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Study to Identify *Acinetobacter* Species from Various Clinical Samples and Antimicrobial Resistance Pattern to Demonstrate it with Special Emphasis on Molecular Characterization of Carbapenemase Producing Strains at a Tertiary Hospital

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ABSTRACT

Acinetobacter is an opportunistic bacterial pathogen primarily associated with Hospital-acquired infections. One of the most striking features of *Acinetobacter* species is their capacity to produce multiple drug resistant mechanisms against major antibiotic classes. Present study was conducted to study the prevalence of MBL among carbapenem resistant strains of *Acinetobacter* species in our hospital and to investigate the presence of OXA type beta-lactamases in eropenem-resistant *Acinetobacter* isolates using PCR. Material and present study was single-center, prospective, observational study, conducted in patients of all groups, who had prolonged hospitalization in the intensive care unit, medical wards, surgical wards, orthopaedic wards, paediatric wards and gynaecology wards. Out of the total 622 samples, 351 (56%) were found to be culture positive. Out of 103 isolates, 59 (57%) isolates were between the age group of 51-60 years. Among them 70 were males and 33 were females with the ratio of 2:1. A.baumannii 100 (97%) was the most common isolate among. The highest sensitivity was for colistin (100%) and lowest sensitivity for cefotaxime (15%). 45 (62%) isolates were confirmed as ESBL producers. Among the 100 isolates, 20 (100%) of them were positive for bla OXA-51 and bla OXA-23 genes. blaVIM coexisted with blaOXA-23 and blaOXA51 in 10 (50%) isolates and no isolate was positive for blaIMP. Simultaneous existence of different classes of carbapenemases is a major problem to encounter with and hence detection methods are required for each of these. In outbreaks, an initial screening of the carbapenemase producers will help to organize early therapeutic interventions.

INTRODUCTION

Acinetobacter is an opportunistic bacterial pathogen primarily associated with Hospital-acquired infections. Multi-drug resistant (MDR) *Acinetobacter* has increasingly become a formidable organism in nosocomial and community acquired infections. So much so, that in recent years it has been designated as a red alert human pathogen generating alarm among the medical personnel. Antibiotic resistant bacteria currently imply an impending disaster worldwide and therefore constitute a strong challenge when treating patients in hospital settings[1].

WHO has recently identified antimicrobial resistance as one of the important causes of life-threatening nosocomial infections among critically ill and immunocompromised individuals in treating human diseases. The most common and serious MDR pathogens have been encompassed within the acronym"ESKAPE" standing for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species[2].

Carbapenems have emerged as the agent of choice for managing *Acinetobacter* species, as they are resistant to aminoglycosides, fluoroquinolones, penicillins and third generation cephalosporins. But increased use of these antibiotics have in turn resulted in the emergence of carbapenem resistant strains[3]. Emergence of such antibiotic resistant strains has made the infection untreatable. Hence characterization of the species and identification of antibiotic resistant strains is necessary for treatment and management of infections.

One of the most striking features of *Acinetobacter* species is their capacity to produce multiple drug resistant mechanisms against major antibiotic classes. The aim of this study is to determine the prevalence of MBL among carbapenem resistant strains of *Acinetobacter* species in our hospital and to investigate the presence of OXA type beta-lactamases in Meropenem-resistant *Acinetobacter* isolates using PCR.

MATERIALS AND METHODS

Present study was single-center, prospective, observational study, conducted in department of Department of Microbiology, Coimbatore Medical College Hospital, Coimbatore, India. Study duration was of 1 year (August 2015 to July 2016). Study approval was obtained from institutional ethical committee.

Inclusion criteria:

- Patients of all groups, had prolonged hospitalization in the intensive care unit, on ventilator, indwelling catheters, medical/surgical/ orthopaedic implants and suspected sepsis/open injury following accidents/history of malignancy/ with long-term ulcer due to diabetes mellitus, willing to participate in present study

Exclusion criteria:

- Other species of *Acinetobacter* except *A. baumannii*
- Patients already on antibiotics and admitted less than 48 hrs

Study was conducted in the among patients with prolonged hospitalization in intensive care units, medical wards, surgical wards, orthopaedic wards, paediatric wards and gynaecology wards. In the present study 103 *Acinetobacter* species were isolated from different clinical samples like pus, urine, blood, wound swab, catheter tip, endotracheal aspirates and sputum. All samples were collected under strict aseptic precautions under standard microbiology procedures and processed. Two swabs were taken, one was for gram's stain and the other for culture.

- **Direct gram's stain :** For microscopic examination of pus cells and bacteria
- All the samples except blood, were inoculated into the Nutrient agar, MacConkey agar and blood agar and incubated for 18-24 hrs at 37°C
- **Blood and catheter tip culture:** BHI broth was used in which the samples were inoculated and incubated at 37°C for 48 hrs. The broth was examined for turbidity regularly and subculture was done on blood agar plate as well as MacConkey Agar. Any growth was further processed for identification
- Endotracheal aspirates with >10 epithelial cells per low power field were accepted for processing. Cultures with colony counts $>10^4$ CFU mL^{-1} were accepted for identification. For BAL specimen cultures with colony count of $>10^5$ CFU mL^{-1} were accepted
- Non-lactose fermenting colonies on macConkey agar which were oxidase negative was processed for identification

Biochemical reactions: The non-lactose fermenting colonies were subjected to the following set of tests at first:

- Gram stain
- Motility
- Catalase test
- Oxidase test

If the organism is a gram-negative coccobacilli, non-motile, catalase positive and Oxidase negative, then it was subjected to the following biochemical reactions (Table 1).

Antibiotic susceptibility testing was done by modified Kirby-Bauer disc diffusion technique using mueller hinton agar. A single colony from the plate was

Table 1: Biochemical tests

Biochemical tests	Results
Indole test	Negative
Simmon's citrate test	Positive
Christensen's urease test	Negative
Triple sugar iron agar	alkaline slant/ no change
Arginine dihydrolase test	Positive
Nitrate reduction test	Negative
Oxidation fermentation test (hugh and leifson's)	Oxidative utilization of 10% glucose

taken with a wire loop, and inoculated into the peptone water and incubated and the result matched with mc farland turbidity standard. The antibiotics were selected based on CLSI guidelines. Antibiotics like amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), ampicillin-sulbactam (10 µg), meropenem (10 µg) and piperacillin-tazobactam (100 µg) were used.

Since majority of *Acinetobacter* isolates were of *A. baumannii* species which are well known for their antibiotic resistance (MDR/XDR/PDR) the study to detect resistance pattern and genes was confined to these 100 isolates of *A. baumannii*. Conventional PCR was done to confirm the presence of marker genes OXA-51 OXA-23, bla IMP and bla VIM which code for drug resistance.

Data was collected and compiled using Microsoft Excel, analysed using SPSS 23.0 version. Frequency, percentage, means and standard deviations (SD) was calculated for the continuous variables, while ratios and proportions were calculated for the categorical variables. Difference of proportions between qualitative variables were tested using chi- square test or fisher exact test as applicable. p-value less than 0.5 was considered as statistically significant.

RESULTS

The *Acinetobacter* species isolated from various clinical samples like endotracheal aspirates, sputum, BAL blood and pus were included in this study and the antibiotic resistance pattern of these isolates were analyzed. Extended spectrum beta-lactamases (ESBL) and Carbapenemase (OXA-BL+MBL) production were detected by phenotypic methods. Molecular identification by conventional PCR to identify the genes coding for resistance mechanism was also done. Out of the total 622 samples, 351 (56%) were found to be culture positive. Among the culture positive isolates, the organisms identified were *Pseudomonas aeruginosa* (36%), *Acinetobacter* species (29%), *Escherichia coli* (16%), *Klebsiella pneumoniae* (12%), proteus vulgaris (3%), *Staphylococcus aureus* (2%), CoNS (2%) (Table 2).

Out of 103 isolates, 59(57%) isolates were between the age group of 51-60 years. Among them 70 were males and 33 were females with the ratio of 2:1 (Table 3).

For all risk factors for acquiring *Acinetobacter* infection. Age, gender, duration of hospital stay for more than 7 days, presence of invasive procedures like mechanical ventilation, urinary and IV catheterization and presence of underlying comorbid illnesses like diabetes, ischemic heart disease, hypertension, patients on chemotherapy, smoking all prove to be important and independent risk factors (Table 4).

Majority of the specimens were from patients on ventilators especially those with an endotracheal tube in situ (endotracheal aspirate) (36%) followed by sputum (33%), pus (22%), blood (6%) and BAL (3%). The p-value was statistically significant (0.001) (Table 5).

A. baumannii 100 (97%) was the most common isolate among *Acinetobacter* species. The only other identified species was *A. lwoffii* 3 (3%) (Table 6).

Table 2: distribution of various isolates (n = 103)

Name of the organism	No.	Percentage
Pseudomonas aeruginosa	124	36
Acinetobacter species	103	29
Escherichia coli	56	16
Klebsiella pneumonia	43	12
Proteus vulgaris	11	3
Staphylococcus aureus	7	2
CoNS	7	2
Total	351	100

Table 3: Age and sex-wise distribution of *Acinetobacter* species (n = 103)

Age	Male		Female		Total	
(years)	No.	%	No.	%	No.	%
1-10	1	1	1	3	2	2
11-20	4	6	2	7	6	6
21-30	6	9	5	15	11	10
31- 40	8	11	4	12	12	12
41- 50	10	14	3	9	13	13
>50 years	41	59	18	54	59	57
Total	70	100	33	100	103	100

Table 4: risk factor analysis (n = 103)

Risk factors	Variables	No.	Relative risk	p-value
Age	>50 years	57	6.01	0.001
	<50 years	46		
Gender	Male	70	2.67	0.001
	Female	33		
Duration of hospital stay	<7 days	46	6.95	0.001
	>7 days	57		
Invasive procedures	Present	59	4.9	0.001
	Absent	44		
Co-morbid illness	Present	55	4.4	0.001
	Absent	48		

Table 5: Distribution of *Acinetobacter* species among various clinical specimens

Specimen	Male		Female		Grand total	
	No.	%	No.	%	No.	%
Endotracheal aspirate	28	40	9	27	37	36
Sputum	22	31	12	37	34	33
Pus	14	20	9	27	23	22
Blood	4	6	2	6	6	6
BAL	2	3	1	3	3	3

Table 6: species distribution of *Acinetobacter* isolates (n = 103)

Species	No.	Percentage
A. baumannii	100	97
A. lwoffii	3	3

Table 7: Antimicrobial sensitivity against *A. baumannii* isolates

	Acinetobacter baumannii n = 100			
	Sensitive		Resistant	
Antibiotic disc	No.	Percentage	No.	Percentage
Gentamicin G	29	29	71	71
Amikacin AK	33	33	67	67
Netilmicin NET	74	74	26	26
Ciprofloxacin CIP	36	36	64	64
Levofloxacin LVX	80	80	20	20
Ceftazidime CAZ	28	28	72	72
Cefotaxime CTX	15	15	85	85
Cefepime CPM	27	27	73	73
Ampicillin-sulbactum A/S	68	68	32	32
Cefaperazone-sulbactum CFS	78	78	22	22
Piperacillin-tazobactam PIT	39	39	61	61
Meropenem MRP	80	80	20	20
Tigecycline TGL	96	96	49	4
Colistin COL	100	100	-	-

Table 8: Detection of ESBL production in a *Acinetobacter* isolated by DDST

Total no. of resistant Isolates	ESBL producer	NON ESBL
73	45 (62%)	28 (38%)

Table 9: Comparison of oxa-carbapenamase detection by phenotypic and molecular methods

Total carbapenem resistant isolate	MHT (%)	OXA-51 and OXA-23 (%)
20	15 (75%)	20 (100)

Table 10: Comparison of MBL detection by phenotypic and molecular methods

carbapenem	DDST (%)	VIM (%)	IMP
20	19(95)	10(50)	-

Table 11: Mortality in patients infected with carbapenem resistant/susceptible strains

Carbapenem AST	No.	Total
Resistant (n = 80)	20	6
Susceptible (n = 20)	80	3

The highest sensitivity was for Colistin (100%) and lowest sensitivity for cefotaxime (15%). The sensitivity to all other antibiotics were as follows: Tigecycline (96%), meropenem (80%), levofloxacin (80%), cefoperazone sulbactum (78%), netilmicin (74%), ampicillin-sulbactum (68%), piperacillin-tazobactam (39%), ciprofloxacin (36%), amikacin (33%), gentamicin (29%), ceftazidime (28%) and cefepime (27%) (Table 7).

All the Cefotaxime resistant isolates of *Acinetobacter* were subjected to Double disc synergy test for ESBL production. Among this 45(62%) isolates were confirmed as ESBL producers (Table 8).

Out of 100 isolates, 20 were found to be carbapenem resistant. Modified hodge test was performed for detection of class D OXA carbapenemases.15 (75%) were positive for modified hodge test. Conventional PCR was done for the detection of the OXA-type carbapenemases found in *Acinetobacter baumannii*. Among the 100 isolates, 20 (100%) of them were positive for bla OXA-51 and bla OXA-23 genes. The blaOXA-51 gene cluster is unique in that, it is intrinsically present in all *Acinetobacter* species. The most frequent and most common type of carbapenemase identified among the carbapenem resistant *A. baumannii* is blaOXA-23 (Table 9).

Metallo-betalactamase was identified using double disc synergy test. Out of 20 carbapenem resistant isolates 19 (95%) were positive for double disc synergy test. Conventional PCR was done for the detection of VIM, IMP gene. In this study blaVIM coexisted with blaOXA-23 and blaOXA51 in 10 (50%) isolates and no isolate was positive for blaIMP (Table 10).

The mortality rate of patients is almost double when infected with carbapenem resistant strains when compared to the sensitive strains. The p-value (0.00181) is statistically significant (Table 11).

DISCUSSIONS

Acinetobacter is an emerging nosocomial pathogen and has been increasingly associated with a wide variety of illnesses in hospitalized patients, especially patients in intensive care units. *Acinetobacter* species are often resistant to a wide range of antimicrobial agents including carbapenems due to both intrinsic and acquired mechanisms as well as their complex epidemiology. The various mechanisms include ESBL, MBL production and Carbapenem resistance due to class B and D Carbapenemases[5].

In a study conducted by Preeti *et al*.[6] and Dimple *et al*.[7] prevalence rate of acinetobacter was 20-18.7%, respectively. Age predilection of more than 50 years (57%) in our study may be attributed to one of the following reasons. The patients in this age group had history of chronic obstructive pulmonary diseases, bronchial asthma and respiratory failure, Diabetes mellitus, end stage renal disease, congestive heart failure and coronary artery disease. One or a combination of these factors compromises the immune system of the patients, facilitating initial colonization and subsequent progression to severe infection[8]. These observations correlate with Gupta *et al*.[9] (28%) in whose studies the most common age group involved was 51-60 years. Similar

observation was made by Preeti *et al*.[6] males (68%) were affected predominantly as shown in our study because of habitual smoking that leads to COPD and other lung disorders. Factors such as occupational exposure, air pollution and genetic factors also play an important role in *Acinetobacter* infections in males. The male is to female ratio in our study is 2:1. All these factors indicate that the prevalence of infection was less among female patients (32%) than males. This is similar to various studies done by Dimple *et al*.[7], Benifla[8] and Gupta *et al*.[9] in whose study predominant isolates were from males.

Patients who stay for long period in hospital, acquire infection from other persons who are colonized with *Acinetobacter* species. Longer duration of stay in the intensive care unit further increases risk due to contamination caused by health care workers and from equipments such asventilators, catheters, IV lines. Other risk factors include un scheduled admissions, immunosuppression, use of broad-spectrum antimicrobials, sepsis in the ICU and invasive procedures like urinary and intravenous catheterizations and patients on ventilators due to respiratory failure.

In our study, most isolates of *Acinetobacter* were from endotracheal aspirates (36%) and mechanical ventilation was the most important risk factor for the infection. Patients with chronic lung disease are at increased risk of airway colonization and pneumonia, especially when they require intubation. *Acinetobacter* can colonize and infect the respiratory tract and this accounts for more isolates being from Endotracheal aspirates in our study[10,11].

A study by Gupta *et al*.[9] proved maximum resistance observed to piperacillin (55%) followed by ceftriaxone (46%) and ceftazidime (46%). In the present study out of 100 isolates of *A. baumannii* 45% were found to be ESBL producers as detected by DDST using one or more of cephalosporins. Therefore, cefepime and cefotaxime used together with aqmoxicillin-clavulanate in DDST, increased ESBL detection to 95%. On the other hand, ceftazidime and ceftriaxone with amoxiclav was found to have lower sensitivity in detecting ESBLs in acinetobacter species. High prevalence of ESBL producers in our study indicates the selective pressure due to extensive use of antibiotics. Taneja *et al*.[12] found 48% of ESBL producing isolates in their study. In contrast, Pandey *et al*.[13] and Kumar *et al*.[14] found 75% of ESBL producing and 21% of MBL producing isolates in their studies, respectively.

The gene OXA 23 were found in 100 % of the isolates (100%) and found to be co-existing with OXA 51. In a similar study Amudhan *et al*.[15] found OXA 23 in 89.09% of their isolates and it co-existed with OXA 51 in 83% of their isolates. Amudhan *et al*.[15] observed only 10% of isolates with OXA 24 and OXA In all the above studies, it is shown that OXA 51 and OXA 23 are the most prevalent genes in acinetobacter baumannii and hence detection methods are of utmost importance to prevent the spread of these organisms in the hospital. Among various MBL encoding genes thus far, blaIMP appears to be most clinically relevant due to its ability to spread among other major pathogens. In this study blaVIM gene was most frequently carried by meropenem-resistant isolates. This finding in our study suggests that there is lesser risk of spread of resistance to other isolates. In our study it was observed that mortality rate was doubled in meropenem resistant isolates. Similar observation was seen in various studies by Kim *et al*.[16] and Rimoldi *et al*.[17].

Early detection is critical, the benefits of which include timely execution of strict infection control practices, formulating an effective antibiotic policy to prevent the spread of these MBL producing strains, and treatment with alternative last-line antimicrobials, thereby arresting the spread of antibiotic resistant strains to improve the clinical outcome among patients harbouring these organisms. Continued careful attention to hand hygiene, contact isolation, barrier precautions, adequate environmental cleaning and careful disinfection of patient care equipments along with antibiotic policy and epidemiological surveillance are essential to prevent the outbreak of infections caused by these multi-drug resistant and extremely drug resistant strains.

CONCLUSION

Simultaneous existence of different classes of carbapenemases is a major problem to encounter with and hence detection methods are required for each of these. In outbreaks, an initial screening of the carbapenemase producers will help to organize early therapeutic interventions. Further awareness should be created regarding good housekeeping and equipment decontamination strict attention to hand washing should be undertaken to control the spread of acinetobacter in hospitals.

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