

1 **Direct determination and differentiation of carbapenemases of *A. baumannii* from**
2 **uncultured tracheal samples**

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9 **Running title:** CarbaNP of *A. baumannii* in tracheal aspirates

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13 **Abstract**

14 The emergence of multiple carbapenemases and the consequent multi drug resistance in bacteria
15 constitute a grave concern in the management of critical care patients and also in community
16 acquired infections. Detection of carbapenemase activity helps to understand the possible
17 mechanism(s) of carbapenem resistance in the microorganism. Identification of carbapenemases
18 is currently being done by various phenotypic methods and molecular methods. However,
19 innovative biochemical and spectrophotometric methods are desirable as they will be easy to
20 perform, affordable and rapid. Recently a novel chromogenic method called CarbaNP test was
21 introduced to screen for carbapenemases in clinical isolates of gram negative pathogens. We
22 adopted this assay: (i) to detect the total carbapenemase activity (ii) to measure the relative rates
23 of hydrolysis of imipenem by class A, B and D carbapenemases with inhibitors (iii) to confirm
24 the genotype by PCR and (iv) for direct differential detection of various carbapenemases in
25 uncultured clinical sample like tracheal aspirate. The study included 75 culture isolates and 153
26 purulent tracheal aspirates. All isolates were screened by our optimized protocol and also
27 genotyped by PCR. This adopted assay showed good sensitivity and correlation with
28 conventional phenotyping and genotyping. Our protocol offers the fastest way to identify the
29 pathogen by PCR but also its carbapenemase profile directly from uncultured clinical samples in

30 less than 4h. Our protocol is currently being validated on other types of clinical specimens in our
31 laboratory.

32 **Keywords:** Multidrug resistance, *A.baumannii*, carbapenemases, CarbaNP, tracheal aspirates

33 **Introduction**

34 The emergence of multidrug resistance (MDR) among gram negative pathogens is severely
35 hampering the management of infections in the hospitals as well as in the community.
36 Carbapenemases hydrolyse beta lactam antibiotics and all types of carbapenems. Their
37 production is one of the most important traits which confers resistance to beta lactam antibiotics
38 including carbapenems [1, 2]. In recent years laboratory detection of carbapenemase producers
39 has become more challenging due to the emergence of diverse carbapenemases [3, 4]. Rapid
40 detection and carbapenem sensitivity profiling of these pathogens have become imperative for
41 successful management of infections and also to control dissemination. Antimicrobial
42 susceptibility tests are usually performed on agar plates or in automated systems.
43 Carbapenemases are not the sole cause of resistance, other mechanisms such as porin loss or
44 increased efflux pump activity in the bacterial membrane may also influence the drug sensitivity
45 [5, 6, 7 & 8]. Therefore a definite method is required to distinguish enzymatic and non-
46 enzymatic resistance mechanisms for effective management of infection control and epidemic
47 outbreaks [2, 9]. Phenotypic methods like Modified Hodge Test (MHT) and disk diffusion tests
48 with inhibitors are commonly used in microbiology laboratory to test the carbapenemase activity
49 of isolated bacterial cultures. However, these methods show lower specificity and sensitivity
50 compared to molecular methods [10]. Identification of the resistance gene is considered as gold
51 standard for the confirmation of drug resistance, though they are economically and technically
52 beyond the reach for many clinical laboratories [3, 5]. Thus, there has been an unmet need for a
53 simple, affordable and yet direct demonstration of carbapenemase activity in any given bacterial
54 pathogen. To fill this important gap, Nordmann group designed and developed a novel yet simple
55 phenotypic method called CarbaNP test, for early detection of carbapenemases in
56 *Enterobacteriaceae* [2]. This phenotypic assay is based on the change in pH caused due to
57 hydrolysis of imipenem by carbapenemase(s) present in the cell free bacterial lysate. Phenol red
58 is used as the indicator to monitor this change in pH, which turns from red (alkaline) to yellow
59 (acid) [2, 3, 11 and 12]. This test has become a boon for rapid identification of carbapenemase

60 production in gram negative bacterial pathogens, considering the difficulties in the interpretation
61 of results and false positivity with various other phenotypic methods. CarbaNP is a huge
62 improvement over other phenotypic methods with almost 100% sensitivity and its specificity is
63 comparable to molecular methods [2]. Several modifications have been made to this assay in
64 order to improve its performance characteristics and avoid false negatives [10, 13 and 14].
65 Different classes of carbapenemases have been reported in gram negative pathogens. There are
66 three major classes of carbapenemases, grouped according to their amino acid composition and
67 identity [15]. Based on the action of beta-lactamase inhibitors (tazobactam, clavulanic acid and
68 divalent chelator such as EDTA) CarbaNP assay has been modified to identify different classes
69 of carbapenemases [12] in gram negative bacterial isolates. Later, “Blue-Carba”, was designed
70 using bromothymol blue as an indicator for direct colony approach instead of bacterial extracts
71 [16]. Originally CarbaNP assay was employed for members of *Enterobacteriaceae* isolates, later
72 it was applied to isolates of *Acinetobacter* sp. with minor changes in inoculum size and lysis
73 conditions [17]. The main objective of this study is to adopt and evaluate this CarbaNP assay for
74 the rapid identification and differentiation of various classes of carbapenemases on clinical
75 isolates of *A.baumannii* and extend it to uncultured clinical specimens. In this study we adopted
76 CarbaNP test of Nordmann’s with some modifications in sample processing and assay protocols.
77 In addition, we validated our protocol directly on uncultured tracheal aspirates also. We included
78 specific inhibitors for differential measurement of carbapenemases belonging to Class A, B, and
79 D. We believe that these modifications simplify the assay for application to both culture isolates
80 and directly to clinical samples also. These uncultured tracheal aspirates were further subjected
81 to species identification and “carbapenamase” genotyping by multiplex PCR.

82 **Materials and Methods**

83 The study included 75 clinical isolates of *Acinetobacter baumannii* and 153 purulent tracheal
84 aspirates were used for the development and validation of our assay. The clinical isolates and the
85 tracheal aspirates, which formed part of routine work, were provided by the clinical
86 microbiology laboratory at two tertiary care hospitals in Hyderabad. Species identification and
87 antimicrobial susceptibility testing were done in the microbiology laboratories on VITEK-2 in
88 accordance with CLSI guidelines and results were decoded later for comparative analysis with
89 our CarbaNP and genotyping studies.

90 **Preparation of genomic DNA of *A. baumannii*:** Pure single colony of each isolate was
91 suspended in 100 μ l of TEX buffer (10 mM Tris-HCl pH 8.5, 1 mM EDTA, 1% (w/v) Triton X-
92 100). The suspension was vortexed to achieve a uniform suspension and centrifuged. The pellet
93 was subjected to another wash in TEX buffer. Finally the pellet was resuspended in 100 μ l of
94 TEX buffer and lysed by heating in a dry bath at 95°C for 15min [18]. The lysate was used as the
95 DNA template for PCR amplifications. All isolates were screened by PCR using primers
96 designed for the carbapenemase genes-*bla*_{KPC}, *bla*_{NDM-1}, *bla*_{OXA-23-like}, *bla*_{OXA-51-like} and the
97 species marker (Ab-ITS) of *A. baumannii* as described in “Table-1” [19-22]. Each selected target
98 in this assay is the most common among carbapenemases described.
99 The species marker (Ab-ITS), *bla*_{OXA-23-like}, *bla*_{OXA-51-like} were identified by multiplex PCR and
100 amplification conditions are as follows; initial denaturation at 95°C for 10min, followed by 35
101 cycles at 94°C for 30s, 50°C for 40s and 72°C for 50s and a final extension at 72°C for 5 min.
102 Whereas *bla*_{KPC}, *bla*_{NDM} PCR conditions consisted of an initial denaturation at 95°C for 10min,
103 followed by 35 cycles of 94°C for 30s, 58°C for 40s, 72°C for 50s (*bla*_{NDM}) and 94°C for 30s,
104 55°C for 30s (*bla*_{KPC}) and 72°C for 50s, and a final extension at 72°C for 10min for both gene
105 targets. The multiplex PCR products of 501bp (*bla*_{OXA-51-like}), 353bp (*bla*_{OXA-23-like}), 208bp (Ab-
106 ITS) and amplicons of 660bp (*bla*_{NDM-1}), 246bp (*bla*_{KPC}) were visualized in a 2% agarose gel
107 after electrophoresis (w/v) and staining with ethidium bromide.

108 **CarbaNP Assay:**

109 The CarbaNP assay (Nordmann *et al*, 2012) was performed as described below with some
110 modifications to the original protocol. Colonies of *A. baumannii* grown on Muller Hinton Agar
111 plates with imipenem disks for 24h were used. A single calibrated loop (5 μ L) of bacterial mass
112 was suspended in 100 μ L of TX buffer (10 mM Tris-HCl pH 8.5, 1% w/v Triton X-100) vortexed
113 for one minute and further incubated at 37°C for 1hour. The lysed bacteria were centrifuged at
114 10,000 rpm at room temperature for 5 min and the cell free supernatant was used for
115 carbapenamase assay. Tracheal aspirates were processed as follows (Figure-1): 150 μ L of the
116 tracheal aspirate was taken into 1.5mL eppendorf tube and centrifuged for 5 min at 10,000rpm.
117 The supernatant was discarded and the pellet was washed as before with TX buffer twice; finally
118 100 μ L of TX lysis buffer was added to the pellet and subjected to vortex in pulses of 30 s for 2
119 min, incubated at 37°C for 1h for maximum lysis of bacteria and centrifuged for 5 min at
120 10,000rpm. The cell free supernatant was used for the CarbaNP assay. The assay was performed

121 in sterile 96 well microtiter plate. 30 μ L of cell free supernatant was mixed with 100 μ L assay
122 solution containing 3mg/L of imipenem, 40 μ L of 0.1 mM ZnSO₄ and 30 μ L of phenol red
123 indicator (pH8.5) in respective wells. 5 μ L of 3mM of Ethylene Diamine Tetra Acetic acid
124 disodium salt (EDTA for Metallo β -lactamases) or 1 μ L of 8 mg/ml of Phenyl Boronic Acid
125 (PBA for Class A enzymes) or 1 μ L of 100 mM of NaCl (for Class D enzymes) was added
126 depending on which enzyme was being assayed. After addition of inhibitor, 40 μ L of 0.1 mM
127 ZnSO₄ and 30 μ L of phenol red (pH8.5) were added to the wells and incubated at 37⁰C up to 2 h.
128 The carbapenemase activity was identified by a change in color of phenol red to yellow. The
129 absorbance of the solution in the microtiter wells was measured at different time intervals (T₀,
130 T₁₅, T₃₀, T₆₀ and T₁₂₀ min) at 546 nm.

131 RESULTS

132 Carbapenemase detection:

133 To extend the chromogenic assay protocol to uncultured clinical samples: first we optimized the
134 assay on a few culture isolates (n= 75) of *A. baumannii*. These isolates were screened for species
135 specific marker Ab-ITS (16S-23S rRNA spacer region) and carbapenemase genes through PCR.
136 The distribution of carbapenemase genes among these culture isolates of *A. baumannii* were as
137 follows: most of them, 97.3% (73/75), carried the *bla*_{OXA-51-like} gene, and *bla*_{OXA-23-like} gene,
138 85.3% (64/75). Further, *bla*_{NDM-1} and *bla*_{KPC} were detected in 44% (33/75) 30.6% (23/75) of the
139 isolates. A total of 153 purulent tracheal aspirates were directly processed for CarbaNP test and
140 genotyping. The phenotypic (VITEK-2) data were blinded to us. Hence, we screened them first
141 for the species genetic marker (Ab-ITS) and subsequently were subjected to carbapenemase
142 genotyping. Out of the 153 purulent tracheal aspirate samples, 103 (65.1 %) were identified as *A.*
143 *baumannii* by species specific marker. 52 (32.9%) of them carried *bla*_{OXA-51-like} and 37 (23.4%)
144 carried *bla*_{OXA-23-like}. *bla*_{KPC} and *bla*_{NDM-1} were present in 26 (17%) and 39(25.4%) tracheal
145 aspirate samples respectively. Genotypic results are presented in Table-2.

146 Validation of CarbaNP assay:

147 To validate CarbaNP assay *Escherichia coli* (NCTC 10418), *Pseudomonas aeruginosa* (NCTC
148 10662), *Klebsiella pneumonia* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606) strains
149 as positive controls and *Staphylococcus aureus* (ATCC 25923, ATCC 43300) strains were used
150 as negative controls. The assay mixture contained buffered cell free extract, imipenem and

151 ZnSO₄ which when incubated at 37⁰C turned from red to orange or yellow depending on the
152 enzyme activity or remained red for bacterial extracts which did not contain any carbapenemase.
153 Our CarbaNP test results showed absolute correlation to our genotype results. Out of 75 culture
154 isolates 73 contained *bla*_{OXA-51}, 24 contained *bla*_{OXA-23} plus *bla*_{OXA-51} and 3 were NDM producers
155 (*bla*_{OXA-51} plus *bla*_{NDM-1}). Multiple carbapenemase producers were 16 (*bla*_{OXA-51}, *bla*_{NDM-1},
156 *bla*_{OXA-23}), 2 (*bla*_{OXA-51}, *bla*_{NDM}, *bla*_{KPC}), 10 (*bla*_{OXA-51}, *bla*_{OXA-23} and *bla*_{KPC}) and 12 (*bla*_{OXA-51},
157 *bla*_{OXA-23}, *bla*_{NDM} and *bla*_{KPC}) respectively. 6 isolates were completely negative for
158 carbapenemases other than *bla*_{OXA-51}. The rate of hydrolysis of imipenem was monitored at
159 different time periods *i. e* 0 min, 15min, 30min, 60min and 120min with and without the
160 appropriate carbapenemase inhibitor. The rate of hydrolysis was different for single
161 carbapenemase, two carbapenemase and multiple carbapenemase producers. The results of these
162 enzyme assays are represented in fig-2. EDTA was used as inhibitor for Class B (Metallo Beta
163 Lactamase, MBL) type carbapenemases, NaCl for Class D (Oxacillinases, OXA) and Phenyl
164 Boronic Acid (PBA) was used as specific inhibitor of Class A type (*K. pneumoniae*
165 carbapenemase, KPC) enzymes. CarbaNP assay results “(see table 3)” were interpreted as
166 follows: 1) if the color turned from red to orange or yellow in the presence of 3mM of EDTA
167 (inhibits MBL) and 100mM of NaCl (inhibits OXA type) whereas wells containing PBA
168 remained red in color, the isolate was KPC producer; 2) if the color in the wells turned from red
169 to orange or yellow in the presence of PBA and 100mM of NaCl whereas wells containing
170 EDTA remained red in color, the isolate was MBL (*bla*_{NDM-1}) producer; 3) if the color in the
171 wells turned from red to orange or yellow in the presence of PBA and EDTA whereas wells
172 containing NaCl remained red in color, the isolate was oxacillinase (*bla*_{OXA-23} and *bla*_{OXA-51})
173 producer; and 4) if the color of the wells remained red under all the above conditions, the isolate
174 was considered a non-carbapenemase producer. Assay results were categorized based on
175 carbapenemase genotype data into 4 groups: (i) all positive, (ii) OXA-51, OXA-23 positive and
176 others negative, (iii) NDM negative and others positive and (iv) KPC negative and others
177 positive. Colorimetric changes after 30 min incubation at 37⁰C are presented in fig-3.
178 Absorbance at 546nm of all categories were noted and represented in graph. 30 min incubation
179 was used for screening and for inhibitor assays. Results were depicted in fig-4.
180 The relative orders of rate of imipenem hydrolysis (total carbapenem activity) by these groups of
181 clinical isolates of *A.baumannii* were as follows:

182 Class A, B and D (+) (**all positive**) > **Class A (-) and B, D (+)** > **Class D (+) and B, D (-)** >
183 **Class B (-) and A, D (+).**

184 This summary of correlation between CarbaNP test and genotyping results may need further
185 validation in other laboratories and for other genotypes of carbapenemases.

186 A total of 153 tracheal aspirates were included in this study. Phenotypic data of these clinical
187 samples was blinded to our laboratory and the results were decoded after genotyping and
188 CarbaNP study. Subsequently modified chromogenic assay was performed on the cell free
189 extracts from these tracheal aspirates. These samples were processed as described earlier. Results
190 of CarbaNP assay were compared with both VITEK-2 results as well as our genotype PCR
191 results.

192

193 **Discussion**

194 The massive dissemination of carbapenemase producers among *Enterobacteriaceae* demands
195 that methods to quickly determine the microbial species and also the drug sensitivity profile are
196 available in the microbiology laboratory. High sensitivity and specificity together with a rapid
197 workflow has become mandatory to devise therapeutic strategies to treat dangerous pathogens
198 and to control their spread [11, 12]. The application of CarbaNP in this study shows that it is
199 capable of detecting different carbapenemases in Gram negative pathogens allowing distinction
200 between enzymatic and non-enzymatic mechanisms of carbapenem resistance. The adaptation of
201 CarbaNP method showed multiple advantages over the other phenotypic screening methods.
202 CarbaNP has high sensitivity, specificity and is easily available for prompt identification of
203 carbapenemases [11, 23]. Original CarbaNP test was validated on clinical isolates of various
204 *Enterobacterial species* [2] and subsequently on clinical isolates of *A. baumannii* [17]. Recently
205 the assay was adopted to detect “carbapenemase” activity in spiked blood cultures [24]. This
206 method eliminates false positives compared to boronic acid synergy test, the modified Hodge test
207 (MHT), and drastically reduces time compared to disc diffusion/E-Test methods [25, 26]. The
208 carbapenem inactivation method (CIM), and the modified carbapenem inactivation method
209 (mCIM) reported recently require at least 24-72h for results even though it costs less than <1\$ [5,
210 27]. Rapidec CarbaNP test kit (BioMerieux, France), Neo-Rapid Carb screen kit, Rapid Carb
211 Blue screen (ROSCO Diagnostics Denmark) are commercial kits available for screening clinical
212 isolates for carbapenemase activity [14, 28, 29]. MALDI-TOF MS is effective method compared

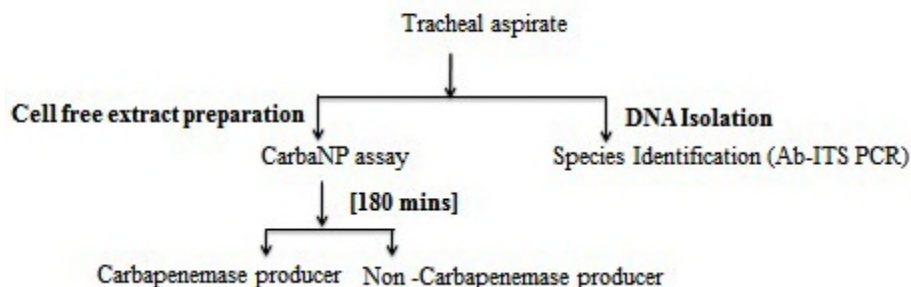
213 to Rapidec CarbaNP test kit to discriminate between carbapenemase and non-carbapenemase
214 producers [30, 31]. As an alternative to molecular assay, antibody based methods such as lateral
215 flow immunoassay (LFIA) has been developed for detection carbapenemase production. Lateral
216 flow assay yield results from cultured strains within 15min with 100% sensitivity and specificity
217 [32, 33]. The manual versions of rapid calorimetric assays includes manual Carba NP CLSI
218 method, manual Blue Carba, and modified Carba NP which requires reagent preparation
219 compared to ready to use kit methods [16,29, 34]. These CarbaNP tests available till date help to
220 reduce the time taken to identify carbapenemase activity in clinical isolates. However, the full
221 potential of the test could be realised only when the test is applicable directly to uncultured
222 clinical samples so that therapeutic decisions can be made quickly. We attempted to fulfill this
223 gap. We have simplified the cell free extract preparation and validated it directly on purulent
224 tracheal aspirates to detect, quantitate and also differentially demonstrate various carbapenemase
225 activities using specific inhibitors. Remarkable correlation was observed in our study between
226 the genotypic test results (PCR) and CarbaNP assay results. CarbaNP method greatly reduces
227 cost and labor with excellent accuracy in results even with uncultured tracheal aspirates. The
228 convenience of CarbaNP test is notable as it doesn't require any specialized equipment and needs
229 a short hands-on time to perform and requires only single colony from the agar plate or 150 μ L
230 of the tracheal aspirate. It is easily scalable to a high throughput format for large number of
231 Gram negative isolates and adaptable in many laboratories. This adapted assay can be used for
232 rapid differential diagnosis of carbapenemases directly in clinical samples and clinical isolates.
233 Blindfolding the tracheal aspirate (n=153) samples for the genotyping/CarbaNP screening by the
234 microbiology laboratory shows that our CarbaNP test format is applicable and adaptable as a
235 routine in a clinical microbiology laboratory. Remarkable correlation between genotyping and
236 CarbaNP test results shows that this colorimetric assay is reliable and accurate. We have
237 optimized the average incubation time as 30 min. Inclusion of inhibitors seem to be an attractive
238 option to narrow down the drug sensitivity profile compared to expensive, time consuming disc
239 diffusion or E-test. *bla*_{KPC}, *bla*_{NDM} were used as typical member of the Class A and B enzymes in
240 PCR genotyping. Considering the prevalence in *A. baumannii*, both *bla*_{OXA-23} and *bla*_{OXA-51} were
241 screened for Class D (CHD) in culture isolates. Whereas in tracheal aspirates Class D enzymes
242 (*bla*_{OXA-23} and *bla*_{OXA-51}), Class B (*bla*_{NDM}) and Class A (*bla*_{KPC}) were screened to correlate with
243 phenotyping results along with species identification. The data from these genotyping were used

244 to evaluate the merit of the CarbaNP test for differentiating various classes of carbapenemases.
245 We introduced certain modifications to Nordmann's protocol to make it more user friendly, to
246 reduce the quantity of cells required and to apply it directly to uncultured tracheal aspirates.
247 These modifications include (i) extraction buffer i.e TX buffer (10 mM Tris- HCl pH 8.5, Triton
248 X 100, 1% w/v) used for bacterial cell lysis (ii) Extraction was performed by vigorous vortexing
249 and incubation at 37⁰C for one hour for cell lysis (iii) Rate of imipenem hydrolysis was
250 monitored at 546 nm for different time periods: 0m, 15m, 30m, 60m and 120m. This assay
251 clearly differentiated various classes of carbapenemases in culture isolates and uncultured
252 clinical samples. We are validating our protocol on other types of clinical samples currently and
253 also for other gram negative pathogens. All the carbapenem resistant isolates showed some
254 (intrinsic) carbapenemase activity due to the presence of *bla*_{OXA-51} and other isolates showed
255 incrementally higher hydrolysis depending on whether they contained one, two or more
256 carbapenemases. Whereas reaction is completely inhibited in the presence of inhibitors resulting
257 in no change in the absorbance at 546 nm at different time periods. Graphical representation of
258 rate of hydrolysis with or without inhibitors clearly indicates that identification and
259 differentiation of carbapenemase activity would be a valuable tool in characterization of isolates
260 and monitoring their spread. The sensitivity and specificity of this assay were found to be
261 excellent when compared with VITEK-2 (sensitivity and specificity was 100%). This CarbaNP
262 test offers an economical and faster way to profile the carbapenemases in isolates of *A.*
263 *baumannii* and also in uncultured tracheal aspirates faster than even molecular techniques. The
264 format can be easily adopted for a high throughput screening of carbapenemases in clinical
265 samples or isolates. We hope it will become a valuable and useful tool in clinical microbiology
266 screening and to study the epidemiology of carbapenemase producers [12].

267 **Conclusion**

268 We propose the following algorithm to screen uncultured clinical samples directly for
269 carbapenemase activity:

Algorithm for screening clinical specimens for carbapenemase activity



270

271 This algorithm will intensify the quick identification of carbapenemases for proper infection
272 control measures. The conventional microbiology results are made available in 3-4 days for
273 species identification and drug sensitivity profile. Our protocol which includes a simple one step
274 PCR for species identification and CarbaNP test determination of carbapenemase activity
275 promises to make the same information available to the clinician the same day, both together
276 precisely in less than 4 h. This is potentially a remarkable improvement and it is hoped that will
277 revolutionalise the way multidrug resistance will be identified and used for the management of
278 patients and to control its spread in the society.

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Table-1: Primers used in this study:

Target	Primer name	Sequence (5'-3')	Amplicons size(bp)	Reference
Species specific marker (Ab- ITS)	Ab- ITS F	CAT TAT CAC GGT AAT TAG TG	208	19
	Ab- ITS R	AGA GCA CTG TGC ACT TAA G		
<i>bla</i> _{OXA-51}	OXA-51 F	TAA TGC TTT GAT CGG CCT TG	353	20
	OXA-51 R	TGG ATT GCA CTT CAT CTT GG		
<i>bla</i> _{OXA-23}	OXA-23 F	GAT CGG ATTGGA GAA CCA GA	501	20
	OXA-23 R	ATT TCT GAC CGC ATT TCC AT		
<i>bla</i> _{KPC}	KPC-F	GAT ACC ACG TTC CGT CTGG	246	21
	KPC-R	GCA GGT TCC GGT TTT GTC TC		
<i>bla</i> _{NDM}	NDM-F	GGT GCA TGC CCG GTG AAA TC	660	22
	NDM-R	ATG CTG GCC TTG GGG AAC G		

Table -2: Results of genotyping and modified CarbaNP on both carbapenem resistant isolates and clinical samples.

Sample	Species marker	Class A	Class B	Class D		Modified carbaNP assay	
				OXA-51	OXA-23	CarbaNP	Inhibitor
Culture isolates (n=75)	75	23	33	73	64	68	64
purulent aspirates (n=153)	103	26	39	52	37	85	48

Table 3: Modified CarbaNP results with inhibitors for different classes of carbapenemases

S. No	Reaction mixture + Inhibitor	Color change	Enzyme category
1	1. EDTA +NaCl	Red → Orange /Yellow	CLASS A
	2. PBA	Red (No change)	
2	1. PBA + NaCl	Red → Orange /Yellow	CLASS B
	2. EDTA	Red (No change)	
3	1. PBA + EDTA	Red → Orange /Yellow	CLASS D
	2. NaCl	Red (No change)	
4	1. EDTA +NaCl	Red (No change)	NO CARBAPENEMASE ACTIVITY
	2. PBA + EDTA	Red (No change)	
	3. PBA + NaCl	Red (No change)	

Fig 1: Flow diagram of processing of tracheal aspirates

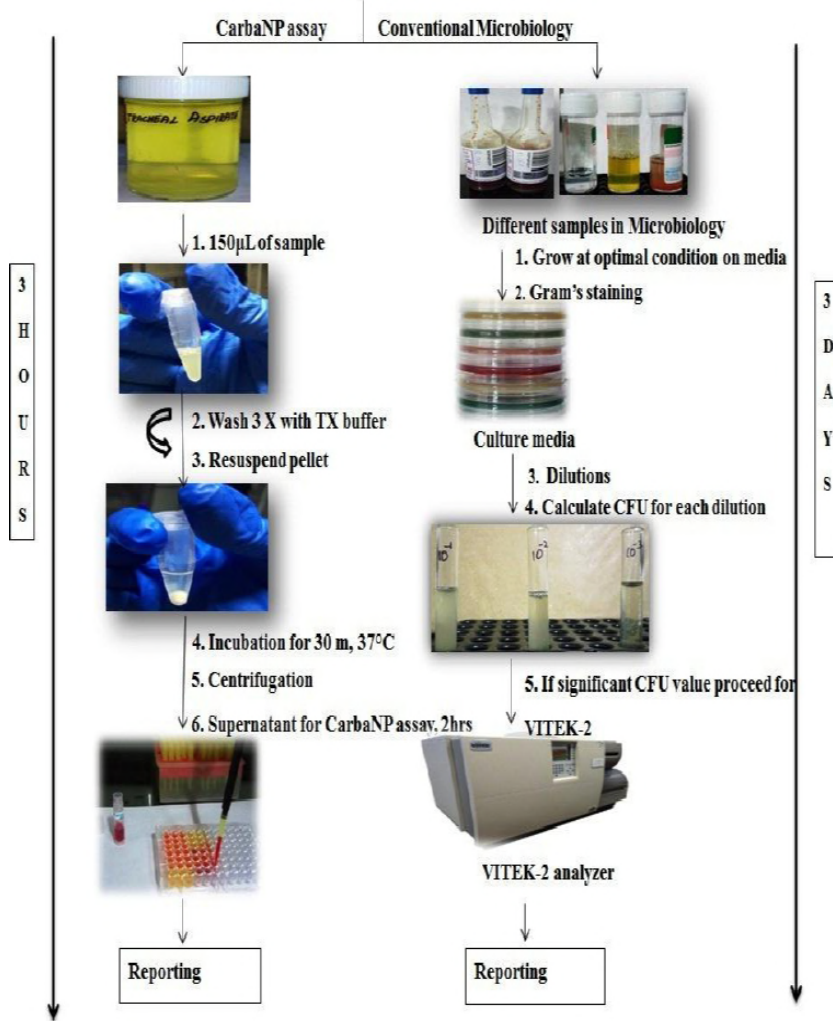


Fig -2: Rate of hydrolysis between single, double and multiple carbapenemase producers

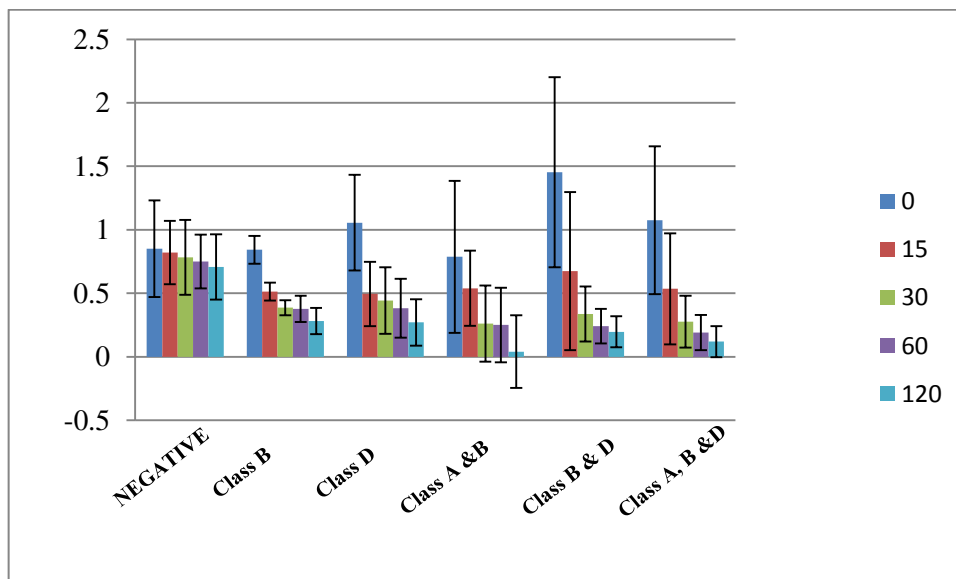


Fig -2: Graphical representation of rate of hydrolysis at T₀, T₁₅, T₃₀, T₆₀ and T₁₂₀

1. Negative: non carbapenemase producers
2. Class B: *bla*_{NDM}
3. Class D: *bla*_{OXA-51}; *bla*_{OXA-23}
4. Class A & B: *bla*_{KPC} *bla*_{NDM}
5. Class B & D: *bla*_{NDM}, *bla*_{OXA-51}, *bla*_{OXA-23}
6. Class A, B & D: *bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA-51}, *bla*_{OXA-23}
7. Error bars represent standard deviation of calorimetric measurements at 546nm.

Fig 3: Colorimetric changes during modified carbaNP assay in clinical isolates of *A. baumannii*

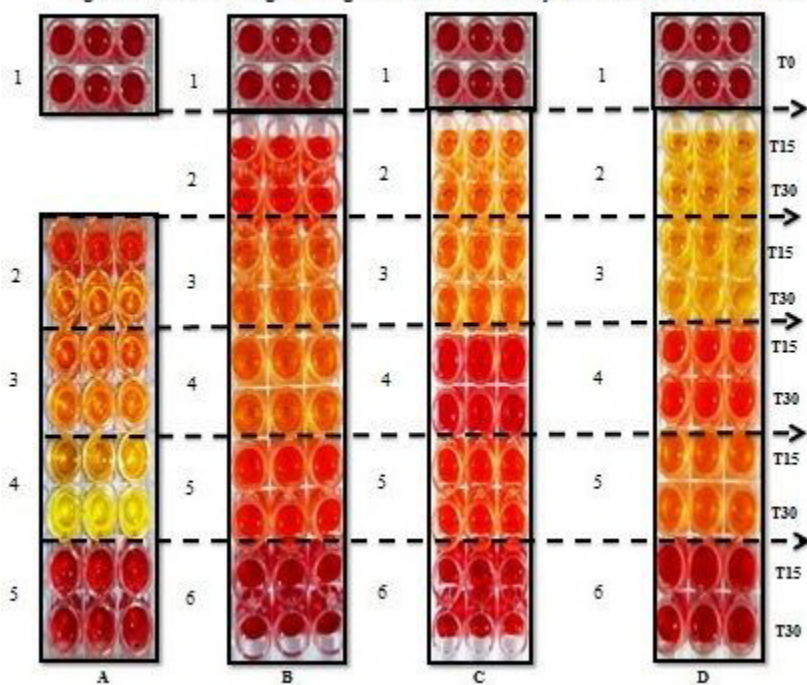


Fig -3: A:ALL POSITIVE; 1. Reaction mixture at T₀, 2. Imipenem solution + ZnSO₄+ PBA, 3. Imipenem solution + ZnSO₄+ EDTA, 4. Imipenem solution + ZnSO₄+ NaCl , 5. No imipenem

B: Class B Negative and others positive; 1. Reaction mixture at T₀, 2. Imipenem solution + ZnSO₄+ PBA, 3. Imipenem solution + ZnSO₄+ EDTA, 4. Imipenem solution + ZnSO₄+ NaCl, 5. Imipenem solution + ZnSO₄+ EDTA, 6. No imipenem.

C: Class D positive and others negative; 1. Reaction mixture at T₀, 2. Imipenem solution + ZnSO₄+ PBA, 3. Imipenem solution + ZnSO₄+ EDTA, 4. Imipenem solution + ZnSO₄+ NaCl, 5. Imipenem solution + ZnSO₄+ NaCl, 6. No imipenem.

D: Class A negative and others positive; 1. Reaction mixture at T₀, 2. Imipenem solution + ZnSO₄+ PBA, 3. Imipenem solution + ZnSO₄+ EDTA, 4. Imipenem solution + ZnSO₄+ EDTA, 5. Imipenem solution + ZnSO₄+ NaCl, 6. No imipenem.

Fig -4: Rate of hydrolysis at T₃₀ with inhibitors

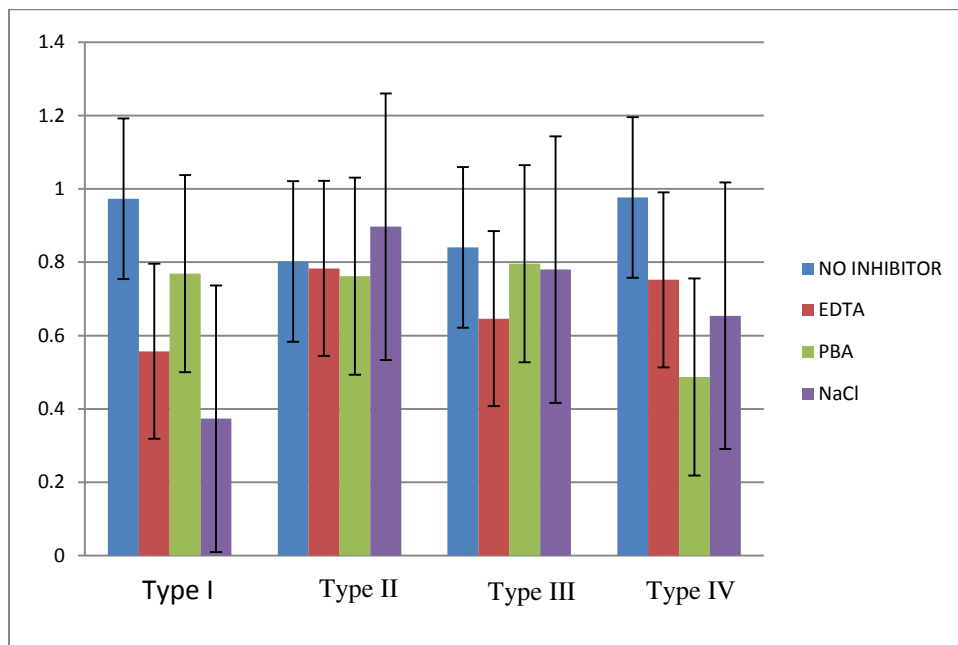


Fig-4: The carbapenemase activity and the effect of inhibitors.

The effect of inhibitors (Control, EDTA, PBA & NaCl) on carbapenem resistant isolates of *Acinetobacter baumannii*. The bacterial extract was incubated with different inhibitors at room temperature for 2hrs. The enzyme activity was assayed here for 30 mins for all 4 categories. They are Type I: Class A (bla_{KPC}), B(bla_{NDM}) and D(bla_{OXA-51} , bla_{OXA-23}) (+); Type II: Class A(bla_{KPC}) (-) and B(bla_{NDM}) and D(bla_{OXA-51} , bla_{OXA-23}) (+); Type III: Class A (bla_{KPC}) (+) and B (bla_{NDM}) and D(bla_{OXA-51} , bla_{OXA-23}) (-); and Type IV: Class B (bla_{NDM}) (-) and A(bla_{KPC}) and D(bla_{OXA-51} , bla_{OXA-23}) (+). Error bars represent standard deviation of triplicate measurements.