RESEARCH ARTICLE

Colistin Resistant Gram-Negative Bacteria Isolated from Various Clinical Samples in North Indian Tertiary Care Center

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ABSTRACT

Background: Antibiotic resistance and their mischaracterization often lead to treatment failure. In the present scenario, resistance to colistin is emerging. Poor testing methods for colistin resistance detection can increase the risk of treatment failures and even mortality. In this study, we analyzed the burden of multidrug-resistant (MDR), Extensively drug-resistant (XDR), pandrug resistant (PDR), and colistin resistant Gram-negative bacteria and also evaluated Vitek-2 compact and Broth Microdilution (BMD) for the detection of colistin resistance at North Indian Tertiary-Care Hospital.

Methods: A total of 11237 samples were processed in which 1822 (16.21%) were gram-negative bacilli (GNB). All GNB after identification by Vitek-2 compact, were subjected to antibiotic sensitivity testing by Vitek-2 and characterized as MDR, XDR, and PDR as per guidelines of Centers for Disease Control and Prevention US (CDC) and European Centre for Disease Prevention and Control (ECDC). Identification of colistin resistance in Enterobacteriaceae family, *Pseudomonas aeruginosa, Acinetobacter baumannii* was made by broth microdilution (BMD) method. BMD was used as a standard gold method for colistin resistance.

Results: A total of 43.57% MDR, 9.49% XDR, and 3.5% PDR gram-negative bacteria was isolated from various departments and from various sites. Vitek-2 compact detected 47 (2.75%) and MBD method detected 38 (2.23%) colistin-resistant gram-negative bacilli. Vitek-2 compact failed to detect nine colistin-resistant organisms. The highest resistance towards colistin was seen in *A. baumannii* (6%), followed by *P. aeruginosa* (5%), *Escherichia coli* (1.07%), *Klebsiella pneumonia* (1%).

Conclusions: There is a need for quick and efficient antibiotic resistance management efforts against colistin resistance. Vitek-2 compact gives unsatisfactory and inaccurate results in the detection of colistin susceptibility

Keywords: Broth Microdilution (BMD), Colistin Resistance, Superbugs, Vitek-2.

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INTRODUCTION

Antibiotic resistance (AMR) is a great threat because of the decreasing development of new antibiotics. According to World Health Organization, with rising treatment failure in many health institutions throughout the world AMR had been prioritized at the top of the list.[1,2] Neglecting of antibiotic regimens and poor prescriptions help the target bacteria evolve. Due to this evolution, bacteria may become multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR).[1,3]

In a collaborative effort, US Centers for Disease Control and Prevention (CDC) and the European Centre for Disease Prevention and Control (ECDC) addressed a proposal for categorizing these resistant isolates and those isolates that were resistant to at least one antibiotic from three separate groups of first-line antibiotic were considered as multidrug-resistant (MDR), isolates those were resistant to at least one antibacterial agent in all categories except two or fewer were considered as Extensively drug-resistant (XDR). Pan drug resistance (PDR) were those isolates resistant to all antibiotics tested with all agents in all antimicrobial categories listed in the Clinical and Laboratory Standards Institute (CLSI) guidelines for each bacteria.[4] Gram-negative bacteria were now emerging resistant to colistin, a last-resort antibiotic used to treat multidrug-resistant infections.[5]

Colistin was launched in the late 1950s as a Penta- cationic

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antibiotic that directly acts on lipopolysaccharides of gram-negative bacteria. Resistance towards last colistin is increasing by alteration in Lipopolysaccharides. Colistin resistance can be chromosomal or plasmid-mediated, and there are few species of bacteria inherently resistant, like *Morganella, proteus, Providencia*. Chromosomal resistance is may be due to misuse of colistin, while plasmid-mediated resistance occurs due to MCR gene transfer which was discovered in china and now reported all around the globe.[5]

Even in developing nations, automated methods have formed the backbone of diagnostic microbiology labs. It is very difficult to perform BMD tests because of the lack of skilled person to perform BMD, all of which encourage the deployment of semi or completely-automated identification/antimicrobial susceptibility testing (ID/AST) systems. According to a recent study, Vitek-2 may not be as trustworthy as it appears, with a very significant error rate of 36% for colistin testing.[6]

In 2016, CLSI and EUCAST jointly recommended only the broth microdilution (BMD) methodology to perform the colistin susceptibility tests.[7]

The lack of knowledge on the resistance trend of these "superbugs" and difficulties in testing colistin susceptibility are diverse, including poor diffusion of polymyxins into agar, colistin inherent cationic properties, the occurrence of colistin heteroresistance in many species, and the lack of a reliable reference method.[8]

This study investigates the status of MDR, XDR, PDR, and colistin-resistant Gram-negative bacteria in a tertiary care hospital in Ambala, India.

MATERIAL AND METHOD

A short cross-sectional study was carried out at the Department of Microbiology in Maharishi Markandeshwar Institute of Medical Science and Research, Mullana, Ambala, India, from May 2021 to April 2022. All clinical samples such as blood, urine, endotracheal aspirate, bronchoalveolar lavage fluid, pleural fluid, pus, peritoneal fluid, cerebrospinal fluid, etc. were collected from patients suspected of infections. The Ethical clearance was taken from the Institutional Ethical Committee (IEC) vide letter no. - MMIMSR /IEC/1916. Before enrollment, each patient or their guardian provided written consent to participate in this study. Specimens were properly collected by skilled nurses with their consent and transferred to the microbiology laboratory. Specimens that precisely satisfied the American Society for Microbiology (ASM) criteria were chosen for further processing and analysis.[9] Specimens that did not meet the ASM criteria and duplicate specimens from the same patient, Sterile/No growth and gram-positive isolates were excluded from the study. The intrinsic colistin-resistant organism was also excluded from colistin-resistance detection. A computer system was used to enter information about patient demographics, Health care-associated, bacterial isolates, antimicrobial susceptibilities, and resistance determinants. The data were analyzed with Microsoft excel and interpreted using frequency distribution and percentage.

Identification of Isolates

Clinical specimens were cultured into 5% Sheep Blood Agar (BA) and MacConkey Agar (MA) plates (HiMedia, Mumbai, India). The BA and MA plates were incubated for 24 hours in an aerobic environment at 37°C. The identification of isolates was done by Vitek-2 automated system (Vitek-2 GN-card) according to the manufacturer guidelines.

Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was done by the Vitek-2 Compact (bioMérieux) using N280 (for lactose fermenters) and AST-N281 (for non-lactose fermenters) to estimate the minimum inhibitory concentration (MIC) for all isolates as per manufacturer guidelines and interpreted as per CLSI 2020 guidelines After that Kirby Bauer method also used to asses antibiotic sensitivity testing.[10] The colistin MIC was also evaluated in uncoated 96-well polystyrene microtitre plates using the standard BMD protocol (MIC range: 0.25–16 mg/L) colistin sulfate salt (HiMedia, Mumbai, India) dissolved in CAMHB (HiMedia, Mumbai, India), and according to CLSI guidelines.[10] For Colistin MIC in Enterobacteriaceae family, *Pseudomonas aeruginosa,* and *Acinetobacter baumannii*, EUCAST MIC breakpoints of >2 mg/L for resistance and <2 mg/L for susceptibility were Applied.[7] For each run of the colistin MIC testing, *Proteus mirabilis* (colistin MIC >16 mg/L) was selected as a colistin-resistant quality-control strain.

Detection of MCR Genes

In each PCR experiment, 12.5 µL Dream Taq Green PCR Master Mix, 5.5µL nuclease-free water, 0.5µL, 2 µL of each of the 10 priming solutions (10 L)*, and 2 µLDNA lysate were employed. The running conditions were as follows: 15 minutes of denaturation at 94°C, followed by 25 cycles of denaturation at 94°C for 30 seconds, 90 seconds of annealing at 58°C, 60 seconds of elongation at 72°C, and a final cycle of 10 minutes of elongation at 72°C. The amplified product was electrophoresed on 1.5% agarose gel to visualize the results. At 130V, ethidium-bromide was used to stain the paper.[11]

Identification of MDR, Extensively drug-resistant (XDR), and PDR from gram-negative bacteria

As per the Center for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC), the isolates that were resistant to at least one antibiotic from three separate groups of first-line antibiotics were considered as MDR isolates were resistant to at least one antibacterial agent in all categories except two or fewer considered as Extensively drug-resistant (XDR). Pan drug resistance (PDR) is named as resistant to all antibiotics tested.[4]

RESULTS

A total of 11237 samples which includes urine (12%), pus (7.22%), blood (6.39%), sputum (4.46%), endotracheal tube (2.06%) vigainal swab (2.3%), ascitic fluid (1.3%), pleural fluid (1.3%), bile fluid (.1%), wound swab (1.3%), tissue (0.09%), tracheostomy (0.08%), ear swab (0.2%), BAL (1.3%), CSF

(0.4%), nasal swab (0.20%), peritoneal (0.24%), stool (0.07%), stool (0.19%), throat swab (0.24%) and unknown (0.48%) were taken from clinically suspected cases of infection. In which 7004 (62.32%) samples were sterile and 2411 (21.45%) were gram-positive bacteria, 1822 (16.21%) were gram-negative bacteria. *Escherichia coli were* the predominant isolates. 794 (43.57%), 173 (9.49%), and 65 (3.5%) organisms were recorded as MDR, XDR, and PDR, respectively. Lactose fermenter gram-negative bacteria accounted for a higher percentage of MDR and XDR, while the PDR percentage was high in Non-lactose fermenters. Non-lactose fermenter gram-negative bacteria had more colistin resistance isolates (5.35%) than lactose fermenter gram-negative bacteria (1%). Vitek-2 compact detected nine more colistin-resistant gram-negative bacteria as compared to broth microdilution test. The sensitivity and specificity of Vitek-2 compact for the detection of colistin resistance when broth microdilution test was considered as the gold standard was 80.8 and 99.4%, while positive predictive value and negative predictive value was 80 and 99%, respectively. Most of the colistin-resistant organisms were isolated from the oncology department (23%) and surgery department (18%). Pus (36%) and urine (31%) were the major sources of colistin-resistant organisms**.** There was not any MCR gene detected in colistin-resistant gram-negative bacteria.

DISCUSSION

With the increasing antibiotic resistance, the post-antibiotic era will be more dreadful than COVID-19 pandemic. Colistin resistance is also emerging, which should be detected by every laboratory so as to slow down the speed. In this study, we assessed all the gram-negative bacteria for MDR, XDR, and PDR, which were also subjected to the detection of colistin resistance in gram-negative bacteria.

A total of 11237 (100%) were processed, of which 1822 (16.21%) gram-negative bacteria were isolated (Table 1). *Escherichia coli* (40%) were the most predominant organism-isolated gram-negative bacteria, followed by *Klebsiella pneumoniae* (26.8%), *Pseudomonas aeruginosa* (19%), *Acinetobacter baumannii* (6%), *Acinetobacter baumannii* complex (3%), *Stenotrophomonas maltophilia* (1%) and others (Table 2). Few studies from India and other countries reported *Escherichia coli* as the most predominant bacteria.[12,13] A Total of 1822 (16.21%) gram-negative bacteria were processed for antibiotic sensitivity by Vitek-2 compact. According to a joint endeavor of the ECDC and the CDC, a group of worldwide specialists convened to debate and describe various resistance patterns discovered in healthcare-associated, antimicrobial-resistant bacteria. In our study, 794 (43.57%), 173 (9.49%), and 65 (3.5%) organisms were recorded as MDR, XDR, and PDR, respectively. A study done by Mohapatra DP *et al.* (2018) in eastern India documented 41.3% (n = 285) as XDR, whereas 8.1% (n = 56) were PDR. Colistin and tigecycline resistance rates were 16 and 51.9%, respectively.[13] The Higher rate of MDR, XDR, and PDR may be attributed to antibiotic misuse and abuse, which includes poor antibiotic selection, an unsuitable dosage regimen, failure to finish therapy, dose skipping, and use of antibiotics for non-bacterial diseases, is still a widespread and prevalent across the world.[14,15] *E. coli* was the most common species with highest percentage of MDR, XDR, and PDR i.e, 440 (58%),51 (6.82%), and 15 (2%) followed by *K. pneumoniae, P. aeruginosa,* and *A. baumannii.* (Table 3), an approximately similar result was observed by Mohapatra DP *et al.* (2018) in eastern India, in which few bacteria were exclusively sensitive to colistin (4%). In contrast, others were only sensitive to tigecycline (2%).[13] In our study samples were collected from various sites and wards and were divided into OPD and IPD based upon their admission which showed IPD patients having more number of MDR (47%), XDR (11.23%) & PDR (5%) as compared to OPD patients which showed MDR (35.5%), XDR (5.4%) and PDR (0.18%) (Table 4). A similar study conducted by Bhatt P. *et al.* (2015) showed more XDR and PDR from IPD patients.[16] Gram-negative bacteria are becoming highly resistant to frequently used antibiotics, So previously abandoned drugs such as colistin are being reintroduced as a last-resort therapy option. However, because colistin is a neurotoxic and nephrotoxic drug, it has its own set of drawbacks.[17] In invasive bloodstream infections caused by carbapenemase-producing gram-negative bacteria, colistin is the only effective treatment, and colistin resistance

Table 1: Distribution of samples and bacterial isolate.

Total Samples	*Sterile/No growth*	*Growth (37.67%)*	
		Gram-positive bacteria	*Gram-negative bacteria*
11237 (100%)	7004 (62.32%)	2411 (21.46%)	1822 (16.21%)

Table 2: Gram-negative bacteria isolated from various specimens.

Sr. no	*Isolated organism gram-negative bacteria*	*N = 1822 (%)*
1	*E. coli*	747 (40.99**%**)
2	*K. pneumoniae*	490 (26.89**%**)
3	*P. aeruginosa*	348 (19.09**%**)
4	*A. baumannii*	110 (6.04**%**)
5	*A. baumannii complex*	67 (3.68**%**)
6	*Stenotrophomonas maltophilia*	19 (1.04**%**)
7	*P. s mirabilis*	16 (0.88**%**)
8	*Burkholderia cepacia*	9 (0.49**%**)
9	*Sphingomonas Paucimobilis*	5 (0.27**%**)
10	*Serratia marcescens*	3 (0.16**%**)
11	*Citrobacter amalonaticus*	1 (0.55**%**)
12	*Citrobacter freundii*	1 (0.55**%**)
13	*Citrobacter koseri*	1 (0.55**%**)
14	*Kocuria rosea*	1 (0.55**%**)
15	*Morganella morganii*	1 (0.55**%**)
16	*Providencia rettgeri*	1(0.55**%**)
17	*Salmonella Typhi*	1 (0.55**%**)
18	*Shigella species*	1(0.55**%**)

Table 3: Distribution of multidrug resistance, extensively resistant, and pandrug-resistant gram-negative bacteria.

Sr. no	*Gram-negative bacteria (N)*	*MDR (%)*	*XDR (%)*	*PDR (%)*
1	*E. coli (747)*	440 (58%)	51 (6.82%)	15 (2%)
2	*K. pneumonia(490)*	196 (40%)	82 (16.73%)	14 (2.8)
3	*P. aeruginosa (348)*	94 (27.01%)	13 (3.7%)	19 (5.45%)
4	*A. baumannii (110)*	34 (30.90%)	13(11.81%)	12 (10.9%)
5	A. baumannii complex (67)	11 (10%)	12 (10%)	05 (7.4%)
6	*P. mirabilis (19)*	9 (8.18%)	2 (1.8%)	00 (00%)
7	*B. cepacia (16)*	4 (25%)	0 (00%)	01 (6.25%)
8	*C. freundii (9)*	1(11.11%)	0 (00%)	00 (00%)
9	*M. morganii (5)*	1 (20%)	0 (00%)	00 (00%)
10	*Pantoea* species *(3)*	1 (60%)	0 (00%)	00 (00%)
11	*Serratia marcescens (1)*	1 (100%)	0 (00%)	00 (00%)
12	*Shigella* species *(1)*	1 (100%)	0(00%)	00 (00%)
13	*S. Paucimobilis (1)*	1 (100%)	0 (00%)	00 (00%)
	Total=1822	794 (43.57%)	173 (9.49%)	65 (3.5%)

Table 5: Detection and Evaluation of colistin resistant organism by Vitek-2 compact and broth microdilution (BMD) test

Sr. No	*Organism (N)*	*No. of colistin resistant given by the Vitek-2 compact*	*No. colistin resistant given by broth microdilution test*
1.	*P. aeruginosa (348)*	20 (5.7%)	18 (5.17%)
2.	*E. coli (747)*	11 (1.47%)	8 (1.07%)
3.	*K. pneumonia (490)*	6 (1.22%)	5 (1%)
5.	*A. baumannii (110)*	9 (8.18%)	7 (6.36%)
7.	*C. freundii (9)*	1 (11.11%)	0 (00%)
	Total=1704	47 (2.75%)	38 (2.23%)

Table 4: Demographic characteristics of MDR, extensively resistant and PDR gram-negative bacteria.

Sr.no	*Segment N = 1822*	*MDR (%)*	*XDR (%)*	*PDR (%)*
1.	OPD (N = 549)	195 (35.5%)	30 (5.4%)	01 (0.18%)
2.	IPD (N = 1273)	599 (47%)	143 (11.23%)	64 (5%)

has emerged recently due to the overuse of this lifesaving drug.[17,18] Colistin-resistant organisms by broth microdilution method were 38 (2.20%) from gram-negative bacteria. We isolated more non-lactose fermenters with colistin resistance than lactose fermenting gram-negative bacteria. The highest resistance towards colistin was seen in *A. baumannii* (6%) followed by *P. aeruginosa* (5%), *E. coli (1.07%), K. pneumonia* (1%). Colistin resistance in gram-negative bacteria also been reported by several scientists in the last 10 years.[14,19,20] Sensitivity and specificity of the Vitek-2 were low for the detection of colistin-resistant gram-negative bacteria as per our study, similar finding was seen by Tan TY *et al.* (2007) (Table 5).[21-23] Only IPD patients had colistin resistance while no colistin resistance seen in OPD patients. The highest number of colistin-resistant organisms were isolated from the oncology department, followed by the surgery ward, while pus and urine were the most common sources of colistin-resistant organisms.

Table 6: Demographic characteristics between colistin resistant gram-negative isolates.

Segment		*Isolates (N = 38)*
OPD		00 (00%)
IPD	EMG	02 (5.26%)
	RESP. MED	05 (13.57%)
	ONCOLOGY	09 (23.68%)
	Surgery	07 (18.42%)
	Gastroenterology	01 (2.63%)
	Others	14 (36.84%)
Specimen	BLOOD	02 (5.26%)
	PUS	14 (36.84%)
	SPUTUM	03 (7.89%)
	URINE	12 (31.57%)
	ASCITIC FLUID	02 (5.26%)
	WOUND SWAB	5 (13.57%)

Multiplex PCR was used to screen for mcr-1 to mar-5 genes in all colistin-resistant gram-negative bacteria. All of the isolates tested negative for the MCR gene. Colistin is not included in urine penal by CLSI guidelines, but emerging resistance and MCR gene in colistin-resistant organisms pressurized scientists to include urine samples for assessing colistin resistance.[22,23] A similar study was conducted by Karade S et al. (2021) in which MCR-1 gene was documented in organisms isolated from urine.[24]

Study Limitations

However, one drawback of this study is the limited number of isolates for the detection of colistin resistance and evaluation of Vitek-2 with BMD. Molecular characterization of colistin resistance is not done in this study. Due to the increasing rate of MCR genes in gram-negative bacteria, molecular characterization of colistin-resistant isolates should be done. The synergistic effect of other antibiotics and natural molecules or nanoparticles should be examined to overcome the colistin resistance of gram-negative bacteria.

CONCLUSION

Resistance in gram-negative bacteria is increasing day by day. Gram-negative bacteria show 2.23% resistance toward colistin. There is a need of quick and efficient antibiotic resistance management efforts against colistin resistance. Performing BMD for antibiotic sensitivity is demanding in every laboratory. Laboratories must train their staff to perform BMD for colistin. The detection of MCR genes in these bacteria would also give molecular epidemiological data. Most of the studies on colistin resistance have involved one or a few Enterobacterace species, but this is one of the few studies demonstrating colistin resistance in a significant number of gram-negative bacteria.

Ethics Statement

The Ethical clearance was taken from the Institutional Ethical Committee (IEC) vide letter no. - MMIMSR /IEC/1916.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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