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Dynamics of Antimicrobial Susceptibility and Risk Factors Associated with Infections Caused by Colistin-Resistant Bacteria: A Study from the Northern Region of Haryana, India

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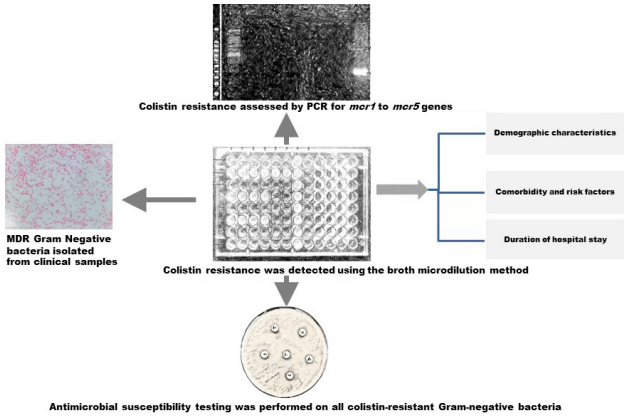
Abstract

Antimicrobial resistance poses a significant threat to global health, with colistin as a last-resort antibiotic against multidrug-resistant (MDR) microorganisms. The present study aimed to investigate the dynamics of antimicrobial susceptibility patterns and risk factors associated with infections caused by colistin-resistant bacteria in the Northern region of Haryana, India. Clinical samples (n = 12,652) collected from a single hospital in Haryana were subjected to microbiological analysis for five months. Among the total samples (n = 12,652) processed, 24% (n = 3,061) showed growth of pathogenic bacteria. Within the Gram-negative isolates, 56% (n = 1,242) were non-MDR, while 44% (n = 995) were MDR. Among MDR isolates (n = 995), 6% (n = 57) showed resistance to colistin. Notably, *Pseudomonas* spp. (12%, n = 19) and *Acinetobacter* spp. (11%, n = 8) demonstrated the highest resistance to colistin, followed by *Klebsiella* spp. (5%, n = 13), *Escherichia coli* (3%, n = 16), and *Citrobacter freundii* (1%, n = 1), respectively. The study revealed significant associations between the level of education (demographic variable) and the occurrence of colistin resistance. Prolonged hospital stays (> 5 days) and specific comorbidities, including diabetes ($p < 0.01$) and chronic obstructive pulmonary disease ($p < 0.01$), were identified as risk factors for colistin-resistant infections. Importantly, none of the colistin-resistant bacteria harbored *mcr* genes, suggesting alternative resistance mechanisms. Antibiotic sensitivity analysis indicated promising efficacy of antibiotics such as amikacin and gentamicin against colistin-resistant strains, though with variations across bacterial species. In summary, the study emphasizes the urgent need for enhanced surveillance, infection control protocols, and antimicrobial stewardship programs in healthcare settings to minimize the dissemination of MDR and colistin-resistant bacteria.



K e y w o r d s: antimicrobial resistance, multidrug resistance, colistin resistance, *mcr* gene

Introduction

Antimicrobial resistance (AMR) has become a "hidden epidemic" due to the lack of new antimicrobial medication breakthroughs. AMR is receiving the highest priority and attention from the World Health Organization due to its growing importance as a global health concern (Ferrara et al. 2024). Colistin is frequently used as a last-resort antibiotic to treat infections caused by multidrug-resistant Gram-negative bacterial infections (MDR-GNB), mainly when the bacteria are resistant to carbapenem. The possible cause of colistin resistance

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in bacteria may be due to chromosomal mutations, which often cause changes in the target site of lipopolysaccharides (LPS), or it can also occur through the transfer of plasmids carrying *mcr* genes (Andrade et al. 2020; Bostanghadiri et al. 2024). Early and accurate identification of colistin resistance is crucial for effective antimicrobial treatment.

Colistin is a growth stimulant often used in the cattle, poultry, and aquaculture industries, leading to its widespread dissemination in the environment. This leads to the selection and proliferation of bacteria resistant to colistin, which may infect human beings as well (Gharaibeh and Shatnawi 2019). Literature indicates that the prevalence of colistin resistance was relatively low (Chauhan et al. 2022). Nevertheless, antibiotic misuse and overprescription are considered the primary cause of the development of antimicrobial resistance. Further, the ease with which antibiotics can be acquired over the counter exacerbates the problem of antibiotic abuse, resulting in the emergence of antibiotic resistance (Mithuna et al. 2024). The several risk factors that can lead to the development of colistin resistance include colistin usage, previous colistin use, age of the patient, patients undergoing surgical procedures, duration of stay in ICUs, use of monobactams, and use of antifungal agents (Yau et al. 2009; Sharma et al. 2022). Further, the prevalence of colistin resistance among Gram-negative bacteria varies widely depending on the geographic area, duration, antimicrobial susceptibility dynamics, and method of antimicrobial testing. In this study, we hypothesized that the prevalence of colistin resistance in MDR-GNB would be significantly higher in our study population than in the domestic and global average. In addition, demographic factors like age, gender, residence, and educational level would significantly impact the prevalence of colistin resistance in MDR-GNB. Additionally, the presence of underlying comorbidities like diabetes, chronic kidney disease, or chronic obstructive pulmonary diseases would be linked to a higher risk of colistin-resistant MDR-GNB infections. To our knowledge, this is the first study investigating the risk factors and antimicrobial susceptibility dynamics from the Northern region of Haryana, India. In light of the above factors, we undertook a study to examine the dynamics of antimicrobial susceptibility and identify various risk factors associated with infections caused by colistin-resistant bacteria within our study settings.

Experimental

Materials and Methods

Study setting. A descriptive, cross-sectional, and observational study was carried out in the Department of Microbiology, Maharishi Markandeshwar Institute of Medical Science and Research, Ambala, India. The study was carried out in the department from May 2021 to September 2022. Informed consent was recorded at the time of specimen collection. Other demographic details such as gender, age, co-morbidities, unit/ward of the patient, duration of hospital stay, antibiotics intake, and use of invasive devices were also noted.

Laboratory methods. Samples, including pus, blood, sputum, urine, and wound swabs, were collected from every clinic and department of the constituent hospital of Maharishi Markandeshwar Institute of Medical Science and Research. We excluded duplicate samples from the current study, including repetitions or those collected from the same patients. Further, sample collection from a single hospital ensured a controlled environment with consistent data collection procedures and minimized variability in clinical practices, further enhancing the study's internal validity. However, using data from just one hospital limits the generalizability of the findings, as the results may not be fully representative of other healthcare settings with different patient demographics, regional health trends, or hospital protocols. Subsequently, these samples underwent standard direct microscopy and bacteriological culture in the hospital's microbiology laboratory. All the clinical samples were subjected to Gram staining (except the blood sample). At the same time, urine was microscopically prepared (a wet mount of uncentrifuged urine) and observed to detect the pus cells and bacteria. All specimens were inoculated on blood agar and MacConkey agar and incubated at 37°C for 18–24 hours. Blood samples were incubated at 37°C in the BACTEC™ 9050 (Becton, Dickinson and Company, USA) system for five days and processed per the standard laboratory protocols (Isenberg 2004). The identification of Gram-positive and Gram-negative bacteria were performed using VITEK® 2 GP-ID cards (bioMérieux, France) and VITEK® 2 GN-ID cards (bioMérieux, France), respectively. The results were also confirmed through microscopy, colony characteristics, and biochemical tests, following standard microbiological protocols (Isenberg 2004).

In the current study, VITEK® 2 was used to conduct the antibiotic susceptibility testing, however, an agar disk diffusion method (Kirby-Bauer disk diffusion) was also performed parallelly to ensure consistency in automated testing. Further, any discrepant results from VITEK® 2 were primarily resolved using the Kirby-Bauer disk diffusion method. 0.5 McFarland standard was used to standardize the turbidity of bacterial suspensions. Mueller-Hinton Agar was used to perform the agar disk diffusion method. All the antibiotic discs were procured from HiMedia Laboratories Pvt. Ltd. (India). The tested antibiotics included: amoxicillin/clavulanic acid (20/10 μg), piperacillin/tazobactam (100/10 μg), cefotaxime (30 μg), ceftriaxone (30 μg),

cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), and trimethoprim-sulfamethoxazole / cotrimoxazole (1.25/23.75 µg). Further, this study primarily focused on antibiotics commonly used in our geographical region; therefore, other antibiotic combinations, such as imipenem/relebactam, ceftazidime/avibactam, ceftolozane/tazobactam, aztreonam/avibactam, tigecycline, and chloramphenicol were not included. The VITEK® 2 cards used in the current study include; lactose fermenters (LF) – N-405 (antibiotic tested include: amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, cefuroxime axetil, ceftriaxone, cefoperazone/sulbactam, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, tigecycline, fosfomycin, colistin, cotrimoxazole), non-lactose fermenters (NLF) – N-406 (antibiotic tested include: piperacillin/tazobactam, ceftazidime, cefoperazone/sulbactam, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, minocycline, tigecycline, fosfomycin, colistin, cotrimoxazole), and urine – N-235 (antibiotic tested include; ampicillin, amoxicillin/clavulanic acid, ticarcillin, piperacillin/tazobactam, cefalotin, cefoxitin, cefixime, ceftazidime, ceftriaxone, ertapenem, amikacin, gentamicin, naldixic acid, ciprofloxacin, norfloxacin, ofloxacin, fosfomycin, nitrofurantoin, cotrimoxazole). However, for the final analysis, we included only the commonly tested antibiotics against organisms isolated from various samples to ensure consistency in the selection of MDRs. All the tests and the interpretations of the antimicrobial susceptibility testing were performed per the relevant CLSI standards (CLSI 2020). The susceptibility testing of colistin was determined using the broth microdilution (BMD) method with cation-adjusted Mueller Hinton broth (CA-MHB; HiMedia Laboratories Pvt. Ltd. India) (CLSI 2020).

MDR was defined as resistance to at least one antibiotic in three or more different antibiotic classes as recommended by the CDC and ECDC (Magiorakos et al. 2012). Additionally, strains showing the presence of specific resistance mechanisms such as extended-spectrum beta-lactamases, AmpC, or carbapenemase production were included. The isolated multidrug-resistant bacteria were subjected to the detection of colistin resistance by the BMD. The exclusion criteria for this study were as follows: bacterial strains with intrinsic colistin resistance such as *Proteus* spp., *Providencia* spp., *Serratia* spp., and *Morganella morganii* were excluded. Additionally, non-MDR isolates were excluded, as the study specifically focused on colistin resistance among MDR bacteria, given that colistin resistance is more prevalent in MDR strains compared to non-MDR strains. Duplicate samples were also excluded to prevent redundancy in the data, and samples from colonizers were excluded, as the study aimed to focus on clinically relevant infections. All other samples were included in the study. The presence of *mcr* genes (*mcr-1* to *mcr-5* gene) was assessed in colistin-resistant strains using multiplex PCR (M-PCR) and concurrently analyzed their antibiotic susceptibility profile.

Colistin susceptibility profiling. The MIC of colistin was determined in untreated 96-well polystyrene micro-titer plates using the BMD method (MIC range: ≤ 0.5–16 µg/ml). For the preparation of stock solution 12.6 mg (19,000 UN/mg) colistin sulphate salt (HiMedia Laboratories Pvt. Ltd. India) was dissolved in 12.6 ml of sterile distilled water. The master tube was prepared by adding 64 µl colistin solution in 936 µl sterile CA-MHB. Then, 500 µl of CA-MHB was added to each microcentrifuge tube (MCT), and two-fold dilutions were prepared. Then, 25 µl of solution from MCT was added to corresponding wells, which were already containing 50 µl of CA-MHB in each designated well. The bacterial inoculum (0.5 McFarland standard) was prepared in normal saline using the colonies obtained from blood agar. The bacterial inoculum was further diluted to 1:75 (to yield approximately 5×10^4 CFU/ml of concentration), and 25 µl of diluted bacterial inoculum was added to each well. The microtiter plate was further incubated at 37°C for 24 hours. CLSI MIC breakpoints of ≥ 4 µg/ml for colistin-resistance and ≤ 2 µg/ml for intermediate were used for interpreting the MIC of colistin in Enterobacterales, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Proteus mirabilis* (with a colistin MIC > 16 µg/ml) served as the quality control strain for colistin resistance. In contrast, *Escherichia coli* ATCC® 25922™ was used as the quality control strain for colistin susceptibility (CLSI 2020) (Fig. 1).

Multiplex PCR (M-PCR) for the screening of *mcr-1* to *mcr-5* genes. *DNA extraction.* Table I shows the primers used for the targeted gene characterization of bacterial isolates in this study. Plasmid DNA was extracted using the boiling method. For the DNA extraction process, a loopful of colonies from the blood

Table I
The primers used for the targeted gene.

Sr. No.	*mcr* genes	Amplicon size (bp)	Primer sequences (5'–3')*
1.	*mcr-1*	320	F: AGTCCGTTTGTTCTTGTGGC R: AGATCCTTGGTCTCGGCTTG
2.	*mcr-2*	700	F: CAAGTGTGTTGGTCGCAGTT R: TCTAGCCCGACAAGCATACC
3.	*mcr-3*	900	F: AAATAAAAATTGTTCCGCTTATG R: AATGGAGATCCCCGTTTTT
4.	*mcr-4*	1,100	F: TCACTTTCATCACTGCGTTG R: TTGGTCCATGACTACCAATG
5.	*mcr-5*	1,644	F: ATGCGGTTGTCTGCATTTATC R: TCATTGTGGTTGTCCTTTTCTG

* – Primer Source (Eurofins Genomics India Pvt. Ltd., India)

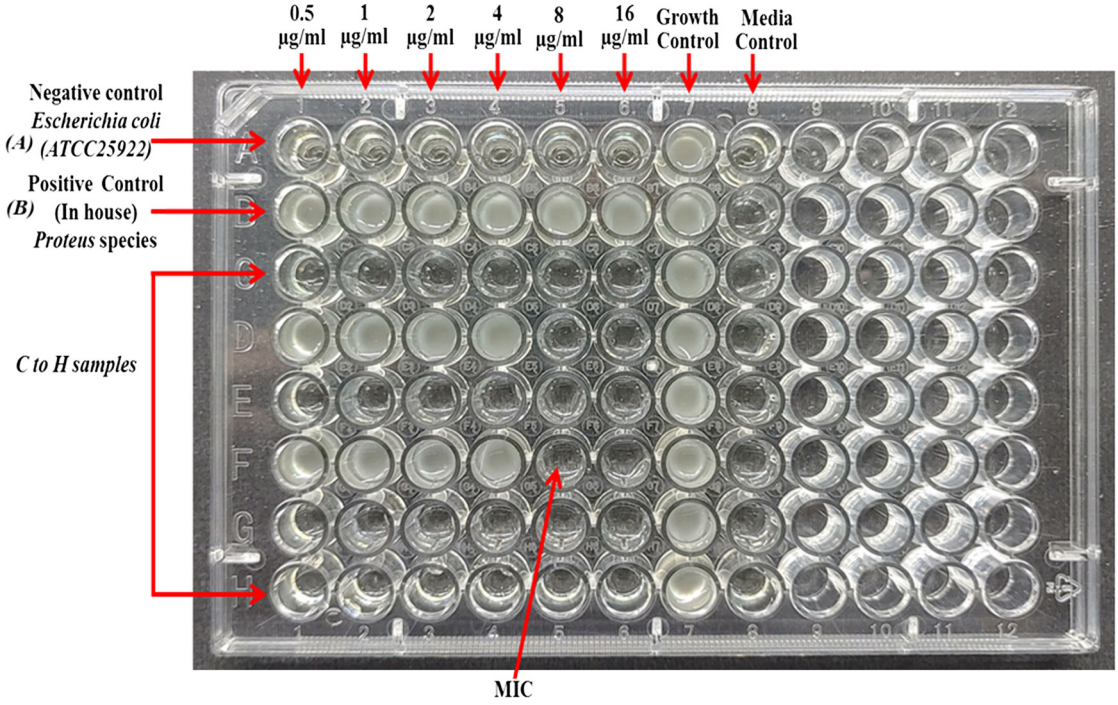

Fig. 1. Broth microdilution method for the detection of colistin-resistant bacteria.

Vertical Lane A is a negative control, B is an in-house positive control, and C-H is processed bacteria for the identification of colistin resistance, while horizontal lanes from 1–8 show the dilution of colistin sulfate from 0.5 µg/ml to 16 µg/ml, lane 7 is growth control and 8 is media control.

culture plate was suspended in 100 µl of sterile TE buffer, boiled for 10 minutes at 100°C, and centrifuged at 600 × *g* for 5 min. Further, this suspension was diluted at 1:10 in Tris-HCL buffer and used as a PCR template (Rebelo et al. 2018).

Multiplex Polymerase Chain Reaction. For M-PCR, 12.5 µl of Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X) (Thermo Fisher Scientific Inc., USA), 5 µl of primer mix (0.5 µl of each primer), 2 µl of DNA lysate (Thermo Fisher Scientific Inc., USA), was mixed with 25 µl nuclease-free water in a PCR tube. Thermal cycling for 40 cycles was performed as described earlier (Rebelo et al. 2018). In this study, primers previously reported by Rebelo et al. (2018) were used to identify *mcr-1* to *mcr-5* genes. The primer sequences, designed by Eurofins Genomics India Pvt. Ltd., (India), were used for the analysis. Further, the internal control was not employed, while a positive control was run, and the isolated DNA was quantified using the Nanodrop™ (Thermo Scientific™, Thermo Fisher Scientific Inc., USA) and verified through gel electrophoresis. However, the study did not include an analysis of the clonal relationship of these strains as it was beyond the scope of the study.

Electrophoresis. The amplified products were electrophoresed on 1.5% agarose gel and stained using ethidium bromide at 130V (Fig. 2).

Statistical Analysis. The descriptive analysis of patient demographics, bacterial isolates, antimicrobial susceptibilities, and risk factors for resistance was analyzed using Statistical Package for the Social Sciences software, IBM SPSS Statistics for Windows v28.0 (IBM Corp., USA). The chi-square test was employed to assess the relationship between colistin resistance in GNB and demographic characteristics, as well as associated risk factors. Additionally, the chi-square test was used to compare the frequency distribution of multidrug and colistin resistance among various GNBs. All statistical analyses were executed using GraphPad Prism v6 (GraphPad Software, USA, www.graphpad.com), with significance determined by p-values < 0.05.

Results

The demographic characteristics and risk factors associated with colistin-resistant Gram-negative bacteria are depicted in Fig. 3 and Table II. Patients aged between 41 and 60 were infected with colistin-resistant bacteria compared to those in other age groups when comparing the patients' age group. Moreover, while comparing the differences between genders, male patients had a more significant percentage of GNB resistant to colistin than females. Additionally, an association between the isolation rate of MDR GNB and colistin resistance was noted among females ($p = 0.2439$). Patients living in urban settings showed a higher proportion of colistin resistance when compared to patients living in rural areas (Table II). Patients with intermediate levels of education had a more significant percentage of colistin resistance compared

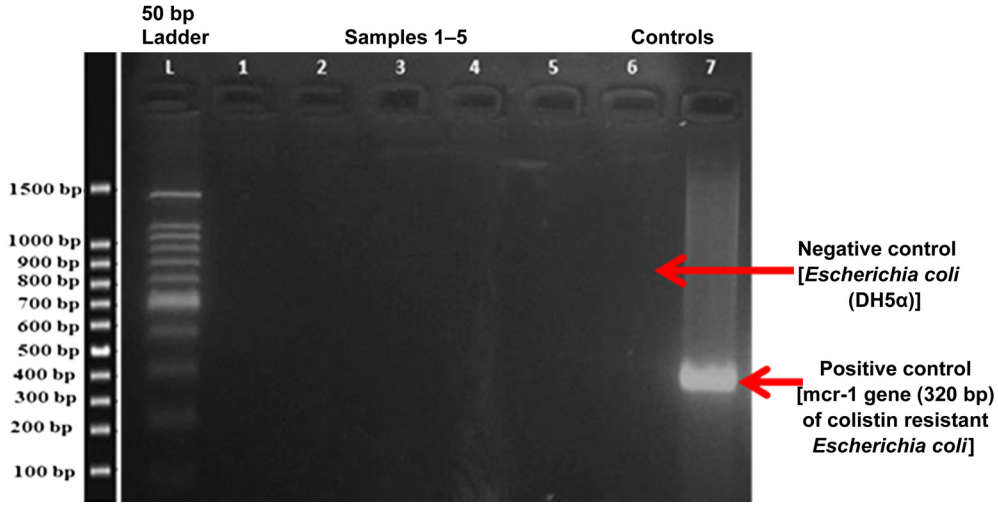

Fig. 2. Identification of *mcr 1–5* genes in colistin-resistant Gram-negative bacteria by multiplex PCR.
Bacterial plasmid DNA was isolated by boiling method and the *mcr 1–5* genes were identified by multiplex PCR and further visualized by gel electrophoresis. Lane (L): 50 bp ladder; Lane (1, 2, 3, 4, 5): samples; Lane (6): negative control; Lane (7): positive control.

to patients with different educational qualifications. Within the risk variables associated with colistin-resistant Gram-negative pathogens, all the colistin-resistant Gram-negative bacteria were found in patients who stayed in the hospital for more than five days. In diabetic individuals, colistin-resistant bacteria were identified at a rate of 56%, followed by chronic obstructive pulmonary disease (19%) and chronic renal disease (7%), respectively.

Out of the 12,652 samples processed, 79% (n = 9,591) were sterile or showed no growth in culture. Conversely, 24% (n = 3,061) showed growth of pathogenic organisms, wherein 7.0% (n = 824) and 18% (n = 2,237) were Gram-positive bacteria and Gram-negative bacteria, respectively. Of 2,237 total Gram-negative bacteria, 45% (n = 995) were MDR-GNB and 56% (n = 1,242) were non-MDR GNB. Table III shows the distribution of multidrug-resistant and colistin-resistant GNB in various samples. The majority (56%) of MDR-GNB isolates were recovered from urine samples, followed by sputum (15%), pus (14%), blood (11%), wound swab (3%), endotracheal aspirate (0.2%), and high vaginal swab (0.01%), respectively. In the current study, 995 MDR GNB isolates were analyzed for colistin resist-

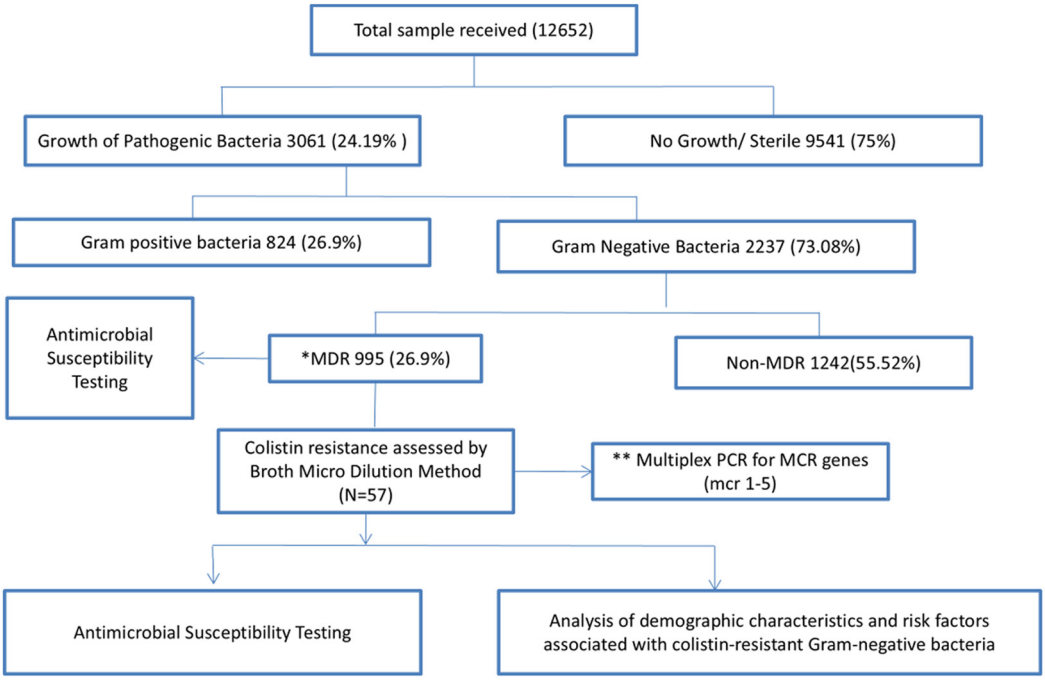

Fig. 3. Flowchart showing the study schedule.
*MDR – multidrug resistance, **PCR – polymerase chain reaction

Table II
Demographic characteristics and risk factors associated with colistin-resistant Gram-negative bacteria.

Demographic characteristics		MDR-GNB (n = 995) (%)	Colistin resistance (n = 57) (%)	Level of significance (Chi-square test)
Age (years)	0–20 years	64 (6%)	08 (13%)	0.0816
	21–40 years	279 (28%)	15 (5%)	
	41–60 years	361 (36%)	23 (6%)	
	Above 60 years	291 (29%)	11 (4%)	
Gender	Male	602 (61%)	46 (8%)	0.2439
	Female	393 (39%)	11 (3%)	
Residence	Rural	724 (73%)	40 (6%)	0.6699
	Urban	271 (27%)	17 (6%)	
Education	aIntermediate level	530 (55%)	47 (9%)	< 0.0001
	bUndergraduate level	357 (37%)	06 (2%)	
	cGraduate level or higher	108 (6%)	01 (2%)	
Risk factors associated with colistin resistance				
Duration of hospital stay	Less than 48 h	19(2%)	–	< 0.001***
	2–5 days	99 (10%)	–	
	5–15 days	368 (37%)	1 (2%)	
	> 15 days	509 (51%)	56 (98%)	
History of previous hospitalization for more than 5 days with beta-lactam antibiotics		448 (45%)	41 (72%)	0.0264*
Diabetes		278 (28%)	32 (56%)	0.0021**
Chronic heart disease (CHD)		139 (14%)	1 (2%)	0.0151*
Chronic obstructive pulmonary disease (COPD)		265 (27%)	11 (19 %)	0.0061**
Chronic renal disease (CRD)		129 (13%)	4 (7%)	0.2358

*, **, *** – denotes significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively
[a] – Intermediate level – 10 + 2, [b] – Undergraduate level-Bachelor's degree,
[c] – Graduate level –Master's degree or higher

Table III
Distribution of multidrug resistance and colistin-resistant Gram-negative bacilli in various samples.

Specimens	Gram-negative isolates (n = 2,237) (%)	*MDR GNB (n = 995) (%)	Colistin resistant bacteria (n = 57) (%)
Blood	402 (18%)	112 (28%)	9 (8%)
Sputum	419 (19%)	151 (36%)	9 (6%)
Urine	811 (34%)	560 (69%)	25 (4%)
Wound swab	165 (10%	32 (19%)	2 (6%)
Pus	322 (14%)	137 (43%)	12 (9%)
Endotracheal swab	46 (2%)	02 (4%)	–
Vaginal Swab	72 (3%)	01 (1%)	–

*MDR GNB – Multidrug resistant Gram-negative bacilli

ance across various bacterial species. It was noted that *E. coli* was the most frequently isolated species, accounting for 48% (481/995) of the total MDR-GNB isolates. Of these, 16 isolates (3%) demonstrated resistance to colistin (chi-square test, $p < 0.0001$). The second most common MDR GNB was *Klebsiella* spp., which represented 28% (280/995) of the MDR-GNB isolates, with 13 isolates (5.0%) showing resistance to colistin (chi-square test, $p < 0.0001$). This is followed by 16% (163/995) of *Pseudomonas* spp. with 19 isolates (12%) being colistin-resistant (chi-square test, $p < 0.0001$), 7% (70/995) of *Acinetobacter* spp. with eight isolates (11%) exhibiting colistin resistance (chi-square test, $p < 0.0001$), and 0.10% (1/995) *Citrobacter freundii,* the least frequent isolate, was completely colistin-resistant (100%), respectively.

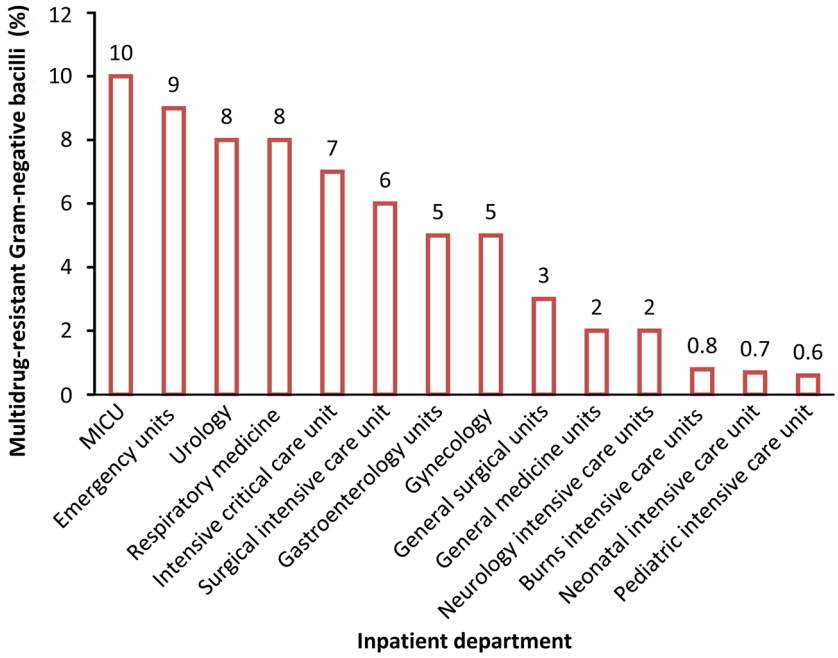

Fig. 4. Distribution of multidrug-resistant Gram-negative bacilli isolated from different clinical specimens collected across various inpatient departments.

In the current study, 29% of MDR-GNB were isolated from outpatient department (OPD) patients, while 71% were sourced from the inpatient department. Figure 4 illustrates the distribution of MDR-GNB isolated from different clinical specimens collected across various inpatient departments. Table IV shows the antibiotic-resistance pattern of MDR-GNB. It was noted that most of the Enterobacterales showed resistance to cefotaxime when the antimicrobial resistance pattern of the MDR-GNB was analyzed. Nevertheless, most of the *P. aeruginosa* strains were resistant to cefepime (Table IV). Conversely, most of the strains of *Acinetobacter* spp. were resistant to imipenem. The colistin minimum inhibitory concentration (MIC) was determined for all the MDR-GNB isolates and ranged between ≤0.5 µg/ml to 16 µg/ml, with the majority of the isolates showing a MIC value of ≥0.5 µg/ml (Table V). Of the total 57 colistin-resistant isolates obtained, the majority of the isolates (n = 39) showed a MIC value of 4 µg/ml. Eleven isolates showed a MIC value of 8 µg/ml, while seven had a MIC of ≥16 µg/ml. The predominant colistin-resistant bacteria were *P. aeru-*

Table IV
Antibiotic-resistance rate (in percentage) among multidrug-resistant Gram-negative bacilli.

Antibiotics	*Escherichia coli* (n = 481) (%)	*Klebsiella pneumoniae* (n = 265) (%)	*Klebsiella oxytoca* (n = 15) (%)	*Pseudomonas aeruginosa* (n = 157) (%)	*Pseudomonas* spp. (n = 06) (%)	*Acinetobacter* spp. (n = 70) (%)	*Citrobacter freundii* (n = 1) (%)
Amoxycillin/ Clavulanic acid	257 (53%)	189 (71%)	13 (87%)	ND	ND	ND	ND
Piperacillin/ tazobactam	200 (42%)	176 (66%)	12 (80%)	85 (54%)	4 (67%)	62 (89%)	–
Cefotaxime	455 (95%)	251 (94%)	14 (93%)	24 (86	ND*	7 (88%)	1 (100%)
Ceftriaxone	405 (84%)	239 (90%)	13 (87%)	110 (70%)	4 (67%)	59 (84%)	–
Cefepime	93 (78%)	198 (75%)	14 (93%)	115 (73%)	4 (67%)	65 (93%)	ND
Imipenem	399 (82%)	223 (84%)	11 (73%)	104 (66%)	5 (83%)	68 (97%)	1 (100%)
Meropenem	193 (40%)	127 (48%)	8 (53%)	82 (52%)	4 (67%)	57 (81%)	1 (100%)
Gentamicin	218 (45%)	150 (7%)	8 (53%)	79 (50%)	4 (67%)	60 (86%)	1 (100%)
Amikacin	79 (16%)	85 (32%)	8 (53%)	72 (46%)	4 (67%)	36 (51%)	–
Ciprofloxacin	450 (94%)	229 (86%)	14 (93%)	110 (70%)	5 (83%)	68 (97%)	1 (100%)
Cotrimoxazole	257 (53%)	175 (66%)	09 (60%)	143 (91%)	05 (83%)	59 (84%)	–

ND – not done

Table V
Determination of colistin minimum inhibitory concentration (MICs) of different MDR Gram-negative isolates by Broth Micro Dilution method.

MICs of colistin (n = 995)	*Klebsiella* spp. (n = 280) (%)	*Escherichia coli* (n = 481) (%)	*Pseudomonas* spp. (n = 163) (%)	*Acinetobacter* spp. (n = 70) (%)	*Citrobacter freundii* (n = 1) (%)
≤ 0.5 µg/ml	207 (74%)	415 (86%)	99 (61%)	28 (40%)	–
1 µg/ml	17 (6%)	23 (5%)	36 (22%)	30 (43%)	–
2 µg/ml	43 (16%)	27 (6%)	09 (6%)	04 (6%)	–
4 µg/ml	09 (3%)	06 (1%)	17 (10%)	06 (9%)	01 (100%)
8 µg/ml	03 (1%)	6 (1%)	01 (0.61%)	01 (1%)	–
≥ 16 µg/ml	01 (0.36%)	04 (0.82%)	01 (0.61%)	01 (14%)	–

Table VI
Antibiotic sensitivity pattern of colistin-resistant bacteria (n = 57).

Antibiotics	*Escherichia coli* (n = 16) (%)	*Klebsiella pneumoniae* (n = 13) (%)	*Pseudomonas aeruginosa* (n = 19) (%)	*Acinetobacter* spp. (n = 8) (%)	*Citrobacter freundii* (n = 1) (%)
Amoxicillin/clavulanic acid	7 (44%)	1 (8%)	ND	ND	–
Piperacillin/tazobactam	5 (31%)	–	6 (32%)	1 (17%)	1 (100%)
Cefotaxime	1 (6%)	–	–	ND	–
Ceftriaxone	2 (13%)	–	3 (16%)	–	1 (100%)
Cefepime	2 (13%)	–	1 (5%)	–	ND
Imipenem	–	–	–	–	–
Meropenem	3 (9%)	–	2 (11%)	4 (50%)	–
Gentamicin	8 (50%)	5 (38%)	7 (37%)	2 (33%)	–
Amikacin	13 (81%)	5 (38%)	10 (53%)	6 (86%)	1 (100%)
Ciprofloxacin	3 (19%)	1 (8%)	2 (11%)	1 (14%)	–
Cotrimoxazole	10 (63%)	4 (30%)	0	1 (13%)	–

ND – not done

ginosa (33%), followed by *E. coli* (28%), *K. pneumonia* (23%), *Acinetobacter* spp. (14%), and *C. freundii* (2%). All colistin-resistant organisms were tested negative for *mcr* genes (*mcr*-1 to *mcr*-5) using multiplex PCR. Table VI demonstrates that most of the colistin-resistant Gram-negative isolates were susceptible to amikacin, followed by gentamycin when comparing the antibiotic susceptibility patterns.

Discussion

Colistin has been considered the last resort for treating multidrug-resistant bacteria, but the emergence of colistin resistance in recent years has alarmed clinicians. Several risk factors have been investigated by researchers, including repeated antibiotic exposure (especially from beta-lactam antibiotics), invasive medical procedures, the excessive and improper application of colistin as a growth promoter in agriculture and animal husbandry, and the isolation of wastewater-borne bacteria resistant to colistin (Sharma et al. 2022; Yuan and Pian 2023). Therefore, it is essential to study the risk factors, co-morbidities, and antimicrobial susceptibility patterns of colistin-resistant bacteria to restrict the further spread of colistin resistance. The findings of our study highlight the significant influence of demographic variables on the prevalence of colistin-resistant GNB (Table II). The current study found an association between age, gender, education level, and colistin resistance. Specifically, the 41–60 age group showed notably higher colistin resistance rates among patients. This was consistent with earlier research, thereby highlighting the vulnerability of this particular age group to MDR bacterial infections (Mondal et al. 2024). The study also noted a gender gap, as males had a higher colistin-resistant GNB prevalence than females. This aligns with findings from another study indicating a consistent gender-related correlation with colistin resistance across multiple studies (Panigrahi et al. 2022). Further, the study found a clear urban-rural divide in colistin resistance, with urban areas showing higher prevalence than rural areas. This would probably be due to the increased availability and over-usage of antibiotics in urban areas. Additionally, we observed a notable association between education level and colistin resistance,

with patients with up to intermediate-level education exhibiting a higher proportion of colistin-resistant GNB than those with higher education levels. This finding underscores the complex interplay between the level of education and antimicrobial resistance; more specifically, patients with higher education are aware of the development of antimicrobial resistance and its serious complications. Moreover, the current study identified prolonged hospital stays (> 5 days) as a significant risk factor for colistin-resistant GNB. Diabetic patients showed the highest incidence, followed by those with chronic obstructive pulmonary disease and chronic renal disease. These findings highlight the importance of considering specific patient populations and clinical contexts in combating the spread of colistin resistance.

Out of 12,652 clinical samples examined, 3,061 (24%) showed growth of pathogenic bacteria. Of these pathogens, 27% (n = 824) were found to be Gram-positive bacteria, while 73% (n = 2,237) were found to be Gram-negative bacteria, respectively. This finding followed earlier research wherein the investigators found that Gram-negative bacteria accounted for a significant portion of the infections and were often resistant to multiple antibiotics (Chakraborty et al. 2023). In the current investigation, 2,237 Gram-negative bacteria were identified from all specimens processed. Among these, 44% were MDR-GNB, and 56% were non-MDR-GNB. This finding validates a previous report from Egyptian patients, indicating a consistent geographical distribution globally (El-Kholy et al. 2021). However, a study conducted among cancer patients in the Eastern part of India reported the distribution of MDR (48%), extensive drug resistance (30%), and non-MDR-GNB (20%), respectively (Shelke et al. 2024), aligning with our findings and highlighting a consistent pattern of antimicrobial resistance across Indian geographies.

The results of the current study demonstrate that *E. coli* is the most often isolated MDR-GNB from clinical samples, followed by *K. pneumoniae* (Table IV). The isolation of MDR-GNB is a significant issue in clinical settings since these bacteria are often resistant to multiple antibiotics and can cause severe life-threatening conditions. Therefore, appropriate and timely investigations are needed to determine the best action to prevent MDR-GNB from spreading throughout hospitals. This study possesses a few limitations: a) single-center study design – the current study was conducted at a single hospital in Haryana, India, limiting the availability of information on colistin resistance across various geographies; b) selection bias of patients since the current study included 71% MDR-GNB from hospitalized patients (usually possess co-morbid conditions or severe MDR bacterial infections) rather than patients treated at outpatient clinics – this could skew the results of the current study toward higher rates of MDR bacterial infections and colistin resistance; c) sampling bias – the current study analyzed samples collected from various departments within a single hospital, this may lead to sampling bias because patients in the critical care units might have been suffering from severe preexisting infections compared to patients in other units/wards; d) the cross-sectional study design of the current study minimizes the insights about the causal relationship between the risk factors and the development of colistin resistance. This is because the cross-sectional study designs analyze the association between risk factors and resistance for a particular period. However, a longitudinal study design that tracks the patients over a while would help in understating the dynamics of resistance acquisition.

Of the total MDR-GNB (n = 995) isolated in the current study, 71% were isolated from the inpatient department. MICU had the most isolations, followed by emergency, urology, and respiratory departments, ICCU, and SICU. On the other hand, a study conducted in a tertiary care teaching hospital in central India revealed most of the GNBs isolated from different medical and surgical wards (57%), followed by ICU (26%) and OPDs (17%), respectively (Soni et al. 2023). This difference in the prevalence of distribution of MDR GNB may be attributed to the difference in the geographical locations. However, we could not compare studies conducted in North Indian settings because of the scarcity of literature. Various studies have consistently reported the isolation of colistin-resistant bacteria from different hospital settings. These studies have also highlighted variations observed across different departments and sample types (Sinha et al. 2019; Panigrahi et al. 2022; Soni et al. 2023). In the current study, the most colistin-resistant bacteria were isolated from pus samples (21%), followed by blood, sputum, urine, and a wound swab, respectively (Table III). Consistent with our findings, another research group also observed a high prevalence of MDR-GNB in a study conducted in central India (Soni et al. 2023). Nonetheless, other studies conducted in two distinct locations in India have demonstrated a higher isolation rate of colistin-resistant bacteria from blood, exudates, and urine, as well as from sputum and pus samples, respectively (Sinha et al. 2019; Panigrahi et al. 2022).

Antibiotic resistance patterns differ from one bacterial species to another, from genus to genus, and also among different families. It is evident from Table IV that most Enterobacterales were resistant to cefotaxime whereas most of the *Pseudomonas* spp. showed resistance to cotrimoxazole followed by cefotaxime. In contrast, the majority of the *Acinetobacter* strains were resistant to imipenem. Further, it was noted that most of the strains in the current study were susceptible to amikacin. These findings were inconsistent with the earlier studies conducted in India wherein the highest

resistance among Gram-negative isolates was found to be against cefotaxime.

In contrast, the lowest resistance was found against imipenem (particularly isolates obtained from blood samples) (Shah et al. 2022). In contrast, substantial levels of resistance to ciprofloxacin and cefotaxime were noted against *E. coli* and *K. pneumoniae*, particularly isolates obtained from UTIs (Verma et al. 2023). This result was coherent with the current investigation, which found that more of the bacterial isolates had ciprofloxacin resistance (Table IV). This probably could be due to the intake of ciprofloxacin as a prophylactic agent in the treatment of recurrent UTIs in communities, which would have spread the resistance genes of ciprofloxacin in the communities to emerge as ciprofloxacin-resistant strains. This finding underlines the significance of improved monitoring of antimicrobial resistance patterns in the hospital. Further, better antimicrobial monitoring and antimicrobial stewardship programs in hospitals can contribute to improved antibiotic usage in the community in several ways, including: a) primarily education of healthcare workers about antimicrobial resistance and proper use of antibiotics, b) reduction of the emergence and spread of antibiotic-resistant bacteria, and c) optimization of antibiotic prescriptions by healthcare workers. Indeed, healthcare professionals' greater understanding of antimicrobial resistance and its use can extend beyond hospital walls, reaching primary care providers and the community. The increased awareness about antibiotic resistance and the importance of responsible antibiotic use can lead to more informed decision-making by patients and healthcare providers in community settings.

Knowledge of the MIC values for colistin against MDR-GNB is essential for optimizing treatment strategies against these bacterial pathogens. In the current study, 6% (n = 57) of colistin-resistant MDR GNB were isolated from the total MDR-GNB (n = 995). However, none of the isolates harbored the *mcr* gene. This suggests that alternate mechanisms like chromosomal mutations or efflux pump proteins may exist in the bacteria to contribute to antimicrobial resistance (Table V). In the current study, the MICs of these bacteria varied between ≥ 0.5 µg/ml and ≤ 16 µg/ml, with most isolates demonstrating MICs ≥ 0.5 µg/ml. These results are consistent with previous studies conducted in India's Southern, Central, and Eastern regions, suggesting a similar pattern in the Northern part of Haryana, India (Aarthi et al. 2020; Panigrahi et al. 2022).

The sensitivity pattern of colistin-resistant Gram-negative bacteria to various antibiotics is critical in combating bacterial infections. The current study presents a comprehensive overview of antibiotic sensitivity across different bacterial strains, highlighting both promising and challenging aspects of treatment. When analyzing the susceptibility pattern of colistin-resistant *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter* spp. and *C. freundii*, amikacin and gentamicin consistently demonstrated high sensitivity across most bacterial strains, while cephalosporins such as cefotaxime, ceftriaxone, and cefepime showed lower efficacy (Table VI). This variation in sensitivity underscores the necessity of modified treatment approaches based on bacterial species. For instance, while amikacin and gentamicin were effective against *E. coli* and *Acinetobacter* spp., their efficacy against *K. pneumoniae* and *P. aeruginosa* was more variable. Despite the promising efficacy of antibiotics like amikacin and gentamicin, there remains a need for more extensive research to validate these findings and optimize treatment strategies. Factors such as variations in bacterial strain and local antibiotic sensitivity patterns can significantly impact treatment outcomes. Future studies should focus on evaluating the efficacy of different antibiotics across diverse bacterial strains to identify the most effective antibiotic regimens for colistin-resistant Gram-negative bacterial infections. This research will enhance our understanding of antibiotic resistance mechanisms, help clinical practice, and improve patient outcomes. In summary, this study underlines a significant presence of multidrug-resistant and colistin-resistant Gram-negative pathogens in inpatient settings, with patterns paralleled across regional hospitals, highlighting the urgent need for robust infection control practices, targeted antibiotic stewardship programs, and comprehensive regional surveillance systems. High resistance rates to key antibiotics like cefotaxime, cefepime, and imipenem reveal the importance of these measures, especially in high-risk hospital areas, to improve treatment protocols and support effective policy decisions. Colistin resistance, particularly in *P. aeruginosa*, is concerning for patients with prolonged hospital stays, although amikacin and gentamicin provide alternative treatment options. Notably, none of the colistin-resistant strains in this study harbored *mcr* genes, indicating possible alternative resistance mechanisms. Therefore, judicious antibiotic use and public education are imperative to control further resistance spread and optimize therapeutic outcomes.

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Ethical statement

This study received approval from the Institutional Ethical Committee at M.M Institute of Medical Science & Research, Mullana (Ambala) (IEC/MMIMSR/1916/dated-15/5/2021).

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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