for CST. The strongest associations were seen for resistance to imipenem and nitrofurantoin, followed by piperacillin/tazobactam, amikacin, ceftolozane/tazobactam, gentamicin, meropenem, ciprofloxacin, levofloxacin, ertapenem, and amoxicillin/clavulanate. Among these, the association with imipenem resistance was especially significant as carbapenem resistance is a major determinant of last-line antimicrobial choice. The observed association between imipenem resistance and colistin-test selection is consistent with the broad clinical rationale that colistin testing is more relevant when carbapenem activity is compromised. This is also supported by the significant association with meropenem resistance, although the magnitude of association was lower than that observed for imipenem.

Results also showed that the resistance to at least one cephalosporin, as a broader cephalosporin-resistance category, was not significantly associated with colistin test selection.

This result is informative in that it suggests that cephalosporin resistance alone may have been insufficient to prompt colistin testing in this dataset. In contrast, carbapenem, aminoglycoside, fluoroquinolone and broader MDR resistance patterns were more strongly associated with testing selection. This discrepancy may reflect laboratory decision making whereby testing for colistin is generally reserved for isolates with more advanced or MDR profiles rather than isolates with resistance to only cephalosporins.

One of the strongest findings from the study came out of the MDR analysis. All the 19 isolates tested for colistin susceptibility were MDR, and no non-MDR isolate was selected for colistin testing. This finding provides strong support for the hypothesis that colistin testing was strongly associated with the presence of multidrug resistance. MDR status was statistically significantly associated with selection of colistin test (high odds ratio and relative risk). However, wide confidence intervals should be interpreted with caution, since one cell of the contingency table contained zero and a continuity correction was used. Despite this limitation, the absolute concentration of colistin testing among MDR isolates is a clinically meaningful finding and suggests that MDR status was a major determinant of testing selection. Considerably, the clinical rationale underlying these test selections is fundamental. Our outcomes imply that laboratory employees employ colistin testing as a targeted diagnostic assay, mainly for isolates exhibiting compromised carbapenem activity. Consequently, microbiological laboratories must establish robust, evidence-based triage criteria for colistin testing. Such findings highlight a probable diagnostic oversight regarding the indiscriminate application of colistin sensitivity testing to intrinsically resistant bacteria.

An important observation was that all 19 isolates selected for CST were classified as MDR, despite MDR status not being used as a predefined criterion for requesting the test. This finding is particularly notable because 95 additional MDR isolates were present among the isolates that were not tested for colistin, indicating that MDR alone did not automatically trigger testing in routine practice. Nevertheless, the complete concentration of colistin testing within the MDR group, together with the observed associations between test selection and resistance to several clinically important antimicrobial agents, particularly carbapenems, suggests that multidrug resistance may represent a useful warning signal when considering the need for CST. Accordingly, Gram-negative isolates exhibiting MDR, particularly those with limited remaining therapeutic options or

resistance to carbapenems, should be strongly considered for CST when clinically relevant. A standardized, risk-based testing strategy incorporating MDR status, bacterial species, resistance phenotype, and clinical context may provide a more consistent approach than relying solely on case-by-case testing requests.

The clustering of the 19 colistin-tested resistant isolates according to the AMR showed that the isolates did not constitute a single homogeneous resistance group. Rather, they were divided into distinct clusters and subclusters, reflecting heterogeneity in antimicrobial susceptibility patterns. Some isolates of the same species group tightly together, with similar resistance patterns, but interspecies clustering was also observed. This implies that the similarity in resistance phenotype was not solely determined by the species identity. Such heterogeneity is to be expected among clinical Gram-negative isolates (Ferenci and Phan, 2015; Mehrotra et al., 2023), as resistance phenotypes can be shaped by different antimicrobial exposure histories, resistance mechanisms and local transmission dynamics.

Biochemical profiling added an additional phenotypic layer to the analysis. The most common biochemical reactions in colistin-resistant isolates tested were A-GLPRB, N-LPROT and A-LTRY, positive in all isolates, and A-LPHET and M-NAG, positive in most isolates. These conserved reactions indicate the existence of common biochemical features among the colistin-tested resistant isolates despite their taxonomic heterogeneity. The biochemical heatmap revealed broad variation across multiple reactions, indicative of species- and isolate-level phenotypic heterogeneity. The dual pattern of conserved and variable biochemical reactions supports the notion that the colistin-tested resistant subset was phenotypically diverse but shared several common biochemical traits.

Biochemical clustering was largely in accordance with taxonomic relationships with several isolates from the same bacterial species clustering together. This result is not surprising, as biochemical tests are mainly used for bacterial identification and indicate metabolic characteristics related to species. However, in the context of the AMR clustering, the biochemical analysis provided useful additional information. Resistance based clustering described similarities in antimicrobial response patterns, whereas biochemical heatmap described phenotypic and metabolic similarity. Altogether, these analyses showed that the colistin-tested resistant isolates

were heterogeneous for resistance profiles and biochemical characteristics but exhibited identifiable species-related patterns.

These results are in line with the known role of colistin as a last resort therapy for difficulty treating MDR Gram-negative infections, compared to previous literature (Falagas et al., 2005; Li et al., 2005). Prior studies and clinical recommendations have stressed the importance of testing colistin for highly resistant Gram-negative organisms, particularly carbapenem-resistant Enterobacterales, *P. aeruginosa*, and *A. baumannii*. This general concept is confirmed in present study where colistin testing was concentrated among MDR isolates and was strongly associated with carbapenem resistance especially imipenem resistance. However, unlike studies aimed at estimating the prevalence or molecular mechanisms of colistin resistance, this study focused on real-world laboratory selection patterns and phenotypic characterization of tested isolates.

This study also differs from molecular epidemiology studies of colistin resistance in that genetic testing for mcr genes, mgrB disruption or other polymyxin resistance mechanisms was not performed. Therefore, the acquired colistin resistance mechanisms could not be confirmed. This limitation is especially relevant for *K. pneumoniae* and *E. coli* where clinically important acquired resistance mechanisms may be present. On the other hand, interpretation of isolates of *Proteus*, *Providencia* and *Serratia* should consider their known intrinsic resistance to colistin. Additional studies using molecular assays or whole genome sequencing would be necessary to discriminate between innate and acquired mechanisms of resistance and to assess clonal relatedness between phenotypically similar isolates.

There are some limitations to the present study. First, the study was retrospective and based on routine laboratory data from a single governmental medical city, which may limit the generalizability. Second, CST was performed selectively and not systematically, and therefore it was not possible to estimate the prevalence of colistin resistance among all Gram-negative isolates. Third, colistin testing was only performed on 19 isolates, which limited the statistical power of subgroup analyses and precluded stable multivariable logistic regression. Fourth, we were unable to directly compare colistin-resistant and colistin-susceptible groups due to the lack of isolates tested for colistin susceptibility. Fifth, the biochemical and antimicrobial cluster analyses were exploratory and descriptive, rather than confirmatory. Finally, molecular mechanisms of colistin resistance were not studied. However, the study gives useful evidence on local laboratory selection

patterns for colistin testing despite these limitations. The findings demonstrate that CST was highly concentrated in MDR isolates, and particularly in Enterobacterales and isolates resistant to carbapenems and other important antimicrobial agents. The study also showed phenotypic heterogeneity of colistin-tested resistant isolates with distinguishable AMR and biochemical profiles. These findings support the need for clearer laboratory criteria for colistin testing, careful interpretation of not-tested results, and the integration of antimicrobial stewardship with microbiology laboratory decision-making.

Conclusion

In conclusion, this study demonstrated that CST was done selectively and was strongly associated with MDR status, Enterobacterales classification, and resistance to several clinically important antimicrobial agents especially imipenem and meropenem. All isolates selected for colistin testing were MDR and the subset tested had a variety of AMR and biochemical profiles. These findings show that colistin testing in routine laboratory practice was not used for universal screening but was targeted to highly resistant Gram-negative isolates. Future studies should consist of prospective sampling, standardized colistin broth microdilution testing for all eligible isolates, molecular characterization of colistin resistance mechanisms, and multicenter validation to clarify the epidemiology and clinical relevance of colistin resistance in the region.

Acknowledgments

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Data availability

The datasets generated and analyzed during the current study are fully documented and included within the manuscript

Author contributions

The conceptualization, methodology, and execution of this research, along with data interpretation and final manuscript drafting, were collectively performed by all authors in an equal capacity.

AI Declaration

The authors acknowledge the use of AI tools strictly for language editing, proofreading, and verifying the accuracy of scientific information and citations to ensure manuscript clarity. All intellectual content, experimental findings, and visual elements remain the original work of the authors.

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