ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Phenotypic and Molecular detection of carbapenem resistant among A.baumannii isolates from hospitalized patient in Coimbatore

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ABSTRACT

The present study was conducted with following objectives to isolate A.baumannii from hospitalized patients and to check for antibiotic susceptibility testing of isolates and screening for multidrug resistance, then to find Phenotypic characterization of carbapenem resistance by Double Disk Synergy Test (DDST) and Modified Hodge Test (MHT) and to determine the genetic profile in the resistant isolates for the presence of bla_{OXA} - type carbapenemases.

Keywords: Acinetobacter, DDST, MHT, genetic profile

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ISSN: 0975-8585

INTRODUCTION

Hospital infections are caused by several microorganisms, being of great relevance the ones caused by bacteria. Some of these represent higher risk for the patient due to a reduced sensibility profile to antimicrobial agents, as observed in glucose non-fermenting Gram negative bacilli. In this group, Acinetobacter baumannii are largely isolated in hospitals worldwide, being associated to high morbidity and mortality rates in seriously ill patients [12]. Of these newer pathogens, Acinetobacter spp., principally Acinetobacter baumannii and a gram negative MRSA are typical opportunistic pathogen frequently involved in triggering the nosocomial outbreaks. It affects the critically ill patients especially in intensive care units, in whom it is associated with a significant increase in mortality [18]. They are ubiquitous, free living gram negative saprophytic bacilli commonly present in the clinical environment, where they can be isolated readily as commensals from the skin of hospital staff and patients. A. baumannii have become increasingly resistant to broad-spectrum cephalosporins used in the hospital setting leading to the use of more powerful β-lactam antibiotics, as the carbapenems [13]. Currently, these agents are important options to treat nosocomial infections due to their high affinity for type 2 (PBP2) penicillin-binding proteins with, stability to many β-lactamases, including broad-spectrum (ESBL) and chromosomal (AmpC) besides showing excellent permeability through bacterial outer membrane [20]. Wide use of carbapenems in the hospital environment can cause more selective pressure on hospital microbiota, thus enhancing the subpopulation of microorganism with decreased sensibility or resistance to these antibiotics. Currently, A. baumannii resistant to most antimicrobial agents and sensitive only to polymyxin B have been isolated in most of the Brazilian hospitals [11]. Resistance to carbapenems are thought to result from the production of metallo-beta-lactamases (MBL). Metallo-β-lactamases (MBLs) are metalloenzymes of Ambler class B and are clavulanic acid-resistant enzymes. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by ethylenediamine tetra-acetic acid (EDTA), as well as other chelating agents of divalent cations [19]. Presently, many reports including few from India are available regarding the prevalence of MBLs [17].

In India, despite many reports of carbapenem resistance in Acinetobacter spp., especially in the nosocomial settings where carbapenems were used as last resort for treatment; the epidemiology of these infections still remains unclear. There is no published report of CHDL genes from our country. As there has been a dramatic increase in carbapenem resistant A.baumannii during the last decade, the present study was undertaken to detect the genes conferring carbapenem resistance to A.baumannii in hospitalized patients.

MATERIALS AND METHODS

Study area

A total 35 isolates of A. baumannii were collected from the various samples like blood, endotracheal aspirate, sputum, urine, catheter tip and pus from patients confined to ICUs admitted in various hospitals in Coimbatore were included for the study.

ISSN: 0975-8585

Phenotypic Identification

Preliminary identification of A.baumannii isolates was done by the gram stain findings, motility testing using mannitol motility medium and the oxidase reaction. Gram negative, non fermenting bacilli that were non-motile and oxidase negative were identified as Acinetobacter spp. Tests for speciating the isolates included citrate test, urease test, gelatin hydrolysis, growth at 37°C and 44°C and glucose oxidation test by Hugh and Leifson's of glucose.

Antibiotic Sensitivity Test

Antimicrobial susceptibility of the isolates was performed using Mueller-Hinton susceptibility agar plates. These plates were swab inoculated with a suspension of each isolate, with the turbidity of 0.5 Mac Farland standard, as recommended for a standard Kirby-Bauer disc diffusion method. Antibiotics used were piperacillin-tazobactam(100/10µg), ceftazidime(30µg), Imipenem(10µg), Meropenem(10µg), ciprofloxacin(5µg) amikacin(30µg), gentamicin(10µg), colistrin(10µg), polymyxin B (300units). Following overnight incubation at 37°C, the diameter of the zone of inhibition around the disc is measured and the result were interpreted as per CLSI guidelines.

Double Disc Synergy Test

Sterile Muller-Hinton agar plates were swab inoculated with the suspension of the isolates adjusted to 0.5 Mac Farland standards. Using sterile forceps, the sterile 10% EDTA(10µg) disc is placed at the centre and the containing 10µg imipenem and 10µg meropenem were placed on either side of the EDTA disc at a distance of 10 mm apart from edge to edge. The plates were incubated at 37°C for about 24 hours. Following overnight incubation, the presence of even a small synergistic zone is interpreted as positive for MBL production. [9]

Modified Hodge Test

0.5 Mc farland dilution of E. coli 25922 is prepared using sterile MHB and inoculated on sterile Mueller-Hinton susceptibility agar plates. The plates were allowed to dry for a span of about 3 – 5 minutes. A sterile disc containing 10 µg imipenem is placed at the centre of the plate using sterile forceps. The test organism is streaked 3mm away from the edge of the disc to the periphery of the plate, in a straight line. Then, the plates were incubated at 37°C for about 24 hours. Following overnight incubation, carbapenemase activity of the isolates was determined based on clover leaf like indentation of E. coli 25922 growing along the test organism growth streak within the disk diffusion zone [9]

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Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) for meropenem was determined using MIC agar diffusion method and results were interpreted as per CLSI guidelines. (Refer Table 5.2)

PCR SCREENING

DNA template were prepared by emulsifying 5 colonies in 100 µL of PCR grade water and adding 1 µL to the PCR reaction mixture prior to thermal cycling. The cycling conditions were: initial DNA release and denaturation at 94^{o}C for 5 min, followed by 36 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 50 s, followed by a single, final, elongation step at 72°C for 5min. The PCR products obtained were analyzed by gel electrophoresis with 2% agarose. Gels were stained with ethidium bromide at 5 µg/ml and visualized by UV transillumination. A 100-bp DNA ladder from Invitrogen was used as a marker.

Table 1: Primers used in this study [21]

Gene	Primer sequence	Product size
bla_{OXA-23} like – F	GAT CGG ATT GGA GAA CCA GA	501bp
R	ATT TCT GAC CGC ATT TCC AT	
bla_{OXA-24} like – F	GGT TAG TTG GCC CCC TTA AA	246bp
R	AGT TGA GCG AAA AGG GGA TT	
bla_{OXA-51} like – F	TAA TGC TTT GAT CGG CCT TG	353bp
R	TGG ATT GCA CTT CAT CTT GG	
bla_{OXA-58} like – F	AAG TAT TGG GGC TTG TGC TG	599bp
R	CCC CTC TGC GCT CTA CAT AC	

RESULTS

Preliminary identification

All the A.baumannii isolates are found to be gram-negative, non-motile, non fermenting bacilli showing oxidase negative and catalase positive. The strains are confirmed as non-motile and non-fermenting by mannitol motility medium, acidification of glucose in triple sugar iron agar test and OF test, negative for indole production and positive for citrate utilization. Growth is observed at both 37°C and 44°C.

Antibiotic Sensitivity Test

Among 35 isolates obtained from the clinical samples, all the isolates showed resistance to ceftazidime (100%), 34 (97%) isolates showed resistance to meropenem , 33 (94%) isolates showed resistance to ciprofloxacin, 30 (86%) isolates showed resistance to gentamicin, 26 (74%) isolates showed resistance to piperacillin-tazobactam and amikacin, 25(71%) isolates showed resistant to imipenem and 16 (46%) isolates were resistance to doxycycline . All the

ISSN: 0975-8585

isolates were found to be sensitive to colistin, tigecycline and polymyxin B, as per CLSI guidelines (Table2).

Table-2 Antibiotic resistance pattern of A.baumannii isolates

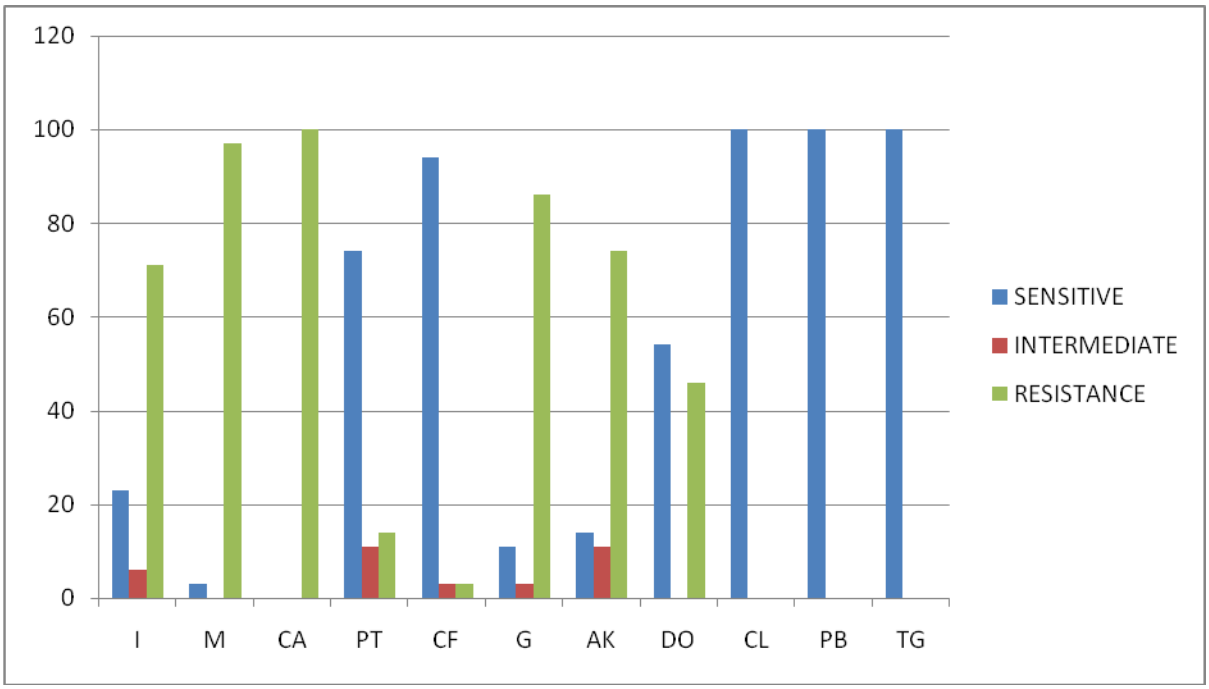
ISOLATE NO	I	M	CA	PT	CF	G	AK	DO	CL	PB	TG
A1	I	R	R	R	R	R	R	S	S	S	S
A2	R	R	R	R	R	R	R	S	S	S	S
A3	R	R	R	R	R	R	R	R	S	S	S
A4	S	R	R	I	R	S	S	R	S	S	S
A5	R	R	R	R	R	R	R	S	S	S	S
A6	R	R	R	R	R	R	I	R	S	S	S
A7	R	R	R	R	R	I	S	S	S	S	S
A8	S	R	R	S	R	S	I	R	S	S	S
A9	R	R	R	R	R	R	R	R	S	S	S
A10	R	R	R	R	R	R	R	S	S	S	S
A11	R	R	R	R	R	R	R	S	S	S	S
A12	R	R	R	S	R	S	R	R	S	S	S
A13	R	R	R	R	R	R	R	R	S	S	S
A14	R	R	R	R	R	R	R	R	S	S	S
A15	R	R	R	R	R	R	R	R	S	S	S
A16	R	R	R	R	R	R	R	S	S	S	S
A17	R	R	R	I	R	R	S	R	S	S	S
A18	R	R	R	R	R	R	R	S	S	S	S
A19	S	R	R	R	R	R	I	S	S	S	S
A20	R	R	R	R	R	R	R	S	S	S	S
A21	R	R	R	I	R	R	S	R	S	S	S
A22	R	R	R	I	R	R	R	S	S	S	S
A23	R	R	R	R	R	R	R	R	S	S	S
A24	S	R	R	S	R	R	R	S	S	S	S
A25	S	R	R	S	R	R	I	S	S	S	S
A26	R	R	R	I	R	R	R	R	S	S	S
A27	S	R	R	R	R	R	R	R	S	S	S
A28	S	R	R	R	R	S	R	S	S	S	S
A29	R	R	R	R	I	R	R	S	S	S	S
A30	R	R	R	R	R	R	R	R	S	S	S
A31	R	R	R	R	R	R	R	S	S	S	S
A32	I	R	R	R	R	R	R	R	S	S	S
A33	R	R	R	R	R	R	R	S	S	S	S
A34	R	R	R	R	R	R	R	S	S	S	S
A35	S	S	R	R	S	R	S	S	S	S	S

KEY: R-Resistant; S-Sensitive; I-Intermediate;

I - Imipenem
M -Meropenem
CA -ceftazidime
PT -Piperacillin-tazobactam
CF -Ciprofloxacin
G -Gentamicin
AK -Amikacin
DO -Doxycycline
CL -Colistin
PB -Polymyxin B
TG -Tigecycline

ISSN: 0975-8585

Fig1. Antibiotic resistant pattern of A. baumannii isolates



Phenotypic characterisation of carbapenem resistance

Double Disc Synergy Test

MBL production was determined by the double- disc synergy test (DDST) in which all the 35 isolates showed negative for the production of metallo-β-lactamases [10]. The presence of MBL gene was sought later by PCR screening for confirmation, as described by [21] (Refer Table 3)

Modified Hodge Test

Carbapenemase activity was screened by the modified Hodge (clover leaf) test, using Mueller-Hinton agar showed a clover leaf like intendation. This was interpreted as positive for all the isolates as described by [10]. (Refer Table 3)

Minimum inhibitory concentration

Minimum Inhibitory Concentration (MIC) for meropenem was determined using MIC method by agar dilution method and interpreted as per CLSI guidelines. (Refer Table 3) MIC_{50} and MIC_{90} were found to be 32 µg/ml and 128 µg/ml.

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Table 3 Phenotypic characterisation of carbapenem resistance

Code of the isolates	Phenotypic characterisation of carbapenem resistance		
	MBL Production (DDST)	Carbapenemase Production (MHT)	Minimum Inhibitory Concentration (MIC)
A1	N	P	64
A2	N	P	32
A3	N	P	32
A4	N	P	32
A5	N	P	32
A6	N	P	32
A7	N	P	32
A8	N	P	32
A9	N	P	32
A10	N	P	32
A11	N	P	32
A12	N	P	16
A13	N	P	64
A14	N	P	128
A15	N	P	16
A16	N	P	256
A17	N	P	128
A18	N	P	512
A19	N	P	32
A20	N	P	32
A21	N	P	32
A22	N	P	64
A23	N	P	128
A24	N	P	32
A25	N	P	16
A26	N	P	32
A27	N	P	16
A28	N	P	16
A29	N	P	64
A30	N	P	64
A31	N	P	256
A32	N	P	128
A33	N	P	256
A34	N	P	32
A35	N	P	2

KEY: DDST- Double Disc Synergy Test; MHT-Modified Hodge Test;
N - Negative; P - Positive.

Molecular methods for the detection of carbapenem resistance

PCR SCREENING

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Among 35 isolates, bla_{OXA-51} and bla_{OXA-23} was detected in all the 35 A.baumannii isolates. [21]

Table 4: Molecular methods for the detection of bla_{OXA-51} and bla_{OXA-23}

Code of the isolates	Molecular characterisation of carbapenem resistance (pcr screening)	
	bla_{OXA-23}	bla_{OXA-51}
A1	P	P
A2	P	P
A3	P	P
A4	P	P
A5	P	P
A6	P	P
A7	P	P
A8	P	P
A9	P	P
A10	P	P
A11	P	P
A12	P	P
A13	P	P
A14	P	P
A15	P	P
A16	P	P
A17	P	P
A18	P	P
A19	P	P
A20	P	P
A21	P	P
A22	P	P
A23	P	P
A24	P	P
A25	P	P
A26	P	P
A27	P	P
A28	P	P
A29	P	P
A30	P	P
A31	P	P
A32	P	P
A33	P	P
A34	P	P
A35	P	P

KEY: P-Positive; N-Negative

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FIG 2: ANTIBIOTIC SENSITIVITY TEST

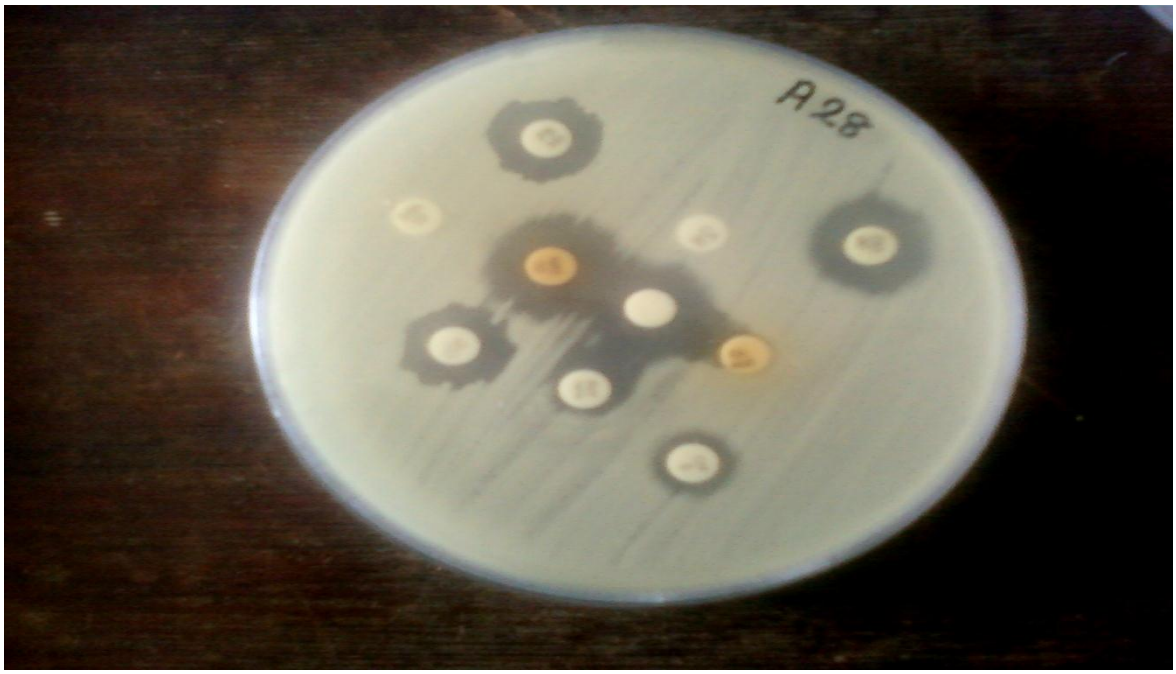
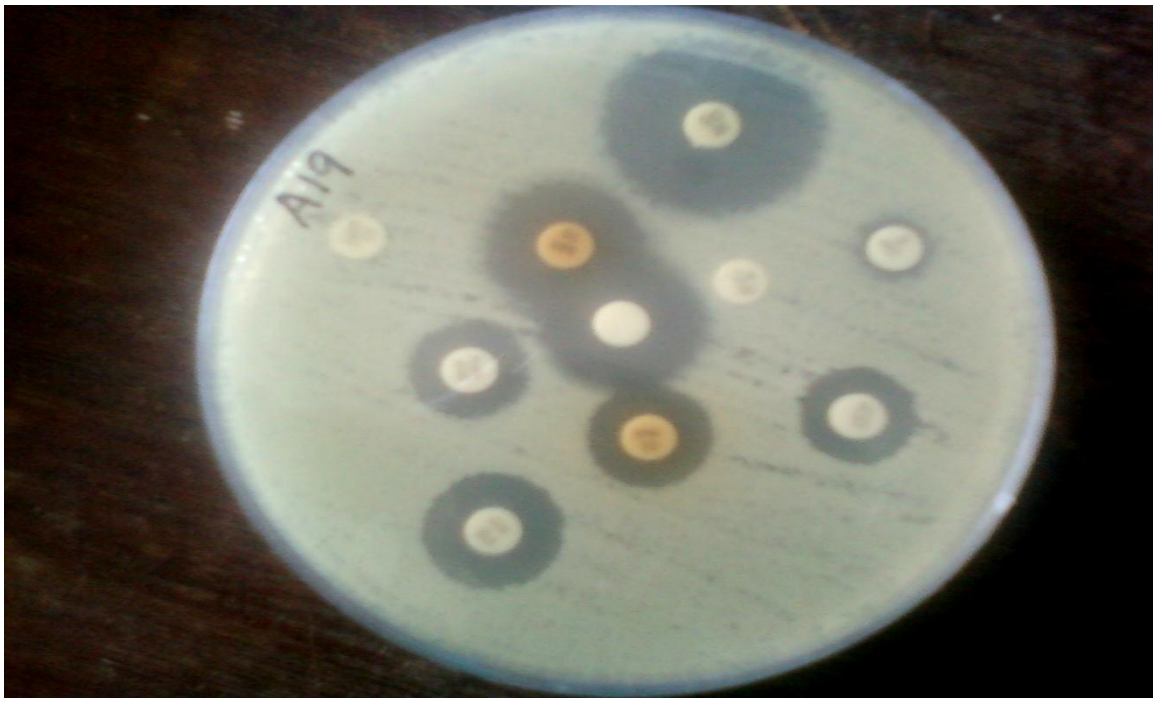


FIG 3: DOUBLE DISC SYNERGY TEST



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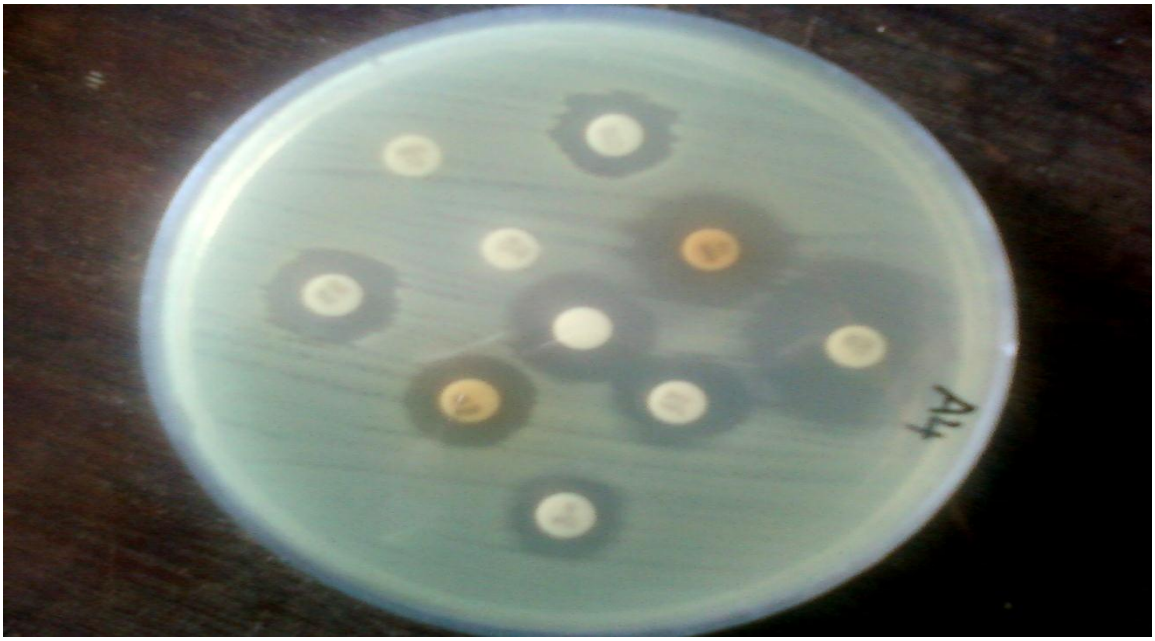

FIG 4: MODIFIED HODGE TEST

PLATE 5.4.1

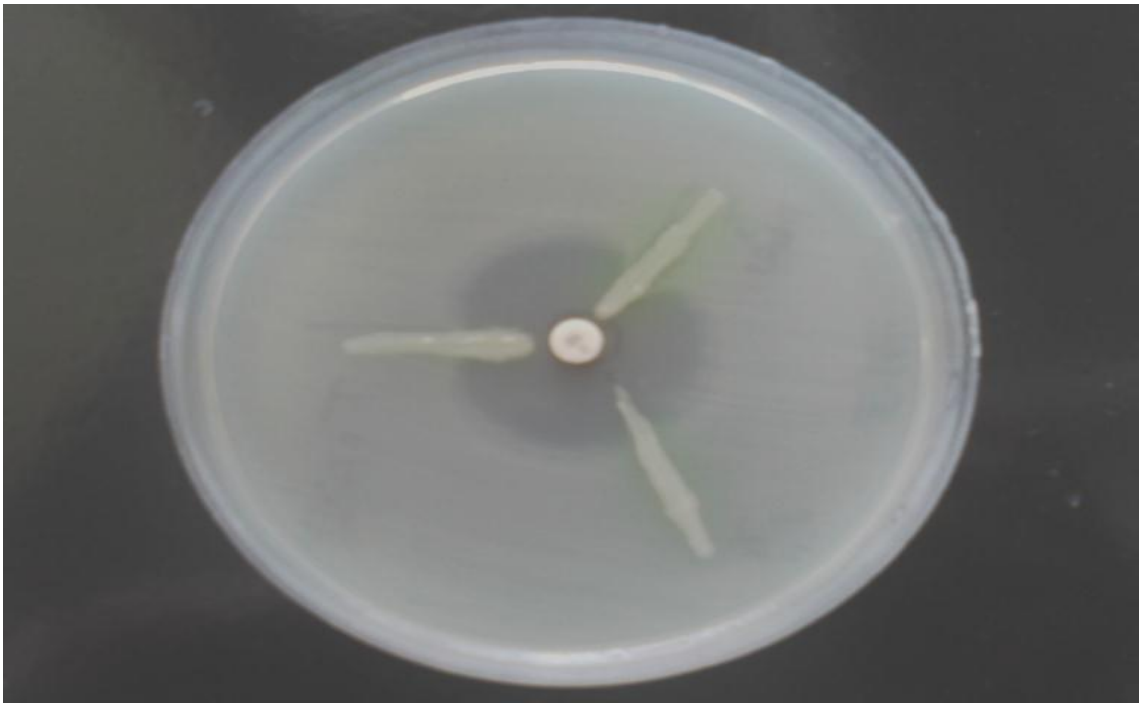

FIG 5: MINIMUM INHIBITORY CONCENTRATION

PLATE5.5.1 (Control plate)

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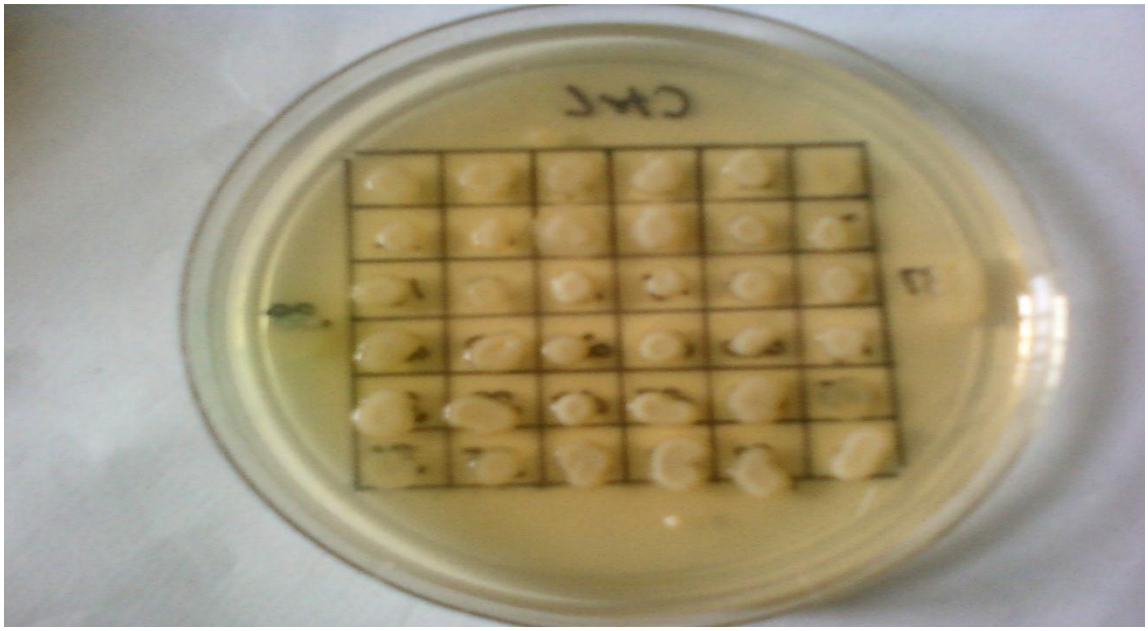
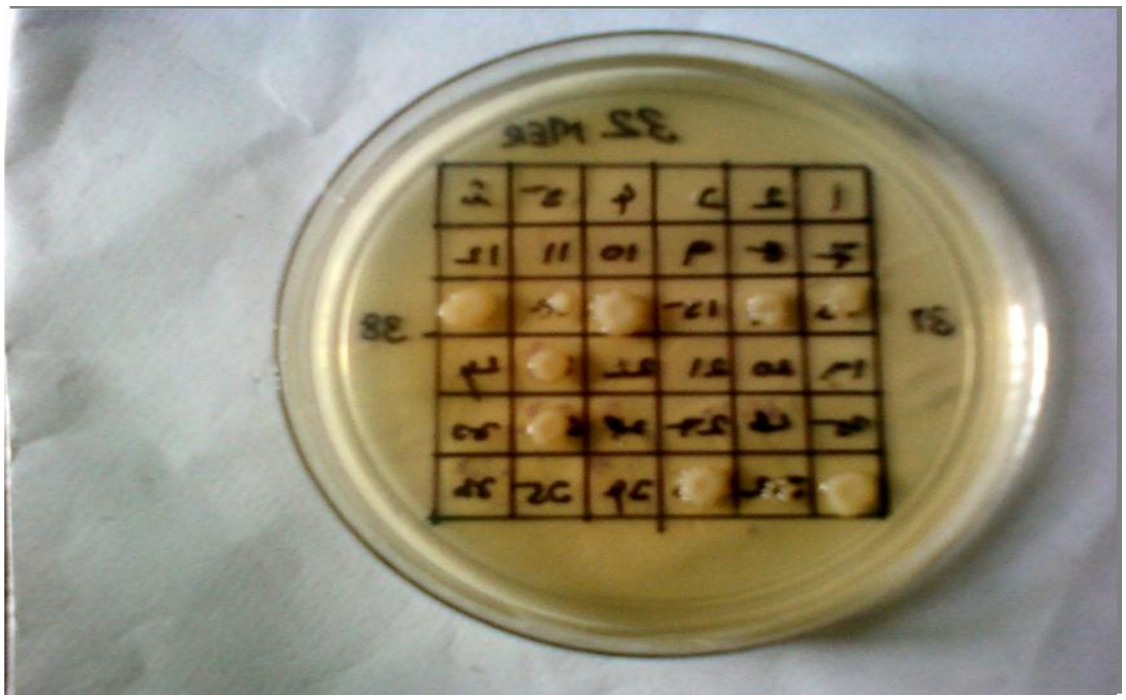


5. 1 MIC FOR 32µg/ml



5.2 MIC FOR 128 µg/ml

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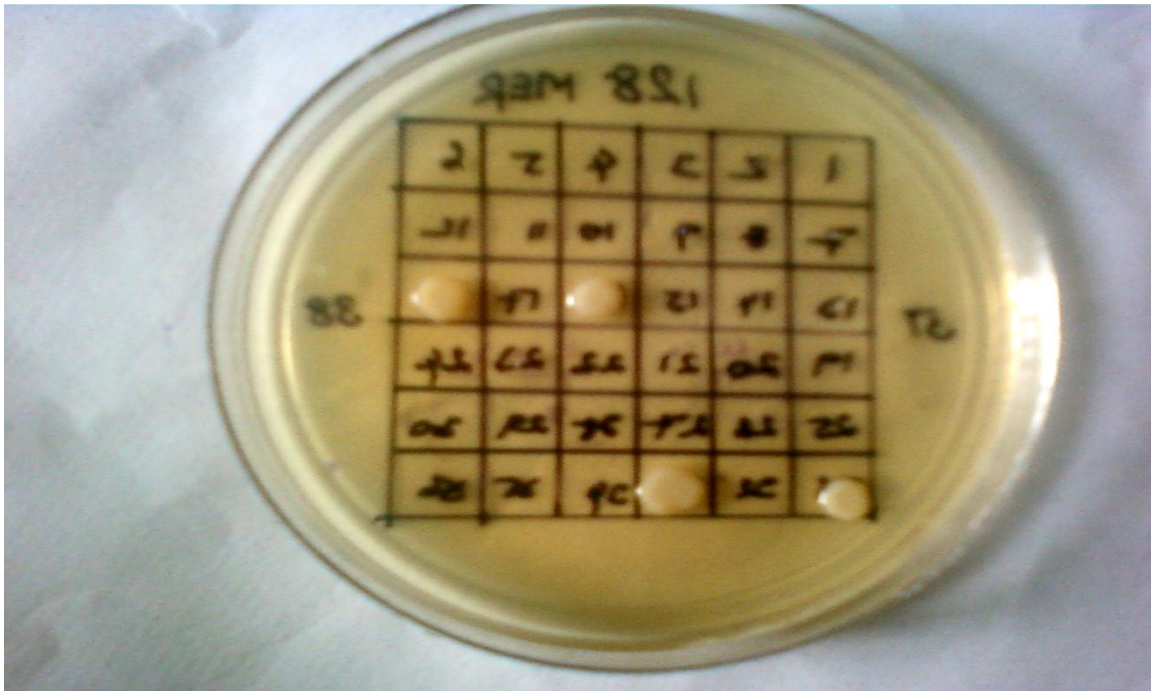
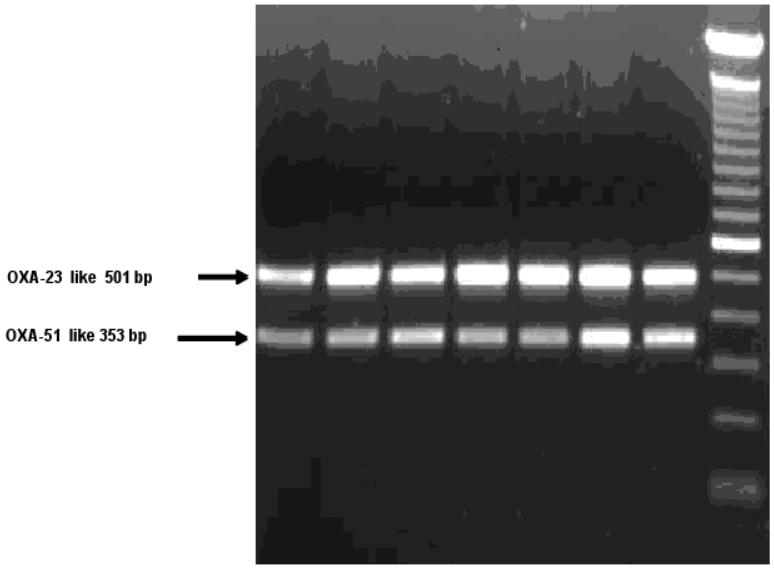


Fig 6: Multiplex PCR for bla$_{OXA}$ type carbapenemases



DISCUSSION

Acinetobacter baumannii has emerged as an important nosocomial pathogen [6]. Hospital outbreaks have been described from various geographic areas, [1] and this organism has become endemic in some of them. The role of the environmental contamination in the transmission of nosocomial infections in general and in A. baumannii infections in particular is well recognized [16]. A. baumannii does not have fastidious growth requirements and is able to grow at various temperatures and pH conditions. [2]

Carbapenems have the most extended antimicrobial spectrum among the ß-lactams. However, carbapenem resistance is emerging and increasing in clinical isolates, especially in A. baumannii. A limited number of antimicrobial agents maintain high activity against OXA-23-producing A. baumannii [5]. Phenotypic identification in the case of Acinetobacter species is very difficult as it was found to be tedious and time consuming using a large panel of 28 tests as described by Bouvet and Grimont [3].

The reliability of this scheme of identification was evaluated by Gerner smidt et al [7] who advocated the use of fewer batteries of tests for the phenotypic identification of A.baumannii. Identification of Acinetobacter spp., based upon growth at 44ºC, 41ºC and 37ºC along with acid production from glucose was found to be very useful from an earlier Indian study [8]. The isolates involved in the present study were confirmed by Gram stain findings, oxidase reaction, citrate test, reaction on mannitol motility medium, TSI reaction, growth observation at both 37ºC and 44ºC. According to one Korean study [10], among 144 carbapenem resistant isolates of Acinetobacter spp., 135 (93.8%) were Hodge test-positive. Of these, 28 were positive in the DDST method. The bla_{OXA} genes were detected in 107 isolates, of which 105 were Hodge-test positive and 2 were Hodge-test negative. 105 isolates were found to be positive for bla_{OXA-51} like genes, whether expressed or not, identifying them as A.baumannii. Of those, 47 had bla_{OXA-23} and 56 had bla_{OXA-51} like genes. In the present study involving 35 clinical A.baumannii isolates, resistance was seen for 25(71%) and 34(97%) isolates towards imepenem and meropenem respectively by Kirby Bauer method. Phenotypic characterization of carbapenem resistance of the isolates included Modified Hodge test and Double Disc Synergy test for the production of carbapenemasas and metallo-β-lactamases respectively [9].

Identification for the presence of MBL is found to be very difficult by phenotypic characterization alone and hence confirmation was done by PCR assay to check the presence of MBL gene which showed negative in all the isolates tested. Investigation was done for the occurrence of bla_{OXA} type β-lactamases (Class D) by multiplex PCR assay by the method of [21] where in they observed that amplified fragments of bla $_{OXA}$ alleles are encoded each into three acquired groups (bla_{OXA-23} like , bla_{OXA-24}like and bla_{OXA-58} like) of OXA-carbapenemases; while the intrinsic bla_{OXA-51} like alleles were seen in all the A.baumannii isolates. Similar findings were seen in the present study, where all the 35 isolates were positive for both bla_{OXA-51} and bla_{OXA-23}, present in the resistant isolates involved in the study.

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