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Research Article

INCIDENCE AND OUTCOME OF MULTIDRUG RESISTANT AEROBIC GRAM-NEGATIVE BACTERIA CAUSING VARIOUS CLINICAL INFECTIONS

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ABSTRACT

Objective: The purpose of the present study was to isolate, characterize, and evaluate Gram-negative antibiotic bacteria isolated from different clinical samples.

Methods: The Clinical and Laboratory Standard Institute guidelines were used to isolate and identify microbial isolates on Muller–Hinton agar using standard bacteriological techniques and to monitor for antibiotic susceptibility by disc diffusion method.

Results: The study involved 129 clinical samples that were obtained from 70 males and 59 females. A maximum number of cases were recorded in the age group 51–60 (33%) followed by 41–50 (16%). The results showed that the common isolates were *Escherichia coli* 49 (37%), *Klebsiella* spp. 37 (28%), *Pseudomonas aeruginosa* 30 (23%), and among 12% microbial isolates, four isolates were Proteus species, seven isolates were Citrobacter species, and two isolates were Providencia species. The most of the isolates were multidrug resistant isolates. However, few isolates showed sensitivity to meropenem and imipenem and most of them were colistin sensitive. Out of 129 isolated microorganisms, 53 isolates were again screened for carbapenemase production through modified Hodge test. It was found that 50 strains were positive for carbapenemase producers (94%) and three strains were negative for carbapenemase production (6%). *E. coli* and *P. aeruginosa* followed by *Klebsiella* species showed carbapenemase production. Among the 50 strains that were positive for the development of carbapenemase, 47 strains were susceptible to colistin that was identified by the "E" strip method and three strains showed resistance to colistin.

Conclusion: The study allows clinicians to select the right antimicrobial agent that not only leads to improved treatment but also helps to avoid the emergence of drug resistance strains which are still sensitive.

Keywords: Gram-negative bacteria, Multidrug resistant bacteria, Modified Hodge test, Colistin.

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INTRODUCTION

The massive spread of multidrug-resistant (MDR) bacteria among humans, livestock, and environmental reservoirs has now created new unexpected epidemiological trends in health-care facilities for these bacteria. Overtime shifts have occurred in global infection epidemiology in patients, marked by a transition from predominant Gram-negative bacteria between the 1960s and 1970s to Gram-positive bacteria 10 years later and, beyond that, a fresh restitution of Gram-negative bacteria in several countries over the past 20 years. Enterobacteriaceae, primarily *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* etc., resistant to different groups of antibiotics, including carbapenems, colistin, and polymyxins, have been increasingly involved as infecting pathogens and are responsible for both population and hospital-related infections and are involved in high morbidity and mortality rates across both sexes and ages. Such species are highly responsible for causing urinary tract infections, pneumonia, peritonitis, meningitis, sepsis, and infections associated with medical devices [1-10]. In general, carbapenem antibiotics such as imipenem, ertapenem, meropenem, and doripenem are used as the medication of choice for infections caused by extended spectrum beta-lactams (ESBL) manufacturers. Today, acquired resistance to beta-lactams is predominantly mediated by the ESBLs, AmpC-type cephalosporinases, and carbapenemases. From the literature survey, it was found that resistant microbial strains increase the worldwide development of β-lactamases, hydrolyzing all β-lactam antibiotics, including carbapenems. Veronica integron Metallo-beta-lactamase (MBL), Imp sort, KPC, and Oxacillinase-48 are the key forms of carbapenemase and MDR or pandrug-resistant and difficult to treat infections usually induced by CRE species. This leads to an increase in morbidity, mortality, and the cost of health care. Since the most CREs are resistant to many antimicrobial groups, colistin, polymyxin-B, aztreonam, tigecycline, fosfomycin, and rifampin are needed for additional antibiotic susceptibility testing [11-15]. Although colistin is used as the last line of medication of choice, nephrotoxicity, which was seen in half of the patients treated with high parenteral doses, but which appears to be reversible in most cases, is the key adverse effect of colistin. These resistant strains, however, are susceptible to colistin and very few studies have recorded the prevalence of resistant strains with colistin. The growing use of colistin, however, explains why acquired resistance to colistin can now be applied to the resistance trait of carbapenem in Enterobacteriaceae. Hence, there is a need for tests that enable rapid detection of carbapenem and polymyxin resistance in Enterobacteriaceae and that may contribute to its containment. Hence, the present study employs some rapid tests for the detection of carbapenem and colistin resistant strains of Enterobacteriaceae isolated from the various infectious patient samples for the disease management therapy and to prevent the dissemination of resistant genes among the GNB.

METHODS

It is a retrospective analysis. Different clinical samples obtained from patients with both inpatient (IP) and outpatient (OP) from tertiary care teaching hospital were the material for this research. Clinical specimens including pus, urine, blood, endotracheal aspirates, sputum, and ascitic fluid. Patient demographic variables were identified including age, sex, type of patient (IP or OP), and samples. Gram's staining was initially conducted to study the patient's demographic variables.

Blood agar, chocolate agar, and MacConkey agar are inoculated in the specimens. With the exception of chocolate agar incubated with increased carbon dioxide, all other inoculated agar plates were aerobically incubated for 24 h at 35–37°C.

For direct gram staining to make a presumptive diagnosis, the second swab was used. Identification of isolates was done using combination of colonial characteristics, gram staining characteristics, and conventional standard biochemical tests. BACT ALERT System (bioMerieux) was used to isolate and identify the bacterial isolates in blood samples. The culture of pathogens enables colonies of pure growth to be isolated for identification and antimicrobial susceptibility testing. The specimen is streaked on the culture plates (Blood agar and Mac Conkey agar) and incubated at 37°C for 24 h. Urine samples were inoculated into cystine–lactose–electrolyte-deficient agar. Following culture methods, biochemical tests are often required to identify pathogens using substrates and sugars to detect their enzymatic and fermentation reactions. The tests include carbohydrate fermentation tests with glucose, lactose, sucrose, xylose, mannitol, and maltose. The organisms were also tested for indole production, methyl red test, Voges-proskauer test, citrate utilization, urease test, oxidase test, catalase test, nitrate reduction test, triple sugar iron agar test, etc.

Antibiotic susceptibility testing

Disc diffusion method: Antibiotic susceptibility was checked by Kirby-Bauer's disc diffusion method. According to the Clinical and Laboratory Standard Institute (CLSI) guidelines, the process was carried out. Using a sterile pair of forceps, selected antibiotic discs were placed aseptically on the surface of the inoculated media after 5 min. The MHA plates were incubated for 16–18 h at 37°C. The antibiotic discs used in this study were Piperacillin (100 μg), Amikacin (30 μg), Gentamicin (10 μg), Ceftazidime (30 μg), Ciprofloxacin (5 μg), Imipenem (10 μg), Ampicillin/Sulbactum (10/10 μg), Colistin E strips, Ampicillin (10 μg), Amoxyclav (20/10 μg), Cotrimoxazole (25 μg), Cefepime (30 μg), Cefuroxime (30 μg), Ceftazidime/Clavulanic acid (30/10 μg), Nitrofurantoin (300 μg), and Tetracycline (30 μg). The inhibition zone was measured according to the CLSI guidelines (CLSI Catalog, 2016) (Collee, 2006) [16].

ESBL confirmation

Bacterial suspensions have been calibrated to 0.5 McFarland turbidity and distributed uniformly on MHA, two plates per strain, in all directions. Ceftazidime (30 μg) and ceftazidime/clavulanic acid (30/10 μg) disks (Himedia Pvt. Ltd) were mounted on the petri dish and then incubated at 37°C for 16–18 h. An increase in the zone diameter of 5 mm for either antimicrobial agent measured in combination with clavulanic acid was reported as ESBL-positive strains according to the CLSI criteria when tested alone [17].

Identification of MBL producers by ethylenediaminetetraacetic acid (EDTA) double synergy test

An overnight culture (0.5 McFarland opacity standards) of the test isolate was inoculated on an MH agar. A 10 μg imipenem disc and a sterile blank disc (Hi-media) were positioned 10 mm apart from edge to edge after drying. Another disc with imipenem was placed far as control. A 0.5 M EDTA (Himedia) solution volume of 10 μl was added to the blank disc. The zone of inhibition around the imipenem disc expands toward the EDTA disc after overnight incubation compared to the other imipenem disc, placed on the far side, and was interpreted as a positive result [17].

Identification of carbapenemase producing organisms by modified Hodge test

The production of carbapenemase was established if the microbial isolate tested was capable of producing an enzyme and allowed the growth of the standard *E. coli* ATCC 25922 strain into the carbapenem disc. Based on the observation of clover leaf-like indentation, the findings were noted [1].

Detection of colistin resistant organisms by E-test method

A suspension of each test bacterial isolate was initially prepared and adjusted to 0.5 McFarland standards in the Mueller–Hinton broth. The suspensions on MHA plates have now been swabbed. When the agar surface was fully dry, a colistin E-strip was applied to each plate (concentration varying from 0.06–1.024 μg/ml and incubated for 16–20 h at 35°C. The results were recorded as MIC where growth inhibition intersected the E-strip. As the breakpoint for select as resistant isolates, a colistin concentration of about 4 μg/ml was used.

Statistical analysis

Data were statistically analyzed using SPPS software version 24.0. Frequency and percentages were calculated for categorical and ordinal variables. Chi-square test was carried out and $p \leq 0.05$ was considered statistically significant.

RESULTS

The study involved 129 clinical samples that were obtained from 70 males and 59 females. A maximum number of cases were recorded in the age group 51–60 (33%) followed by 41–50 (16%) (Table 1). A maximum number of samples were urine 51 (39%), pus 28 (21%), sputum 22 (17%), blood 15 (11%), and other samples 12% constitute six throat swabs, four corneal swabs, two CSF, and oneascitic fluid. The most of the microorganisms isolated were *E. coli* 49 (37%), *Klebsiella* spp. 37 (28%), *P. aeruginosa* 30 (23%), and 12% comprises four isolates of Proteus species, seven isolates of Citrobacter species, and two isolates of Providencia species. Table 2 showed that out of 49 isolates 15 *E. coli* strains were sensitive to amikacin and imipenem and majority are MDR isolates (Fig. 1). Out of four isolates of Proteus species, only one isolate showed sensitivity to imipenem and meropenem. Providencia species showed maximum sensitivity to amikacin. The isolated *Klebsiella* species and Citrobacter species and non-fermentative Gram-negative bacilli like *P. aeruginosa* (Table 3) also showed high resistance to maximum tested antibiotics except meropenem and imipenem. The 53 test isolates which showed resistance to imipenem by Kirby-Bauer disc diffusion method were now tested for the MBL production by EDTA combined disc method. The results showed that out of 53 bacterial isolates, 50 isolates showed MBL production (94%) and 3 non-MBL producers (6%). Maximum MBL production was seen in *E. coli*, *Klebsiella* species and *P. aeruginosa*. All these 53 isolates were again screened for carbapenemase production through modified Hodge test. It was found that 50 strains were positive for carbapenemase producers (94%) and three strains were negative for carbapenemase production (6%). The majority of the strains were *E. coli* and *P. aeruginosa* followed by *Klebsiella* species showed carbapenemase production. Among these 50 strains which were positive for carbapenemase production, 47 strains were sensitive to colistin which was detected by "E" strip method and three strains showed resistance to colistin (Fig. 2).

DISCUSSION

The rise of GNB in hospital acquired infections (HAI) in India over the past decade, with increasingly growing patterns. In the event of a failure to control the pathogen within the first 24–48 h, the risk of increased mortality among patients with HAI could be resistant to certain widely

Table 1: Distribution of clinical cases according to their age groups

Age groups	Frequency
<10	3
11–20	4
21–30	16
31–40	15
41–50	21
51–60	43
61–70	14
71–80	13
Total	129

Table 2: Antibiogram of Gram-negative bacterial isolates

Antibiotics	*E. coli* (n=49)		Proteus species (n=4)		Providencia species (n=2)		*Klebsiella* species (n=37)		Citrobacter species (n=7)	
	Sensitivity (Number and %)	Resistance (Number and %)	Sensitivity (Number and %)	Resistance (Number and %)	Sensitivity (Number and %)	Resistance (Number and %)	Sensitivity (Number and %)	Resistance (Number and %)	Sensitivity (Number and %)	Resistance (Number and %)
Ampicillin	0 (0)	49 (100)	0 (0)	4 (100)	0 (0)	2 (100)	2 (5.4)	35 (94.6)	1 (14.2)	6 (85.8)
Cefipime	3 (7)	46 (93)	0 (0)	4 (100)	0 (0)	2 (100)	8 (21.6)	29 (78.4)	2 (28.5)	5 (71.5)
Ceftriaxone	2 (5)	47 (95)	0 (0)	4 (100)	0 (0)	2 (100)	3 (8.1)	34 (91.9)	0 (0)	7 (100)
Cefuroxime	1 (3)	48 (97)	0 (0)	4 (100)	0 (0)	2 (100)	2 (5.4)	35 (94.6)	0 (0)	7 (100)
Ciprofloxacin	5 (11)	44 (89)	0 (0)	4 (100)	0 (0)	2 (100)	4 (10.9)	33 (89.1)	1 (14.2)	6 (85.8)
Cotrimoxazole	3 (7)	46 (93)	0 (0)	4 (100)	0 (0)	2 (100)	12 (32.4)	25 (67.6)	2 (28.5)	5 (71.5)
Amikacin	15 (31)	34 (69)	0 (0)	4 (100)	2 (100)	0 (0)	20 (54)	17 (46)	4 (57)	3 (43)
Imipenem	15 (31)	34 (69)	1 (25)	3 (75)	1 (50)	1 (50)	30 (81)	7 (19)	6 (85.7)	1 (14.3)
Gentamycin	12 (25)	37 (75)	0 (0)	4 (100)	1 (50)	1 (50)	14 (37.9)	23 (62.1)	5 (71.5)	2 (28.5)
Ceftazidime	0 (0)	49 (100)	0 (0)	4 (100)	0 (0)	2 (100)	5 (13.5)	32 (86.5)	0 (0)	7 (100)
Ceftazidime/ clavulanic acid	1 (3)	48 (97)	0 (0)	4 (100)	1 (50)	1 (50)	7 (19)	30 (81)	0 (0)	7 (100)
Ampicillin/ sulbactam	1 (3)	48 (97)	1 (25)	3 (75)	0 (0)	2 (100)	5 (13.5)	32 (86.5)	2 (28.55)	5 (71.5)
Tetracycline	3 (7)	46 (93)	0 (0)	4 (100)	0 (0)	2 (100)	8 (21.6)	29 (78.4)	1 (14.2)	6 (85.8)
Ertapenem	10 (21)	39 (79)	0 (0)	4 (100)	1 (50)	1 (50)	9 (24.3)	28 (75.7)	3 (42.8)	4 (57.2)
Chloramphenicol	4 (29)	35 (71)	0 (0)	4 (100)	0 (0)	2 (100)	7 (19)	30 (81)	1 (14.2)	6 (85.8)
Meropenem	13 (27)	36 (73)	1 (25)	3 (75)	1 (50)	1 (50)	32 (86.5)	5 (13.5)	7 (100)	0 (0)
p<0.01			<0.01 HS		<0.01 HS		<0.01 HS		<0.01 HS	

HS: Highly significant, *P* value: Probability value. *E. coli*: *Escherichia coli*

Table 3: Antibiogram of non-fermentative Gram-negative bacteria *Pseudomonas* species

Antibiotics (n=30)	Sensitivity (Number and %)	Resistance (Number and %)
Cefipime	6 (20)	24 (80)
Amikacin	14 (46)	16 (54)
Ciprofloxacin	4 (13)	26 (87)
Cotrimoxazole	2 (7)	28 (93)
Ceftazidime/clavulanic acid	7 (24)	23 (76)
Gentamycin	10 (34)	20 (66)
Ceftazidime	2 (7)	28 (93)
Ampicillin/sulbactam	2 (7)	28 (93)
Imipenem	23 (76)	7 (24)
Piperacillin	5 (17)	25 (83)
p-value	<0.01 HS	

HS: Highly significant

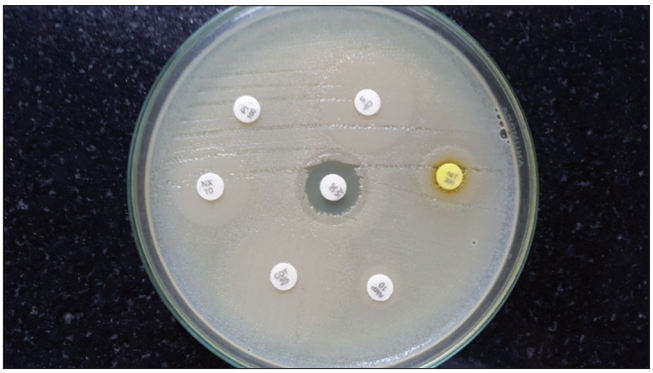

Fig. 1: Antibiotic susceptibility pattern of isolates by Kirby–Bauer method

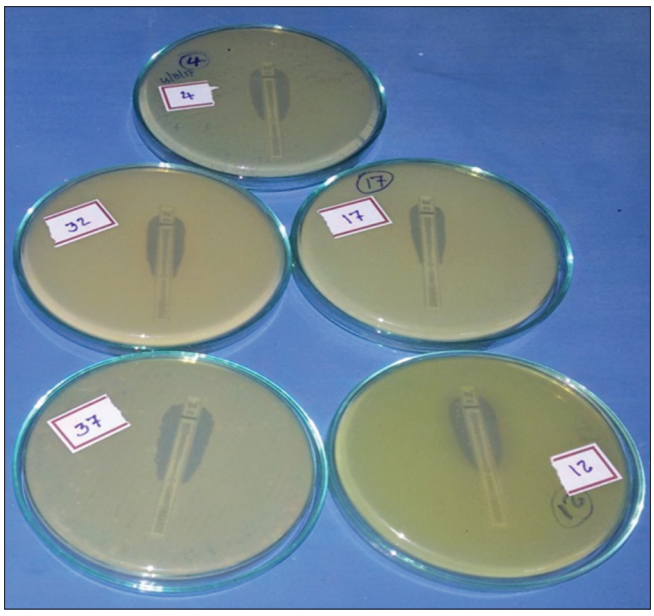

Fig. 2: Detection of colistin sensitivity by "E" strip method

used groups of antibiotics by encoding and expressing some drug resistant genes and therefore being multidrug resistant and restricting the options for the treatment. The results of the present study were in accordance to Shindo *et al*. [18] isolated many MDR isolates in their study and concluded that main risk factors for MDR pathogens were identified: Prior hospitalization, immunosuppression, previous antibiotic use, use of gastric acid-suppressive agents, tube feeding, and non-ambulatory status.

In general, the last line of defense against many pathogenic species immune to other antimicrobial agents is carbapenems. However, there is a rise in the worldwide development of resistant microbial strains of beta-lactamase enzymes, thereby hydrolyzing all β-lactam antibiotics along with carbapenems. However, colistin is one of the older medications, but now has become a common alternative for clinicians facing few choices in the treatment of Gram-negative MDR bacteria. The growing global trend in carbapenemase resistance is a major concern as it reduces the variety of therapeutic options that require clinicians to use costly agents such as colistin that are associated with severe toxicity. In the Tertiary Care Hospital, reports of infections caused by MDR non-fermentative Gram-negative bacteria and Enterobacteriaceae are increasingly reported. For the identification of MDR isolates, different

phenotypic methods and molecular methods were used. Most studies documented dismal outcomes among patients with enterobacteria immune to carbapenem ranging from 40 to 70%, which are in near agreement with the present research. These bad results are due to patient comorbidities, disease severity, and treatment with inadequate empiric antibiotics before the laboratory detects carbapenem resistance. According to the Galani *et al.* [19], MHT is 98% sensitive method keeping polymerase chain reaction as the gold standard assay method. The test isolates of the present study showed high sensitivity to antibiotics like colistin (100%) similar to the reports of Hirsch *et al.* [20], amikacin (54.2%) followed by gentamycin (38.9%) coincides that the findings of Anupurba *et al.* [21] reported least maximum sensitivity to amikacin and gentamycin 45.45%. The majority of the studies had report dismal outcomes among patients with carbapenem resistant enterobacteria ranging from 40% to 70% [22] which is in close agreement to the present study. These poor outcomes are due to patient comorbidities, severity of illness, and treatment with ineffective empiric antibiotics before carbapenem resistance is identified by the laboratory.

CONCLUSION

The present research isolated very high isolates from different clinical samples of drug resistant species. The early identification of such drug-resistant microorganisms can aid at the beginning of prompt antimicrobial therapy and thus avoid the production and dissemination in the hospital as well as in the community of these multidrug resistance strains. The study helps clinicians select the right antimicrobial agent that not only leads to better treatment, but also helps to avoid the development of drug resistant strains that are still susceptible.

AUTHORS' CONTRIBUTION

The main author of the study PUR had performed the work, wrote the first draft of the manuscript, collected the literature, and performed the statistical part of the work.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

SOURCE OF FUNDING

The study was not supported by any grants and funds.

ETHICS CLEARANCE

A proposal regarding the study's aims and objectives was submitted to the Institutional Ethics Committee and Dr. Patnam Mahender Reddy Institute of Medical Sciences (PMRIMS) and permission was obtained from the Institutional Ethics Committee regarding data collection from the university students.

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