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ORIGINAL RESEARCH

Multidrug resistant Acinetobacter species – Emergence of a potent pathogen from clinical isolates of tertiary care teaching centre of Central India

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ABSTRACT

Background: Multidrug resistant (MDR) *Acinetobacter species* reports have sharply increased from India. The study aimed to characterize all MDR *Acinetobacter species* and to evaluate the pattern of antibiotic susceptibility from the various clinical samples. **Method**: This prospective prevalence study conducted from January 2021 to June 2022 in the Department of Microbiologyat our tertiary care teaching institute of central India isolated MDR *Acinetobacter species*from various clinical samples using a standard set ofbiochemical tests and antimicrobialsusceptibility testing performed as per Clinical Laboratory Standards Institute (CLSI) guidelines, (M100; 32th Edition). All colistin resistant isolates(by broth microdilution) were screened for plasmid mediated , mobile colistin resistant, mcr-1, mcr-2 and mcr-3 gene. **Results:** A total 658 *Acinetobacter species* could be cultivated from 21,019-culture positive clinical specimens accounting for an isolationrate of 3.13% .Of these, 292 (44.3%) isolates wereMDR*Acinetobacter*. Out of 292 MDR *Acinetobacter species*, the most common were *Acinetobacter baumannii* complex (59.58%), followed by *Acinetobacter lwoffii*(28.76%) and *Acinetobacter hemolyticus*(11.64%). Majority of MDR *Acinetobacter baumannii complex* were from specimen blood (69.47%), followed by pus (56.52%). Around 4% of MDR *Acinetobacter species* were resistant to colistin. All 6 colistin resistant strains were found to be negative for plasmid mediated mcr-1,2 and 3 genes. **Conclusion:** Our study showed a high prevalence (44.3%) of MDR*Acinetobacter species*. The emergence of multidrug resistant *Acinetobacter species* has become a global public health concern, and ultimately one health concern with a potential to cause serious nosocomial infections. MDR *Acinetobacter species* were 54.5% and 38.2% resistant to carbapenems and aminoglycosides respectively. Early detection is necessary for optimizing antibiotic use and implementation of strict infection control practices to narrow down the development of antimicrobial resistance.

Keywords: antimicrobial resistance, multidrug-resistant Acinetobacter, mcr gene, colistin resistance, broth microdilution

INTRODUCTION

Antimicrobial resistance is a major public health concern for India where the burden of infectious disease is high and consumption of antibiotic is huge and un-regulated[1]. The ever escalating antimicrobial resistance (AMR) has taken the form of a global crisis[2]. AMR caused 1.27 million deaths worldwide in the year 2019[3]. If suitable measures are not immediately implemented to stop the spread of AMR, human fatalities could reach 10 million annually by 2050 [4]. India has been dubbed the global AMR centre[5] and here the causes of antibiotic resistance are varied and multifactorial [6].

ESKAPE bacterial pathogens, i.e. *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacteriaceae* are the most significant bacterial pathogens to contribute to AMR[7] . Of these bacterial pathogens, *A. baumannii* is one of the most challenging because of its unique antibiotic resistance characteristics.

Acinetobacter species has emerged as a prominent cause of nosocomial infections across the globe [8]. The genus *Acinetobacter* consists of strictly aerobic,

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Gram-negative, coccobacilli that are oxidase negative and non-motile. Originally, the genus was placed under the family Neisseriaceae, but now it has been moved to family Moraxellaceae. Currently, at least 41 species are described within the genus *Acinetobacter* [9].

The significant species belonging to this genus are *A. baumannii*, *A. lwoffii*, *A. calcoaceticus*, *A. haemolyticus* and *A. junii*[9,10]. Numerous illnesses, including ventilator-associated pneumonia, wound infections, urinary tract infections, bloodstream infections, and surgical site infections, can be caused by *A. baumannii*. Multidrug-resistant *A. baumannii* is well known to cause life-threatening infections with limited therapeutic options. It has developed resistance to the existing drugs to treat these infections viz, the carbapenem,colistin, polymyxin B, and tigecycline[7].Studies from India have reported a very high resistance rate (40 -75%) among *A.baumannii* for the broadest spectrum beta lactam i.eCarbapenem[11,12].The mechanism of Carbapenem resistance among *A.baumannii* strains include ArmA RNA 16S ribosomal methyltransferase and overexpression of the carbapenem-hydrolyzing oxacillinase (OXA)-51-like-B. *Acinetobacter baumannii* typically develops resistance to most other antibiotics once it demonstrates carbapenem resistance, leaving fewer treatment options [13].

The bacterial outer lipid bilayer permeabilizing agent, colistin is the last resort treatment when other antibiotic options are no longer available for *Acinetobacter baumannii*[14]. This emphasizes the necessity for maintaining colistin as a therapeutic option for resistant Gram- negative bacilli. According to the One Health AMR effort, environmental control is required for this, such as reducing animal diets containing colistin and other antibiotics[15]. Hence, this study was vital in generating regional susceptibility data for ever changing and difficult-to-treat MDR *Acinetobacter species*circulating in central India.

MATERIALS AND METHODS

This prospective study was conducted at the department of microbiology of a tertiary care teaching hospital in central India for a period of 18 months. Institutional ethical committee approval was obtained before the start of the study (letter no.27088/MC/IEC/2021). The various samples taken from patients suspected with bacterial infections were processed for the isolation and characterization of MDR *Acinetobacter* species. All the samples received in the bacteriology laboratory were inoculated on blood agar, chocolate agar, MacConkey agar, and incubated at 37°C in ambient air before being reported as sterile. All the non fermenters isolated were identified using conventional biochemical reactions as per the scheme given by Soni et al[6]. Identification of *Acinetobacter species* was done by Gram's staining, colony morphology, and various conventional biochemical tests[9,10]. (Table 1).

Table 1:-Phenotypic Characterization of *Acinetobacter species*[10]

Test	Acinetobacter baumannii complex	Acinetobacter lwoffii	Acinetobacter hemolyticus	Acinetobacter junii	Acinetobacter Radioresistens
Citrate	+	_	+	+	_
OF glucose	+	_	V	_	_
Hemolysis	_	_	+	_	_
Gelatin hydrolysis	_	_	+	_	_
Growth at 42ºC	+	_	_	_	_
Chloramphenicol Sensitivity	R	S	R	R	R
Arginine hydrolysis	+	_	+	+	+
Malonate	+	_	_	_	+

The antimicrobial susceptibility was performed by Kirby-Bauer disk diffusion method, and colistin minimal inhibitory concentration (MIC) was performed by the broth microdilution method in round bottom polystyrene microtiter plates. Analysis of antibiotic susceptibility testing was done as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100; 32th Edition) [16]. The panel of antibiotics tested included amikacin (30μg), gentamicin (10μg), ceftazidime (30μg), ciprofloxacin (5μg), imipenem (10μg), meropenem (10μg), minocycline (30μg), and piperacillin-tazobactam (100/10μg). For disc diffusion testing the controls used were: *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922. .

In-house *Pseudomonas aeruginosa* (MIC >16 μg/mL) was used as positive control and *Pseudomonas aeruginosa* ATCC 27853 (MIC: 0.5-4 μg/mL) was used as negative control for colistin broth microdilution testing. Multidrug resistance was defined as acquired resistance to at least one agent in three or more antimicrobial categories [17]. Descriptive analysis of all the observation is perforemed.

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Among colistin resistant MDR *Acinetobacter*, the most prevalent plasmid mediated colistin resistance genes mcr-1,mcr-2, and mcr-3 were detected by Polymerase chain reaction (PCR). The alkaline lysis technique was used to extract the DNA. A few colonies were suspended in 100 µL of 25-mM NaOH and kept in a dry bath at 100°C for 10 minutes . Then the suspension was immediately kept in ice bath for 10 minutes. Next, the pH of the suspension was adjusted to 8 by adding 8 µL of 1M tris chloride. Following centrifugation, the DNA template for the PCR was taken from the supernatant. A DNA quality and concentration assay was done using the NanoDrop ND 1000 spectrophotometer(NanoDrop Technologies Inc., Wilmington, Delaware, USA). The primersequences used were taken from the study by Al-Kadmy et al[18].As a positive control, DNA from an *Escherichia coli* strain known to be mcr-1 positive was employed. The ProFlex PCR System (Applied Biosystems, San Francisco, CA, USA) was used for the amplification.Electrophoresis of PCR products was performed using horizontal agarose gels (1-2%) at room temperature.Gels with red stains were processed for around 30 minutes at 100V in a gel electrophoresis tank with 1X TAE buffer. The Gel Doc system was used to visualize the DNA bands following electrophoresis. By comparing the relative mobility of the PCR products to that of 100-bp molecular markers, the PCR product sizes were calculated.

RESULTS

A total 658 *Acinetobacter species* were cultivated from 21,019-culture positive clinical specimens accounting for an isolationrate of 3.13% .Of these, 292 (44.3%) isolates were MDR*Acinetobacter spp*.

Most of the MDR *Acinetobacterspecies*isolated were from Intensive care unit(ICU) patients (62%)followed bymedical andsurgical wards (23%), and thenOut Patient Department (OPD)patients (15%).Maximum number of isolates were from age group 46-65 years (49%), and the least were isolated from neonates (7%). *Acinetobacter baumannii* complex (59.58%) was the most common species isolated, followed by *Acinetobacter lwoffii* (28.76%) and *Acinetobacter hemolyticus* (11.64%).

Blood was the commonest specimen to grow*Acinetobacter baumannii* complex (69.47%), where as *Acinetobacter lwoffii* and *Acinetobacterhemolyticus;*(27.1%) and (16.3%) respectively were commonly isolated from pus specimen. (Table2).

Table 2: Distribution of MDR *Acinetobacter species*across all clinical samples.

S No	MDR Isolates	Type of sample					Total
		Urine	Pus	Blood	Respiratory Sample	Sterile fluids	
		N (%)					
1	*Acinetobacter hemolyticus*	5 (13.88%)	15 (16.30%)	7 (7.36%)	3 (11.53%)	4 (9.30%)	34 (11.64%)
2	*Acinetobacter baumannii*	20 (55.55%)	52 (56.52%)	66 (69.47%)	9 (34.61%)	27 (62.79%)	174 (59.58%)
3	*Acinetobacter lwoffii*	11 (30.55%)	25 (27.17%)	22 (23.15%)	14 (53.84%)	12 (27.90%)	84 (28.76%)
	Total	36(100)	92(100)	95(100)	26(100)	43(100)	292(100)

Table 3: Antibiotic susceptibility profile for *Acinetobacter species*by Kirby Bauer disc diffusion method(as per CLSIM100,32Edition)[16].

S No	*Antimicrobial agents(mcg)*	*Acinetobacter hemolyticus (%susceptibility)*	*Acinetobacter baumannii (%susceptibility)*	*Acinetobacter lwoffii (%susceptibility)*
1	Amikacin(30)	6 (17.6)	50 (28.7)	13 (15.5)
2	Ceftazidime(30)	1 (2.9)	12 (6.9)	4 (4.8)
3	Ciprofloxacin(5)	4 (11.8)	33 (19.0)	18 (21.4)
4	Gentamicin(10)	7 (20.6)	55 (31.6)	20 (23.8)
5	Imipenem(10)	5 (14.7)	36 (20.7)	9 (10.7)
6	Meropenem(10)	3 (8.8)	40 (23.0)	10 (11.9)
7	Piperacillin-Tazobactam(100/10)	5 (14.7)	36 (20.7)	17 (20.2)
8	Minocycline(30)	22 (64.7)	112 (64.4)	51 (60.7)

64.4% of *A. baumannii complex* were susceptible tominocycline (64.4%) but poorly susceptible to ceftazidime (6.9%), *A. lwoffii and A.hemolyticus*also showedleast susceptibility toceftazidime. [Table 3].

Among the 292 MDR *Acinetobacter spp*., 4% were resistant to colistin (MIC ≥ 4 by brothmicrodilution). In these colistin resistant MDR *Acinetobacter species* the plasmid mediated colistin resistance genes mcr-

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1,2 and 3 were not detected. (Table 4).

Table 4: Colistin MIC (by broth microdilution method) of various MDR *Acinetobacter species*

Acinetobacter species	**Total number of MDR isolates**	**Colistin MIC ≤ 2 µg/mL (Intermediate)**	**Colistin MIC ≥4 µg/mL (Resistant)**	**Presence of Mcr-1,2,3 in strains with Colistin MIC≥4 µg/mL**
Acinetobacter hemolyticus	34	32 (94.1%)	2 (5.8%)	Negative
Acinetobacter baumannii	174	170 (97.7%)	4(2.35%)	Negative
Acinetobacter lwoffii	84	84 (100%)	-	-

DISCUSSION

Due to the rampant use of antibiotics, many organisms now exhibit higher levels of multidrug resistance.*Acinetobacter species* are currently becoming significant nosocomial pathogens that cause opportunistic infections and hospital-acquired illnesses.

A total 658 *Acinetobacter species* -were isolated from 21,019-culture positive clinical specimen received during the study duration; accounting for an isolationrate of 3.13%, which were similar to the observations by Gupta et al (3.36%)[19]. Out of these, 292(44.3%) were MDR.

The analysis of study population showed patients most common affected weremales between in the age group of 46-65 years (49%).In the study by Gupta et al, majority of *Acinetobacter* isolates were from age group >50 years[19]. Itmay be due to a weakened immune system and associated chronic diseases in these age groups.

In this study, most of the MDR *Acinetobacter baumannii* were isolated from blood samples (69.47%), followed byvarious body fluids(62.79%) viz. pleural fluid and cerebrospinal fluid ,pus (56.52%), urine (55.55%), and respiratory samples (34.61%). This is similar to a study conducted by Gupta et al, wherein MDR *Acinetobacter baumannii* was most commonly cultured from blood samples(36.9%) [19]. If *Acinetobacter spp.* is isolated from respiratory or wound infections, it is important to know whether the infection is a colonizer in patients who are ill for reasons related to their underlying condition (such as patients who need mechanical ventilation or who have extensive burns), or if it is a genuine pathogen causing increased mortality[3].

MDR *Acinetobacter species* in this study were predominantly from Intensive care unit (62%), followed by medical and surgical wards (23%) and then Out patient department (15%), similar to the observations of Ashuthosh et al, where, isolation rate was high (76%) among patients admitted to intensive care units[20].

Susceptibility to minocycline was 64.4% but was poor to ceftazidime (6.9%). The resistance to reserve drug colistin is 5.9% among MDR *Acinetobacter baumannii*.In the study by Yadav et al MDR *Acinetobacter baumannii* were 100% susceptible to colistin[21] . Also in a study by Sengupta et al on lowerrespiratory tract infections, all MDR *Acinetobacter* isolates were 100%susceptible to colistin [22]. (Table5).

Table 5: Percentage antibiotic susceptibility of MDR *Acinetobacter baumannii* across various studies from India

Antibiotics(mcg)	**Yadav et al (20)**	**Raut et al(7)**	**Gupta et al (18)**	**Sengupta et al (21)**	**Our Study**
Amikacin(30)	-	60.5	36	0	28.7
Ceftazidime(30)	6.2	72.7	43	0	6.9
Ciprofloxacin(5)	11.3	10.5	69	0	19.0
Gentamicin(10)	14.1	14	-	0	31.6
Imipenem(10)	20.3	58.7	64	0	20.7
Meropenem(10)	18.1	61.9	-	0	23.0
Piperacillin-Tazobactam(100/10)	12.4	13.3	29	0	20.7
Minocycline(30)	-	-	-	-	64.4
Colistin MIC(by BMD).	100	100	-	100	94

The widespread use of third-generation cephalosporins in hospital settings may be to blame for the high level of resistance in *Acinetobacter baumannii* (85.4%), whilst the sparing use of colistin as last resort of drug (also due to associated nephrotoxicity) is responsible for the higher level of susceptibility.

MDR *Acinetobacter lwoffii* and MDR *Acinetobacter hemolyticus* were poorly susceptible to ceftazidime. A study conducted in Saudi Arabia at intensive care

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unit by Al Bshabshe et al presented 50% resistance to ceftazidime among these two species [23].

Presence of carbapenemases: OXA24/40-like, OXA-23-like, serine carbapenemases and metallo- β-lactamases, are possible causes of carbapenem resistance in non fermenters (13). In our study carbapenem resistance was found to be 54.5%.

In our study, resistance to aminoglycoside and quinolones is found to be (38.2% and 47.8%) respectively. Aminoglycoside resistance is brought on by aminoglycoside modifying enzymes(AME) or the 16S rRNA methyltransferases. Cause of quinolone resistance is mutations in the chromosomally encoded overexpression of efflux pumps [22].

Polymyxins interacts with lipopolysaccharide's (LPS) lipid A, modification of which results in acquired polymyxin resistance. Addition of a phosphoethanolamine residue to the hepta-acylated form of lipid A, removes negative charges and reduces the affinity of LPS for polymyxins, this is the most common reported mechanism [12]. Another mechanism through which *A. baumannii* develops colistin resistance is by complete loss of the initial LPS [24]. According to Laishram S et al, the combination of meropenem plus colistin and rifampicin plus colistin showed promising results against *Acinetobacter baumannii*[25].

The *mcr-1*, *mcr-2*, *mcr-3* geneswere found to be negative on conventional PCR. Epidemiological analysis of Mcr gene in *Acinetobacter species* worldwide showed variable prevalence across the globe (China, Korea, Brazil, Egypt, Iraq, Pakistan)[14]. Further evaluation of our strains is needed to determine if the cause of colistin resistance could be other genes in the *mcr* family (*mcr-4* to *mcr-10)* or might be due to presence of other colistinresistance mechanism such as chromosomal mediated or modification of LPS.

Although CLSI (M100; 33rd Edition) since January 2023 guidelines discourages the use of colistin and colistin testing even as reserve drug, but looking at widespread use of colistin throughout our country for treating health care associated infections and also unregulated use of colistin to treat, prevent, and promote growth in animals raised for human consumption [15]; it is suggested that complete cessation of colistin resistance monitoringseems impractical as of now.

Limitation of the study

The study's shortcomings were that screening of all the genes responsible for resistant bacteria were outside its purview. The therapeutic response of the patients was also not taken into account in this investigation.Clinical efficacy studies that connect patient outcomes to the implementation ofdefinitive antimicrobial therapy would produce even more robust data for patient welfare. *Acinetobacter lwoffii* is recognised as commensal flora in skin, oropharynx and perineum. Also the clinical status of the patients, their antibiotic therapy and clinical and microbiological cure after treatment for these infections could not be correlated.

Therefore, it is difficult to say whether *A. lwoffii* is an emerging pathogen or normal flora in this study. Since this is a single-centre study, regional epidemiological factors had an impact on the results, which is reason it should be performed at multiple other sites throughout country.

CONCLUSION

The prevalence of carbapenem-resistant *A. baumannii* has increased significantly around the world, and this is becoming an issue of concern.The emergence of pathogenic MDR *Acinetobacter baumannii*(some even resistant to colistin) have made infections caused by these bacteria difficult to treat.

Colistin can be used in synergy with other potent antimicrobials in situations when there is multidrug resistance. Clinicians in everydemographic area must be aware of the most recent prevalence and antibiotic susceptibility pattern of the circulating pathogens.

Polymyxins more frequently is advocated to be as the final effective therapy of choice.Checking for plasmid mediated drug resistance mechanism will help in understanding the transmission of colistin resistance in *Acinetobacter baumannii complex.*

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Conflict of Interest
None declared.

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