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Original Research Article

COMPARATIVE STUDY OF PHENOTYPIC BASED DETECTION METHODS FOR CARBAPENEMASE PRODUCTION WITH FOCUS ON ITS IMPACT ON CLINICAL DECISION MAKING IN CARBAPENEM RESISTANT ACINETOBACTER BAUMANNII ISOLATES

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ABSTRACT

Background: Acinetobacter baumannii is an aerobic non-fermentative, gram-negative coccobacilli that has emerged as an important nosocomial opportunistic pathogen, commonly occurring in critically ill intensive care unit patients with chronic illness or prolonged hospitalizations. This species has a strong ability to acquire antimicrobial resistance, thus producing various types of resistant isolates, including multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pandrug-resistant (PDR) strains. Carbapenems were formerly the last-resort antibiotics for the treatment of MDR A.baumannii infections. However, an increasing number of Carbapenem Resistant A. baumannii (CRAB) strains have been found throughout the world, posing one of the greatest known threats to modern medicine. Present study was aimed to compare different phenotypic methods for detecting carbapenemase production with focus on its impact on clinical decision making in the Carbapenem resistant Acinetobacter baumannii isolates.

Materials and Methods: Present study was a cross sectional study, in which clinically significant biochemically confirmed non-duplicate Carbapenem resistant A. baumannii (CRAB) isolates were subjected to different phenotypic methods for detecting carbapenemase production.

Results: As such a total of 169/339 (49.9%) isolates of A. baumannii were carbapenem resistant during the study period. Using PCR as gold standard, Modified Hodge test had the highest sensitivity (76.5%) and modified in-house Carba NP test had the highest specificity (88.7%) for detection of bla$_{OXA-23}$type carbapenemase gene. Highest sensitivity was shown by temocillin disc diffusion (100%) whereas highest specificity was shown by chromID®CARBA SMART OXA medium (bioMerieux) for detection of bla$_{OXA-48}$ type carbapenemase (98.1%).Lowest turnaround time was for RAPIDEC®Carba NP and modified in-house Carba NP tests (30 minutes-2 hours).

Conclusion: Werecommend the use of EDTA combined disc test for detection of MBL in combination with modified in-house Carba NP test and chromID®CARBA SMART agar (bioMerieux) for detection of OXA type carbapenemases as evidenced by comparison with PCR.

Keywords: Acinetobacter baumannii, CRAB, CarbaNP, mCIM, OXA, PCR.

INTRODUCTION

Acinetobacter spp. is an aerobic non-fermentative, gram-negative coccobacilli that has emerged as an important nosocomial opportunistic pathogen, commonly occurring in critically ill intensive care unit (ICU) patients with chronic illness or prolonged hospitalizations.[1,2]A. baumanniiis the most common species to cause infections constituting 80 percent of the total clinical isolates ofAcinetobacter spp. that has been reported worldwide.A. baumannii is ubiquitous in the hospital environment, particularly in the Intensive Care Unit (ICU) setting and they have immense potential to cause extended nosocomial outbreaks. Acinetobacter baumannii has emerged as an extensively-drug resistant pathogen implicated in healthcare associated infections such as ventilator associated pneumonia, urinary tractinfections, bacteremia, septicemia, secondary meningitis, wound and surgical site infections.[1]Major risk factors for multidrug-resistant A. baumannii colonization and infection include lengthened hospital stays, especially admission to the intensive care unit, surgery, invasive procedures, mechanical ventilation, central venous catheterization, urinary catheterization, co-morbidities like diabetes mellitus, acute or chronic renal failure, chronic lung disease, greater severity of illness, inappropriate and frequent usage of antibiotics, long term steroid therapy, immunosuppressive treatment regimens and breaches in infection control protocols.[1]This organism is now universally recognized as one of the six highly virulent and antimicrobial-resistant bacterial pathogens, contributing the 'A' in the 'ESKAPE' pathogens.[1]

Due to increasing inability to treat infections because of limited antibiotic options as well as increasing morbidity and mortality associated with carbapenem resistant A. baumannii infections, it is emerging as a serious therapeutic and infection control challenge. Rapid and worldwide emergence of carbapenem resistance in A. baumannii is increasingly observed and thereby constitutes a signal for immediate investigation and response. The WHO 2024 report identifies CRAB as the number one priority pathogen globally, highlighting the critical need for innovative therapeutic strategies to combat its extensive drug resistance and associated high mortality rates.[2]A number of phenotypic methods for screening carbapenemase production in Gram-negative bacteria have been reported, whereas there are no standard methods recommended for A.baumannii by the Clinical and Laboratory Standards Institute (CLSI). The most frequently used methods recommended by the CLSI to determine carbapenemase production are the older modified Hodge test, the Carba NP test, and the modified carbapenem inactivation method (mCIM). But the modified Hodge test is only recommended for Enterobacterales[3] and is even excluded from the "Performance standards for antimicrobial susceptibility testing" since 2018.[5] The mCIM can be applied forEnterobacterales and Pseudomonas aeruginosa, but not for A.baumannii.[4,5,6]Moreover, although the Carba NP test was performed for Acinetobacter spp. in the previous years, this method is now only recommended for suspected carbapenemase production in Enterobacterales and Pseudomonas aeruginosa.[6]Various modified methods have been described to identify carbapenemase productionincluding CIMTris,[7] the CarbAcineto NP test,[8] adjusted mCIM,[9]the simplified carbapenem inactivation method[10] and the optimized carbapenem inactivation method (oCIM).[11] However, none of them are officially endorsed by CLSI guidelines.Therefore, an appropriate and reliable phenotypic method is necessary for carbapenemase detection inA. baumannii. Present study was aimed to compare different phenotypic methods for detecting carbapenemase production with focus on its impact on clinical decision making in the Carbapenem resistant Acinetobacter baumannii isolates.

MATERIALS AND METHODS

Present study was a cross sectional study, conducted in a tertiary care medical college hospital located at Tiruvalla, Kerala, India from March 2016 to August 2017. Study was approved by Institutional research committee and Institutional ethical committee.

BACTERIAL STRAINS AND CULTURAL CONDITIONS

Inclusion Criteria

- All clinically significant biochemically confirmed non-duplicate carbapenem resistant Acinetobacter baumannii isolates.

Exclusion Criteria

- All A. baumannii isolates that were sensitive to carbapenems.
- All isolates of A. baumanniiclinically proven to be colonizers or commensals.
- Isolates of repeated samples from the same patient were not included in the study.

The isolates were obtained from various clinical specimens such as sputum, bronchoalveolar lavage, blood, urine, body fluids and wound swabs. Samples were inoculated onto appropriate culture media like 5% sheep blood agar, MacConkey agar and Chocolate agar plates as per the standard operating protocol of the laboratory. Preliminary identification of the Acinetobacter baumannii isolates was done based on the routine conventional microbial culture and biochemical tests for bacterial identification.[12,13]The isolated organisms were confirmed using the automated system VITEK 2C (bioMérieux, Marcy l'Etoile, France). All the organisms identified as A. baumannii were subjected to antimicrobial susceptibility testing and screening for the carbapenemaseproduction as per the Clinical and Laboratory Standards Institute (CLSI) 2017 guidelines.[4]

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing was performed for all the isolates by standard Kirby-Bauer disc diffusion method on Mueller-Hinton agar plates and zone diameters were interpreted according to Clinical and Laboratory Standards Institute guidelines and categorized as susceptible, intermediate or resistant.[4]For all the isolates obtained antibiotic susceptibility profiles and MICs were also determined by automated VITEK 2C (bioMerieux). According to CLSI guidelines carbapenem resistant Acinetobacter baumannii was defined as an A. baumannii isolate that was resistant to both imipenem and meropenem with MIC ≥ 8μg/ml.[4]Minimum Inhibitory Concentration (MIC) of colistin was tested using VITEK 2C (bioMérieux). All carbapenem resistant Acinetobacter baumannii (CRAB) strains were further tested for carbapenemase production.

DETECTION OF ANTIMICROBIAL RESISTANCE PHENOTYPIC SCREENING METHODS FOR CARBAPENEMASE DETECTION

Modified Hodge test (MHT)

MHT test was performed as per recommendations of CLSI guidelines.[3,4]

Carba NP (Nordmann-Poirel) test

Carba NP test emerged as a useful alternative to detect carbapenemase production in Enterobacteriaceae, Pseudomonas spp. and Acinetobacter spp. as recommended by the Clinical and Laboratory Standards Institute (CLSI) 2017.[4] But since 2018 CLSI document has deleted the use of this test for detection of carbapenemase in Acinetobacter spp.[5] All carbapenem resistant Acinetobacter baumannii isolates were tested with commercially available RAPIDEC®Carba NP (bioMérieux) and modified in-house Carba NP tests during the present study period.

RAPIDEC® Carba NP test

The procedure was performed using commercially available ready to use test strip from bioMérieux according to manufacturer's instructions.

Modified In-house Carba NP test(Colorimetric microtube assay)

The procedure was performed based on the CLSI guidelines and previous studies. The cost of Bacterial Protein Extraction Reagent and imipenem standard grade powder used in the CLSI 2017[4] described Carba NP test is high and hence in the present study imipenem- cilastatin injectable powder, heavy inoculum and 5M sodium chloride solution (as lysis buffer)are used as suggested by AbelGhani S et al. in 2015,[14] and Rudresh SM et al. from Karnataka in 2017.[15]Quality control (QC) strains used were K. Pneumonia ATCC®BAA-1705™ as positive control and K. Pneumonia ATCC®BAA-1706™ as negative control.

Table 1: Interpretation of results for patient and QC tubes[4]

Tube "a" Solution A (serves as internal control)	Tube "b" Solution B	Interpretation
Red or red - orange	Red or red - orange	Negative
Red or red - orange	Light orange, dark yellow or yellow	Positive
Red or red - orange	Orange	Invalid
Orange, dark orange, light yellow or yellow	Any colour	Invalid

Modified Carbapenem Inactivation Method (mCIM)

Modified carbapenem inactivation method (mCIM) has been developed in CLSI M100-S27 as a phenotypic technique for detecting carbapenemase activity.[4] But as per the latest CLSI guidelines, mCIM is standardized for testing suspected carbapenemase production in Enterobacteriaceae (KPC, NDM, VIM, IMP, IMI, SPM, SME, OXA type carbapenemases) and Pseudomonas aeruginosa (KPC, NDM,VIM, IMP, IMI, SPM, OXA type carbapenemases) and not recommended for testing Acinetobacter species.[6]

Carbapenemase inhibition tests for differentiation of KPC and MBL[16,17,18]

Imipenem-EDTA combined disc test for MBL detection and phenyl boronic acid potentiation test for Klebsiella pneumonia carbapenemase (KPC) detectionwere performed and interpreted according to the CLSI guidelines and previous studies.

Temocillin disc diffusion for OXA-48 detection

Phenotypic detection of OXA-48 production was difficult so far, as no specific inhibitor is available. Temocillin resistance was suggested as an indicator of OXA-48 production but not for OXA-48 confirmation. Standard disc diffusion method was performed using commercially available disc containing 30 µg of temocillin (Rosco Diagnostica, Denmark)and interpreted as previously described.[16,19]A temocillin zone diameter ≤10 mm (i.e. the absence of a zone of inhibition around the temocillin disc) is considered OXA-48 carbapenemase positive.

chromID®CARBA SMART Agar(CARB/OXA)

chromID®CARBA SMART agar (bioMérieux) consists of two chromogenic culture media dispensed into one petri dish containing separate compartments (CARB/OXA). Growth detects mainly KPC and metallo– carbapenemase, for the CARB medium and OXA-48 type CPE for the OXA medium. Direct plating onto chromID® CARBA SMART agar was performed for each specimen. The inverted plates were incubated at 35 ± 2°C in aerobic conditions. The cultures are generally examined after 18 to 24 hours of incubation. [20]

MOLECULAR TESTING FOR OXA TYPE CARBAPENEMASE GENES

OXA gene amplification from bacterial culture

Acinetobacter baumannii strains with a positively phenotypically tested for carbapenemase, the relevant genes were determined. This was carried out by conventional polymerase chain reaction (PCR) using specific oligonucleotide primers encoding two selected class D types (OXA-23 and OXA-48).

Table 2: Primers of target genes used for PCR[21,22]

Primer	5′→3′	Amplicon size
OXA-23 F	GATCGGATTGGAGAACCAGA	501
OXA-23 R	ATTTCTGACCGCATTTCCAT	
OXA-48 F	TTGGTGGCATCGATTATCGG	733
OXA-48 R	GAGCACTTCTTTTGTGATGGC	

Genomic DNA was extracted from the culture sample using PureLink genomic DNA extraction and purification Kit (Invitrogen, Life technologies, USA) following the manufacturer's instructions. After the quantitative analysis, the genomic DNA was utilized for genotyping assay. Partial gene regions were PCR amplified with specific primers.

bla$_{OXA-23}$ and bla$_{OXA-48}$ sequencing analysis - Methodology

DNA was isolated from the culture provided. Electrophoresed the DNA in 1% agarose and visualized under UV. 16S region was PCR amplified with specific primers and amplicon was checked for appropriate size by agarose gel visualisation. Amplicon was gel purified using commercial column based purification kit (Invitrogen, USA). Automated sequencing was performed with forward and reverse primers in ABI 3730 XL cycle Sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. Sequence analysis was performed using online tool BLAST of NCBI database by comparing with known sequences(http://blast.ncbi.nlm.nih.gov) and based on maximum identity score E value top most sequences were utilised for multiple sequence alignment (Clustal W2) and dendrogram was constructed.

The data collected was entered in Microsoft Excel and analyzed using SPSS version 16 software. Descriptive analysis was done by calculating frequencies, proportions, mean, median and standard deviation. Prevalence (95% confidence level) of carbapenemase production among carbapenem resistant A.baumannii was determined. Tests of association such as Chi square tests and Fischer's exact were used to calculate associations. Validity of various phenotypic tests for detection of carbapenemase production was compared with polymerase chain reaction as gold standard using sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

RESULTS

During the study period, a total of 339 non-duplicate, clinically significant and biochemically confirmed A.baumannii isolates were obtained from the various specimens received in the laboratory. As per the sample size calculated in the present study of these, 138 consecutive patients with non-duplicate carbapenem resistant Acinetobacter baumannii isolates were included in present study for further comparison and analysis. As such a total of 169/339 (49.9%) isolates of A. baumannii were carbapenem resistant during the study period.

Table 3: Phenotypic characterization of carbapenemase production among carbapenem-resistant Acinetobacter baumannii isolates(n=138)

Phenotypic tests	No. of isolates positive (%)
Modified Hodge test	107(77.5%)
EDTA Combined disc test for MBL detection	96(69.6%)
PBA potentiation for KPC detection	0
RAPIDEC®Carba NP test	26(18.8%)
Modified In-house Carba NP test	45(32.6%)
Modified carbapenem inactivation method	46(33.3%)
Temocillin disc diffusion for OXA-48 detection	67(48.6%)
ChromID® CARBASMART CARB/OXA	CARB medium - 24(17.4%) OXA 48 medium - 34(24.6%)

Phenotypic characterization of carbapenemase production among carbapenem-resistant Acinetobacter baumannii isolates

107/138(77.5%) and 96/138(69.6%)isolates were phenotypically tested positive by Modified Hodge test and EDTA combined disc test respectively. Only 26/138 (18.8%) and 45/138 (32.6%) of isolates were positive by RAPIDEC® Carba NP test (bioMérieux)and modified in-house Carba NP test respectively. 46/138 (33.3%) of strains were positive for modified carbapenem inactivation method. None of the isolates were positive for phenyl boronic acid potentiation test for the detection ofKPC type carbapenemase.24/138 (17.4%) isolates were tested positive for carbapenemase production by growth on chromID® CARBASMART CARB medium (bioMérieux). 67/138 (48.6%) and 34/138 (24.6%) isolates were tested positive for OXA 48-type production by temocillin disc diffusion test (Rosco Diagnostica) and growth on chromID® CARBASMART OXA medium (bioMérieux).

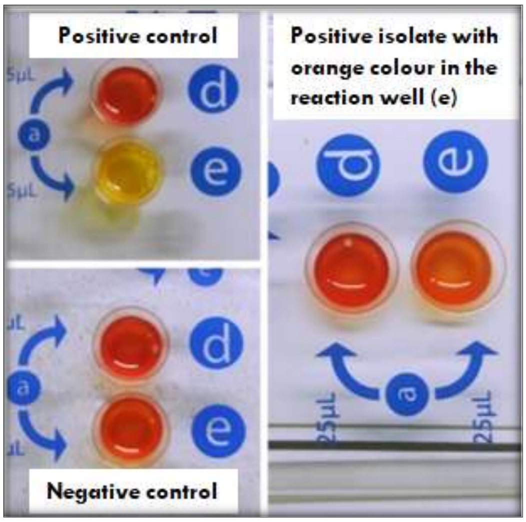

Figure 1: RAPIDEC® Carba NP test results
Note: d = Control well without imipenem, e = Reaction well with imipenem

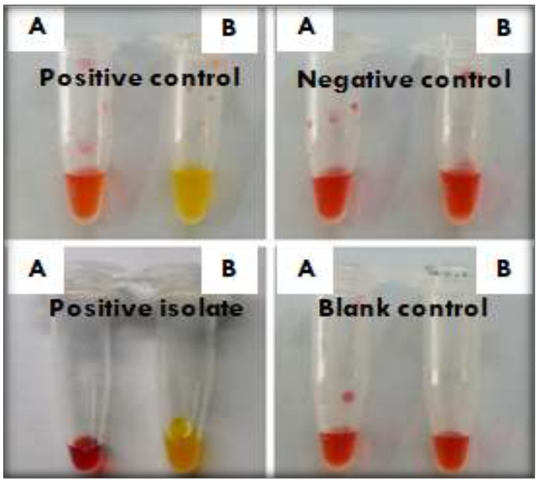

Figure 2: Modified In-house Carba NP test results
Note: A = Control well without imipenem, B = Reaction well with imipenem

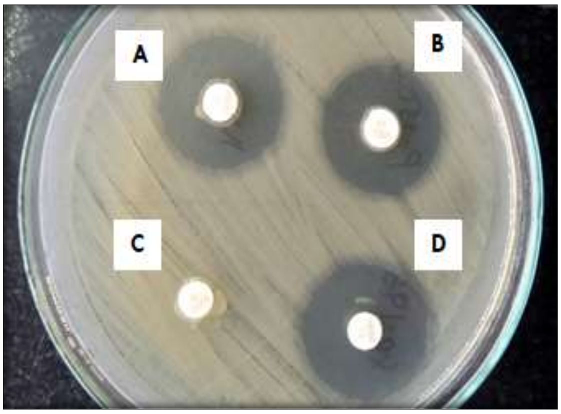

Figure 3: mCIM
Note: A = Negative Control with zone diameter ≥19mm,B = mCIM negative isolate with zone diameter ≥19mm, C = Positive Control with zone diameter 6mm,D = mCIM negative isolate with zone diameter ≥19mm

Figure 4: Temocillin disc diffusion test for OXA-48 detection
Note: A temocillin zone diameter ≤10 mm (i.e. the absence of a zone of inhibition around the temocillin disc) is considered OXA 48 carbapenemase positive.

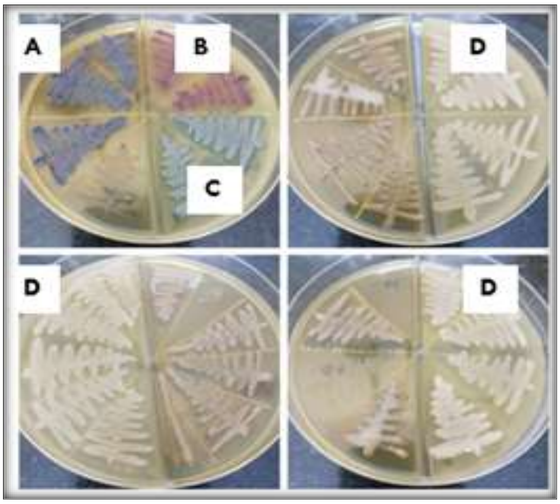

Figure No 5: Growth on chromID®CARBA SMART CARB/OXA(bioMerieux)
Note: A = Bluish-purple colonies of OXA 48 producing K.pneumoniae, B = Pink colonies of carbapenemase producing E.coli, C = Bluish green colonies of K.pneumoniae ATCC® BAA 1705(Positive control), D = Cream colonies of Acinetobacter baumannii

Genotypic characterization of carbapenemase production among carbapenem-resistant Acinetobacter baumannii isolates

Genotypic characterization by conventional polymerase reaction (PCR) among the carbapenemase producing A.baumannii, tested positive by four phenotypic tests, 86(62.3%) were found to bebla$_{OXA-23}$ positive. bla$_{OXA-23}$ was identified as the most prevalent OXA type carbapenemase gene in our hospital setting. Genotypic characterization among the suspected OXA-48 producing A.baumannii, tested positive by temocillin disc diffusion test and chromogenic agar, 35(25.4%) of strains were found to be bla$_{OXA-48}$ positive.

Table 4: Validity of different phenotypic methods for detection of OXA-23 type carbapenemase production among carbapenem-resistant Acinetobacter baumannii

Phenotypic tests	No. of isolates positive (%)n=138	bla$_{OXA-23}$ positive	Sensitivity	Specificity	PPV[a]	NPV[b]
Modified Hodge test	107(77.5%)	86(62.3%)	76.5%	20.8%	60.75%	35.48%
RAPIDEC®Carba NP test	26(18.8%)	86(62.3%)	23.5%	88.6%	76.92%	41.96%
Modified In-house Carba NP test	46(33.3%)	86(62.3%)	47.1%	88.7%	86.96%	51.09%
Modified carbapenem inactivation method	46(33.3%)	86(62.3%)	47.1%	87.7%	86.86%	51.17%

Note : Sensitivity of Modified Hodge test was found to be the highest (76.5%). Modified in-house Carba NP test had the highest specificity (88.7%). (a)Positive Predictive Value(b)Negative Predictive Value

Table 5: Validity of phenotypic methods for detection of OXA-48 type carbapenemase production among carbapenem-resistant Acinetobacter baumannii

Phenotypic tests	No. ofisolates positive (%)n=138	bla OXA-48 positive	Sensitivity	Specificity	PPV[a]	NPV[b]
Temocillin disc diffusion	67(48.6%)	35(25.4%)	100%	68.3%	50.74%	100%
chromID®CARBA SMART OXA 48 medium	34(24.6%)	35(25.4%)	94.1%	98.1%	94.12%	98.08%

Note: Highest sensitivity was shown by temocillin disc diffusion (100%) whereas highest specificity was for chromID®CARBA SMART OXA medium (bioMérieux)for detection of OXA-48 type carbapenemase (98.1%).(a)Positive Predictive Value(b)Negative Predictive Value

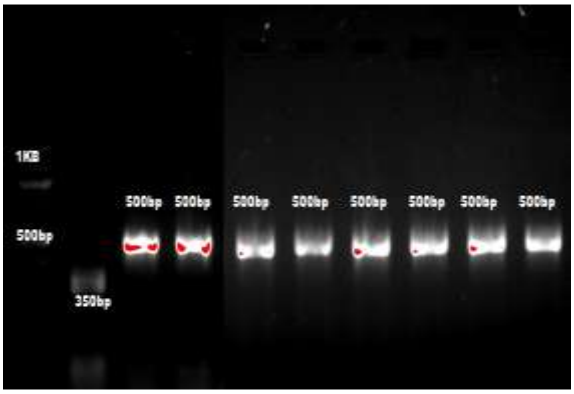

Figure 6: PCR gel showing band at350 base pair and 501 base pair on testing OXA-23 type gene
Note: Gene sequencing to be done to confirm if the non-specific amplicon is a variant of OXA-23

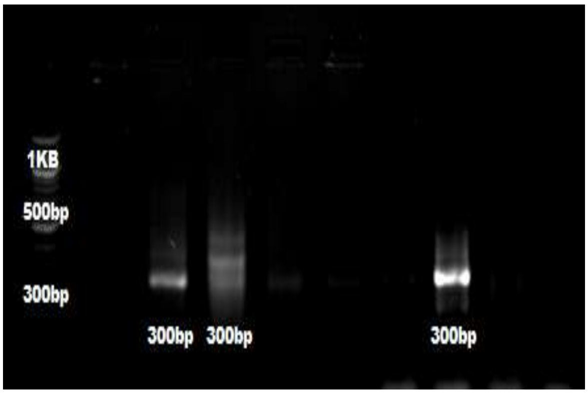

Figure 7: PCR gel showing band at 300 base pair instead of 733 base pair on testing OXA-48 type gene
Note: Gene sequencing to be done to confirm if the non-specific amplicon is a variant of OXA-48

Table 6: Turnaround time of phenotypic and genotypic tests

Tests	Turnaround time
Phenotypic tests	
Modified Hodge Test	Next day (16-18 hours)
EDTA Combined Disc Test	Next day (16-18 hours)
PBA potentiation for KPC detection	Next day (16-18 hours)
RAPIDEC® Carba NP &Modified in-house Carba NP tests	Same day (30 mins -2 hours)
Modified carbapenem inactivation method	Next day (16-18 hours)
Temocillin disc diffusion	Next day (16-18 hours)

chromID®CARBASMART medium	Next day (16-18 hours)
Genotypic tests	
PCR	Same day (2- 4 hours)

Note: Lowest turnaround time was for RAPIDEC® Carba NP and in-house Carba NP tests (30 minutes-2hours).

Results & Interpretation of automated sequencing

Amplification and sequencing were done with respective primers for two representative samples. The gel purified product was sequenced. The OXA-23 (Sample 2) & OXA-48 (Sample 17) region were sequenced very well and was excellent for prediction of the identity of organisms. Contig was obtained for OXA-23 region after trimming off the low quality bases and good consensus was obtained. No good quality sequence obtained for OXA-48 hence no contig was generated. The blast annotation shows 99% identity with Acinetobacter baumannii OXA-23 gene, beta lactamase partial sequence (Strain OXA 23 AB) with 0- E value and 100% query coverage according to the nucleotide homology. Hence 501bp amplified product confirmed to be OXA-23 gene by automated sequencing. 300bp product found after OXA-48 amplification could be any variant of OXA-48 type carbapenemase but was unable to obtain good quality sequence and could not confirm with sequence analysis.

DISCUSSION

The most striking feature of Acinetobacter baumannii is their wide-ranging "armamentarium" of intrinsic antimicrobial resistance mechanisms together with an inherent capability to acquire new resistance determinants. Both intrinsic and acquired resistance mechanisms act synergistically with genes encoding antibiotic inactivating enzymes, efflux pumps, ribosomal binding site mutations and down regulation of porin channels on the cell membrane giving rise to multidrug-resistant isolates.[23]

During the study period, a total of 339 non-duplicate, clinically significant and biochemically confirmed A. baumannii isolates were obtained from the various specimens received in the laboratory. As such a total of 169/339 (49.9%) isolates of A. baumannii were carbapenem resistant during the study period. Thus, the prevalence of carbapenem resistance during the study period was 49.9%.In a review article by Li Yang Hsu et al. it was found that carbapenem resistance rates of > 50% have been reported from large referral hospitals throughout South and Southeast Asia.[24] Our finding also correlates with a study from Kolkata, India, by Somdatta Chatterjee et al. in which the prevalence of carbapenem resistance in Acinetobacter spp. isolated from neonatal ICU was found to be almost 50%.[25]

Phenotypic characterization of carbapenemase production

The Modified Hodge test had been extensively used as a phenotypic technique for carbapenemase detection since it is routinely available in the clinical laboratory and was earlier recommended by the CLSI guidelines. Even though the MHT was found useful for the phenotypic detection of carbapenemase production in Enterobacteriaceae (with greater ability to detect Class A and B carbapenemases than Class D) the test cannot discriminate between different carbapenemases and may give false-positive results among non-carbapenemase-producing isolates.[26,27]Hence this test is even excluded from the CLSI guidelines since 2018.[5] MBL detection is achieved by addition of chelating agents like EDTA, mercaptoacetic acid and dipicolinic acid (DPA) to a carbapenem disc.[16,17,18]In the present study, 107/138(77.5%) and 96/138(69.6%) of our isolates tested positive by Modified Hodge test and EDTA combined disc test respectively for detection of carbapenemases. None of these isolates were positive for phenylboronic acid potentiation test for the detection of KPC type carbapenemase. KPC production in A. baumannii is generally unusual though few case reports have documented the same. In a study by Lee et al. in 2003, for the detection of MBL in Acinetobacter spp., the performance of the Hodge test was improved by addition of zinc sulphate (140 µg/disk) to the imipenem disc.[26] In a study of 19 carbapenemase- producing A. baumannii isolates, by Bonnin RA et al. in 2012, the modified Hodge test gave negative results for all tested NDM-producing strains and only weak positive results for VIM-, IMP- and OXA- type producers.[27] For Double-Disc Synergy Test, Lee et al.2003 found that although EDTA (~1900 µg) discs were better at detecting metallo β lactamase producing strains among Pseudomonas aeruginosa, sodium mercaptoacetic acid (3 mg) and 2-mercaptopropionic acid (3 µl) discs performed better for Acinetobacter spp., while ceftazidime-sodium mercaptoacetic acid double-disc synergy tests failed to detect 22/80 (28%) MBL-producing Acinetobacter spp.[26] They also observed an important practical caution with total or partial loss of MBL-producing cells during room temperature storage of the isolates, indicating the importance of testing imipenem susceptibility and metallo β lactamase production simultaneously. Loss of bla_{IMP-1} from A. baumannii isolates stored at room temperature has been observed by other investigators also.[26]

46/138(33.3%) of our strains were positive for modified carbapenem inactivation method (mCIM). Modified carbapenem inactivation method demonstrated a sensitivity of >99% and specificity of >99% for detection of KPC, NDM, VIM, IMI, IMP, SPM, SME and OXA-type carbapenemases among Enterobacteriaceae isolates and also demonstrated a sensitivity of >97% and specificity of 100% for detection of KPC, NDM, VIM, IMI, IMP, SPM and OXA-type carbapenemases among Pseudomonas aeruginosa isolates as investigated by CLSI.[5] Since 2018 the CLSI has standardized mCIM for suspected carbapenemase production in Enterobacteriaceae and Pseudomonas aeruginosa but not for A.baumannii.[5]Performance for detecting other carbapenemases or testing isolates of non-Enterobacteriaceae other than Pseudomonas aeruginosa has not been established. Performing mCIM with Acinetobacter spp. is not endorsed by CLSI currently due to poor specificity and poor reproducibility between laboratories.

The Carba NP test is a colorimetric assay for detection of carbapenem hydrolysis. It was introduced as a screening test for carbapenemase detection among Enterobacteriaceae, Acinetobacter spp. and P. aeruginosa. Only 26/138 (18.8%) and 45/138 (32.6%) of our isolates were positive by RAPIDEC®Carba NP test (bioMérieux) and modified in-house Carba NP test respectively. In a comparison study, colorimetric assays like RAPIDEC® Carba NP test for detecting carbapenemase - producing bacteria showed a sensitivity of 91.9% and specificity of 83.9% for Enterobacteriaceae and a sensitivity of 90.9% and specificity of 88.2% for Pseudomonas aeruginosa.[28]But the sensitivity and specificity for carbapenemase detection in Acinetobacter spp. was reported to be only 36.4% and 75% respectively.[28] This test is also an accurate and simple biochemical test for the initial screening of carbapenemase production in Enterobacteriaceae, although it may not be as sensitive for detecting OXA-48 producers.[28]Carba NP test is now recommended for testing suspected carbapenemase production in Enterobacteriaceae and Pseudomonas aeruginosa but not for Acinetobacter spp. by the latest CLSI guidelines.[6]Major drawbacks are that invalid results occur with some isolates, certain carbapenemase types (e.g, OXA-type, chromosomally encoded) are not consistently detected and false negative results may occur due to plasmid loss in the isolates for testing, the presence of non-expressed carbapenemase gene or low-level carbapenemase expression.[29] Modified Carba NP test was found to be simple rapid and cost effective method of detecting carbapenemase production in Enterobacteriaceae in the study by Rudresh et al. in 2017[15] and Kumar N et al.in 2018.[30]

In the present study, 67/138(48.6%) and 34/138(24.6%) of our isolates tested positive for OXA-48 type production by temocillin disc diffusion test (Rosco Diagnostica) and growth on chromID® CARBASMART OXA medium (bioMérieux). Optimal screening agents and methods for OXA-48 carbapenemases have yet to be defined. The addition of a temocillin disc has been proposed as a surrogate marker of OXA-48 and functionally related variants.[16,19]The major drawback of temocillin disc diffusion test is that the resistance to temocillin is also observed among organisms producing metallo β lactamases (MBL) and can be exhibited by those expressing KPC or MBL-producing strains as well as for AmpC producers with porin loss or even those not producing any carbapenemase.[19] In our study a 100% sensitivity is shown by temocillin disc diffusion test, but due to the above reason, there is a high chance of some of them being false positive. As production of OXA-48 confers high-level resistance to temocillin compounds, isolates without PBA or DPA synergy and a temocillin inhibition zone 10 mm (absence of inhibition zone) or MIC 128 mg/L were suspected to be OXA-48 producers. It should be noted that the OXA-48 disc test accurately detected OXA-48 producers even in cases of strains that exhibited low carbapenem MICs (imipenem and/or meropenem MICs of ≤2 μg/ml).[19] In this regard, it should be noted that temocillin resistance as a sole diagnostic marker of OXA-48 producers has proved to be insufficiently robust with a positive predictive value of 27.2% for OXA-48 enzyme detection so that it should always be used combined with other markers. Some authors have suggested to supplement the temocillin disc with avibactam (a non β-lactam inhibitor of class A, C and some D carbapenemases) for a better detection of OXA-48 enzymes.[19] Piperacillin-tazobactam in combination with temocillin can also be used to find OXA-48 producers as proved in a study by Huang et al. in 2013.[31] In geographical areas with a high prevalence of CPE, including OXA-48 producers, the use of temocillin discs (30μg) can significantly increase the sensitivity of screening procedures and should be considered in the absence of synergy with inhibitors of class A and B carbapenemases.[31]

Chromogenic screening agars are useful culture-based methods, but have primarily been developed for the recovery of Carbapenem-resistant Enterobacterales(CRE). ChromID® CARBA SMART (CARB/OXA) from bioMérieux, France, is a bi-plate, consisting of OXA-48 like screening media (chromID OXA-48) on one side and CRE screening media (chromID CARBA) on the other side. In our study we tried to determine the accuracy of detecting carbapenemase producing A. baumannii isolates by chromID® CARBA SMART (CARB/OXA). It was observed that only 24/138 (17.4%) were grown on CARB medium whereas 34/138 (24.6%) were grown on the OXA medium. In a comparison study by C Julie et al. in 2016 found out that chromID® CARBASMART (bioMérieux) was the least sensitive agar for the recovery of carbapenemase producing organisms, with a combined sensitivity and specificity of 90.9% and 88.2% when compared to CHROMagar™, mSuperCARBA™(CHROMagar company).[32]In a comparison study by Vrioni Georgia et al. in 2012 to evaluate the performance of chromID CARBA (bioMérieux) for detection of carbapenemase producing Enterobacteriaceae directly from rectal swabs it was observed that the specificity was greater for chromID CARBA (96.9%).[33]

Hence laboratory detection and identification of carbapenemase producing Acinetobacter strains by phenotypic methods is problematic; however, gains have been made with the increasing availability of modern methods, including mass spectrometry and molecular techniques.

Genotypic characterization of carbapenemase production

In our study, of the genotypic characterization among the carbapenemase producing A. baumannii, tested positive by four phenotypic tests such as Modified Hodge test, modified carbapenem inactivation method, RAPIDEC® Carba NP (bioMérieux) and modified in-house Carba NP tests**,** 86/138 (62.3%) were found to be bla_{OXA-23} positive which agreed with the molecular characterization previously observed in other studies globally. Genotypic characterization was not performed for isolates which were positive only for EDTA combined disc test for MBL production, as the gene tested was OXA-23 type carbapenemase alone and not MBL. Genotypic characterization among the suspected OXA-48 producing A. baumannii, tested positive by temocillin disc diffusion test and chromID® CARBASMART OXA (bioMérieux)medium determined that 35/138(25.4%) of strains were found to be bla_{OXA-48} positive. Molecular techniques have become the current mainstay and gold standard for carbapenemase detection. Gene-specific PCR-based techniques and, more recently, multiplex PCR and microarray techniques for detecting several carbapenemase genes in a single test, have been discovered, but are mostly focused on the detection of genes in Enterobacteriaceae. Genotypic methods provide the highest sensitivity and specificity when subtyping carbapenemases and offer clues to exploring antibiotic resistance genes. However, only known, targeted genes can be detected, leaving unknown or rare genes undiscovered.

In a report from SENTRY surveillance program, by Mendes RE et al.2009, the distribution of OXA-type genes among Acinetobacter spp. in Asia-Pacific nations was mainly comprised of OXA-23 type carbapenemase, while OXA-24/40 type and OXA-58 type were less common.[34] In a molecular epidemiology survey by Adams-Haduch JM et al. in 2011 from US, has shown that carbapenemase OXA-23 and OXA-51 were most common.[35] In a study from China by Jia W et al. in 2015, the molecular analysis of 100% of multi-drug resistant A. baumannii isolates demonstrated that the carbapenemase genes bla_{OXA-23} and bla_{OXA-51} were the most predominant genes.[36] bla_{OXA-23} was mostly detected in isolates from Asian countries but was also reported in South America and Europe;bla_{OXA-58} was also frequently found in Europe .$bla_{OXA-24/40}$ was mostly found in the Iberian peninsula and Asia, but also detected in Iran, Belgium, Czech Republic and the United States of America (USA). A molecular study from Pune by Atul Khajuria et al. in 2014, reported bla_{OXA-23} as the most prevalent gene (52.38%) which was similar to our findings.[37]

Validity of phenotypic tests for carbapenemase detection

Validity of Modified Hodge test, modified carbapenem inactivation method, RAPIDEC® Carba NP and modified in-house Carba NP tests were determined by comparing the result with polymerase chain reaction as the gold standard. Since OXA-23 type carbapenemase is the most prevalent carbapenemase detected worldwide in the Acinetobacter baumannii strains, in our study also used conventional PCR to detect bla_{OXA-23} among all carbapenem resistant strains. Sensitivity of Modified Hodge test was found to be the highest (76.5%) while in-house Carba NP test had the highest specificity (88.7%). Modified Hodge test was widely used as a phenotypic technique for detecting carbapenemase activity since it was readily available in clinical microbiology routine settings and was also recommended by CLSI but high rate of false negative results were seen among KPC, NDM-1 and OXA-48 producers and false-positive results can occur in isolates that produce ESBL or AmpC enzymes coupled with porin loss as well. No data exist on the usefulness of these tests for the detection of carbapenemase production in non-fermenting gram-negative bacilli and it is not recommended by CLSI since 2018.[5]In a comprehensive method comparison study to assess the accuracy of 11 carbapenemase detection assays on a collection of well-characterized Enterobacteriaceae isolates only by Tamma et al. in 2016, the sensitivity of the assays ranged from 72% for the boronic acid synergy test for the detection of KPC producers to 99% for the modified Carba NP assay for the detection of all carbapenemase producing Enterobacteriaceae (CPE).[28] In the same study three tests achieved a sensitivity of 98%, including the RAPIDEC® Carba NP,the modified Carba NP, and the mCIM.[28] Moreover, the sensitivity varied by carbapenemase enzyme group such that most of the rapid colorimetric assays tested and the mCIM were less sensitive for the detection of OXA-48type producers. Interestingly, false-negative results were observed with several of the assays for a KPC producers with either low carbapenem MICs (low-level expression) or those produced by organisms that were mucoid in nature.[28,39]

In a comparative study by Rudresh SM et al. 2017 from Karnataka observed a sensitivity of Carba NP, CarbAcineto NP (CANP), and modified Carba NP (m CNP) tests for carbapenemase detection as 77.7%, 80.6%, and 62.1%, respectively with 100% specificity.[15] In a study by Kumar N et al. in 2018, it was observed that modified carba NP test had

91.7% sensitivity and 100% specificity for KPC and 96.7% sensitivity and 100% specificity for MBL detection in Klebsiella species[30]. In a study by Knox J et al. in 2016, a good sensitivity (72.5-100%) and excellent specificity (100%) was reported for Carba NP test for detection of carbapenemase producing Enterobacteriaceae (CPE) and Pseudomonas aeruginosa.[38]Highest sensitivity was shown by temocillin disc diffusion (100%) whereas highest specificity was for chromID® CARBA OXA medium (bioMérieux) (98.1%) in detecting OXA-48 type carbapenemase in our study. In a comparative evaluation study by Girlich et al., in 2013 when they compared chromID CARBA plus chromID OXA-48 (bioMérieux) with SUPERCARBA for the detection of OXA-48 like producing Enterobacteriaceae it was found that chromID OXA-48 medium and SUPERCARBA had the highest sensitivity for detection of OXA-48 producing Enterobacteriaceae (91% and 93%) compared to chromID CARBA (21 %) which correlates with our findings.[40]

Sequence Analysis

501 bp amplified product confirmed to be bla_{OXA-23} gene by automated sequencing. 300 bp product found after bla_{OXA-48} amplification could be any variant of OXA-48 type carbapenemase but was unable to obtain good quality sequence and could not confirm with sequence analysis.

Turnaround time of phenotypic and genotypic tests

In our study, better turnaround time of 30 minutes - 2 hours was observed with various phenotypic tests such as RAPIDEC®Carba NP and modified in-house Carba NP tests. But the major drawbacks of RAPIDEC®Carba NP test is that it has low sensitivity (36.4%) and specificity (75%) for detecting carbapenemse production amongst Acinetobacter species, false negative results are more common with OXA-23 type and OXA-48 type carbapenemases and moreover RAPIDEC® Carba NP is not cost effective. Molecular techniques are considered as more accurate and confirmatory tests for detecting carbapenemase production in A. baumannii.

Limitations of the present study: For genotypic characterization, only OXA-23 type and OXA-48 type primers were used. Presence of other oxacillinases like OXA-72, OXA-40, OXA-51, OXA-58 etc., KPC - type and MBL carbapenemases were not verified by PCR.

CONCLUSION

The rise in the prevalence of carbapenemase producing A. baumannii is creating a therapeutic challenge for clinicians and microbiologists in our hospital. The emergence of resistance to the antibiotics should be constantly monitored so that the occurrence of the newer resistance patterns in the hospital can be determined. There is also a necessity to highlight on the rational use of antibiotics and strict adherence to the concept of reserve drugs to reduce the indiscriminate and inappropriate use of the available antibiotics. Various strategies like hand washing, environmental decontamination, education to the health care professionals especially targeting the nursing staff and continuous surveillance will be helpful in achieving the goal of decreased infection rate with multidrug resistant pathogens. The hospital infection control guidelines should be formulated strictly and all Health care workers should be enforced to follow these guidelines stringently so as to avoid the future spread of these drug resistance strains.

Hence this study helps in identification of Acinetobacter baumannii strains, their resistance pattern, risk assessment and in the prevention and control of these hospital acquired infections thereby reducing mortality among patients in medical and surgical intensive care units. Early detection is always critical the benefits of which include timely execution of strict infection control practices, formulating an effective antibiotic policy to prevent the spread of these carbapenemase producing strains, and treatment with alternative last - resort antimicrobials, thereby arresting the spread of antibiotic resistant strains to improve the clinical outcome among patients harbouring these organisms. Early detection methods were compared for its sensitivity and specificity to diagnose carbapenemase producing Acinetobacter strains and a single phenotypic test by itself cannot be recommended for detection of carbapenemases in Acinetobacter baumannii due to the decreased sensitivity and specificity.

Using PCR as gold standard, Modified Hodge test had the highest sensitivity (76.5%) and modified in-house Carba NP test had the highest specificity (88.7%) for detecting bla_{OXA-23}carbapenemase gene. Genotypic characterization among the suspected OXA-48 producing A. baumannii, tested positive by temocillin disc diffusion test and chromID® CARBA SMART OXA medium (bioMerieux), 35(25.4%) of strains were found to be bla_{OXA-48} positive. Highest sensitivity was shown by temocillin disc diffusion (100%) whereas highest specificity was for chromID®CARBA SMART OXA medium (bioMerieux) for detection of bla_{OXA-48} type carbapenemase (98.1%). Lowest turnaround time was for RAPIDEC®Carba NP and modified in-house Carba NP tests (30 minutes-2 hours). We recommend the use of EDTA combined disc test for detection of MBL in combination with modified in-house Carba NP test and chromID® CARBASMART CARB/OXA (bioMerieux) for detection of OXA type carbapenemases as evidenced by comparison with PCR. In resource limited areas above phenotypic tests can be used for detecting carbapenemase producing isolates.

To conclude, updates on the knowledge of local antimicrobial resistance patterns recognized in the hospital, implementation of easy and reliable phenotypic methods for detecting carbapenemase production in routine clinical microbiology laboratories, continued vigilant attention to hand hygiene, barrier precautions, contact isolation, 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 extensively drug resistant strains of Acinetobacter baumanii.

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