4.0 RESULTS

4.1. General Resistance pattern

A total of 1159 Enterobacteriaceae and 385 non-fermenter Gram-negative bacillus (NFGNB) isolates were screened for resistance to carbapenems by the Kirby-Bauer disc-diffusion method using imipenem, meropenem and ertapenem discs from HiMedia (India). Two hundred of these isolates appeared to be carbapenem-resistant on first screening, giving a carbapenem-resistance rate of 12.9% to begin with, as shown in Figure 1.

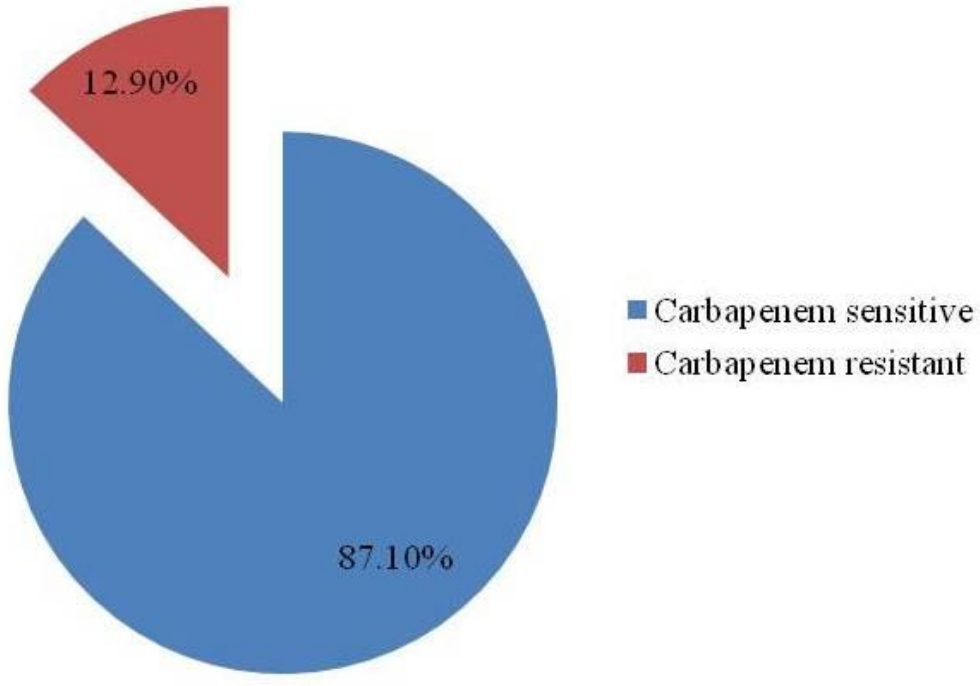


Figure 1: Carbapenem Resistance in GNB on First Testing

4.2. Lack of Agreement between Carbapenem-Resistance Test Results Obtained With Antimicrobial Discs from Different Sources

On primary screening with antimicrobial discs manufactured by HiMedia (India), 200 out of 1544 (12.9%) isolates turned out to be carbapenem-resistant. However, on cross-checking with Neo-Sensitabs antimicrobial discs manufactured by Rosco Diagnostica (Denmark), only 194 of these 200 isolates turned out to be carbapenem-resistant. The results obtained with Neo-Sensitabs from Rosco Diagnostica were corroborated by Etest findings using strips from bioMérieux (France). Performance of all antimicrobial resistance tests was controlled with standard bacterial strains from the American Type Culture Collection; details of individual control strains have already been described in the section on 'Materials and Methods'. An example of such a discordant result set has been shown in Figure 2.

Discordance was observed in three strains of *Klebsiella pneumoniae*, and one strain of *Escherichia coli*, *Proteus mirabilis* and *Proteus vulgaris* each. These isolates were considered falsely resistant, and data from these were not analysed in our study.

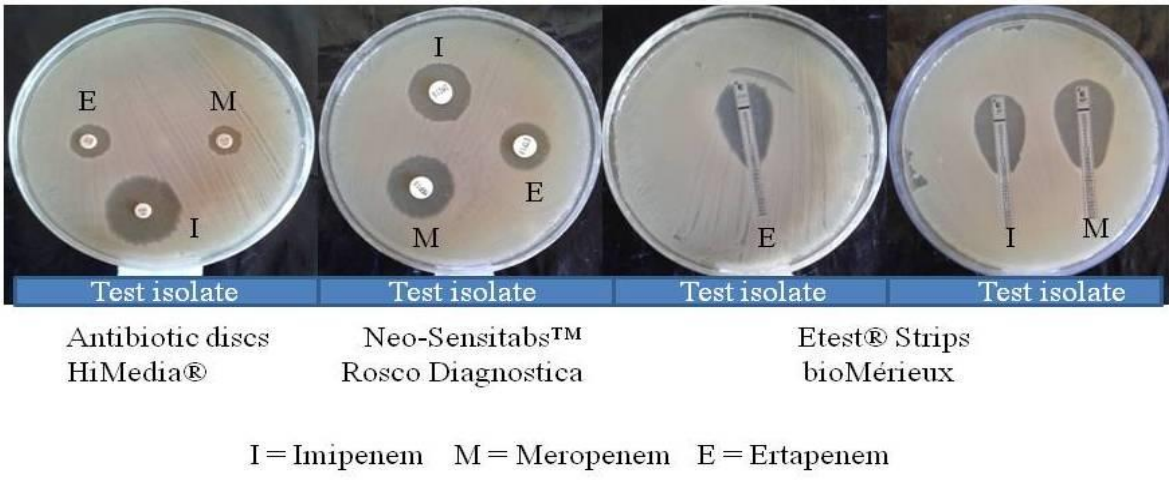


Figure 2: Difference in results of carbapenem-resistance testing when using carbapenem discs from different manufacturers

4.2.1 Excluding Intrinsic Resistance

Six isolates of *Elizabethkingia meningoseptica* and four isolates of *Stenotrophomonas maltophilia* were also excluded from analysis because these species are intrinsically resistant to carbapenems.

4.2.2 Final tally of carbapenem resistant GNB

Finally, 184 cross-checked carbapenem-resistant isolates were selected for further characterization. So the confirmed carbapenem resistance in our GNB isolates was 11.9 %, as shown in Figure 3.

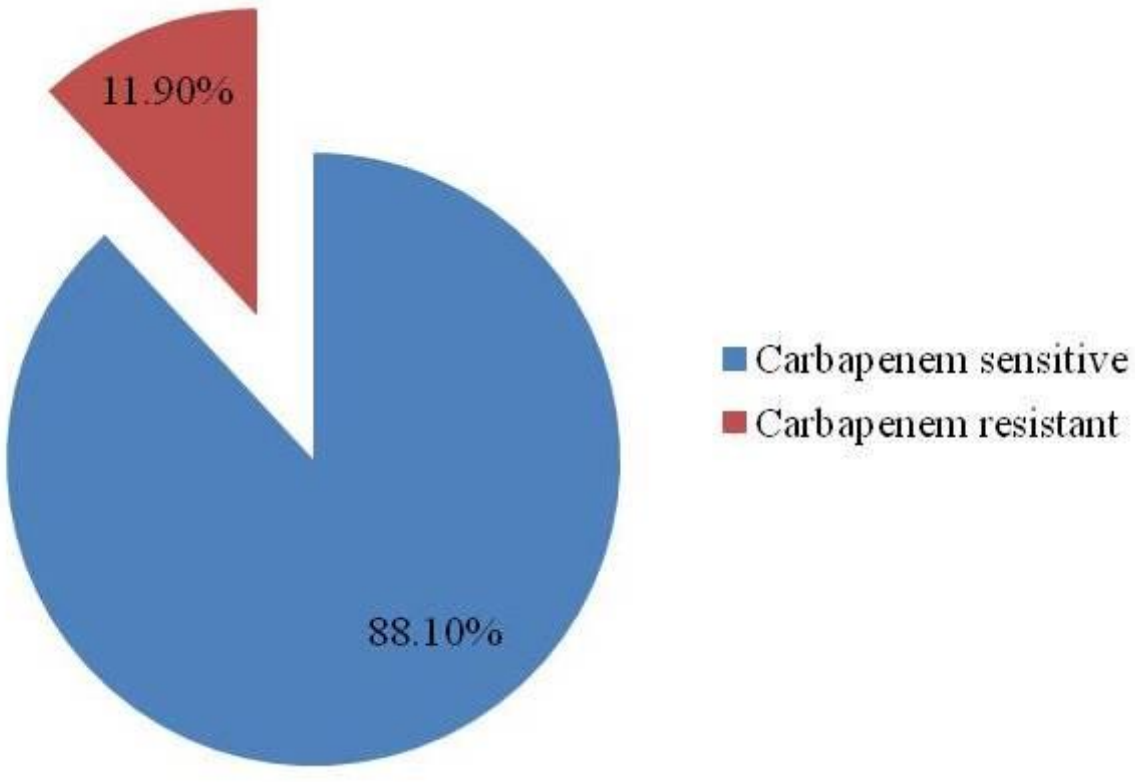


Figure 3: Confirmed carbapenem resistance in studied GNB isolates

Among the 1159 Enterobacteriaceae, 49 (4.2%) were resistant to carbapenems. Of the 385 strains of NFGNB, 149 (38.7%) were resistant to carbapenems, and all of these were either ACBC or *P. aeruginosa.* One hundred and four out of 179 (69.7%) ACBC isolates, and 31 out of 150 *P. aeruginosa* isolates were carbapenem-resistant shown in Figure 4.

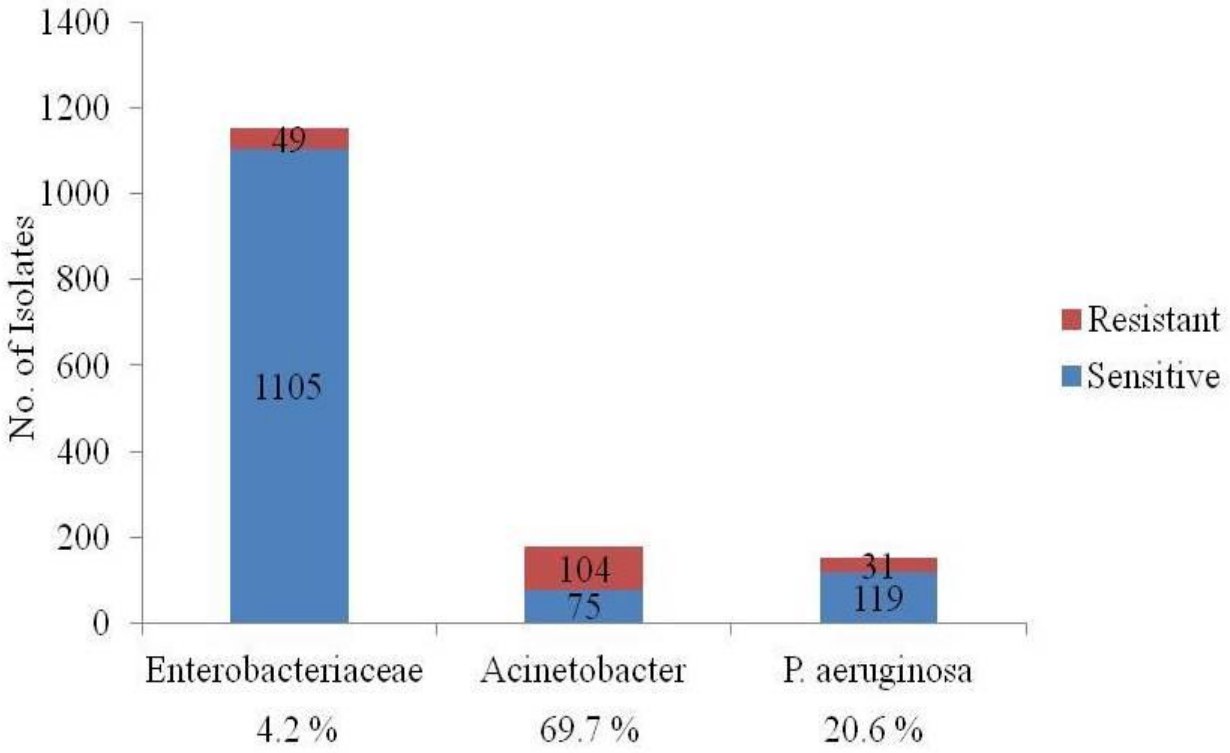


Figure 4: Carbapenem resistance in Enterobacteriaceae, *Acinetobacter* & *P. aeruginosa*

4.3. Carbapenem Resistant Enterobacteriaceae (n=49)

Majority of CRE isolates were *K. pneumoniae* (17), followed by *E. coli* (15). The species distribution of carbapenem-resistant Enterobacteriaceae in our study is shown in Figure 5.

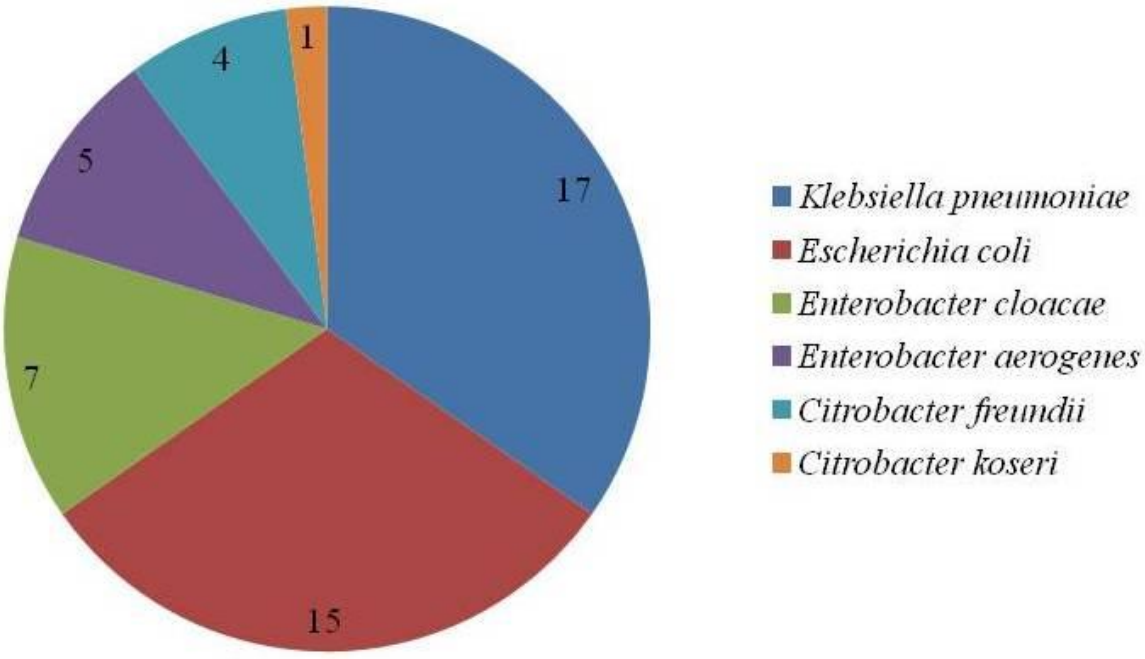


Figure 5: Species distribution of carbapenem-resistant Enterobacteriaceae

4.3.1. Carbapenem-resistant Enterobacteriaceae: Specimen types

The majority of resistant isolates were from pus (18/49; 36 %), followed by urine (16/49; 29 %) and then other specimens, as shown in Figure 6.

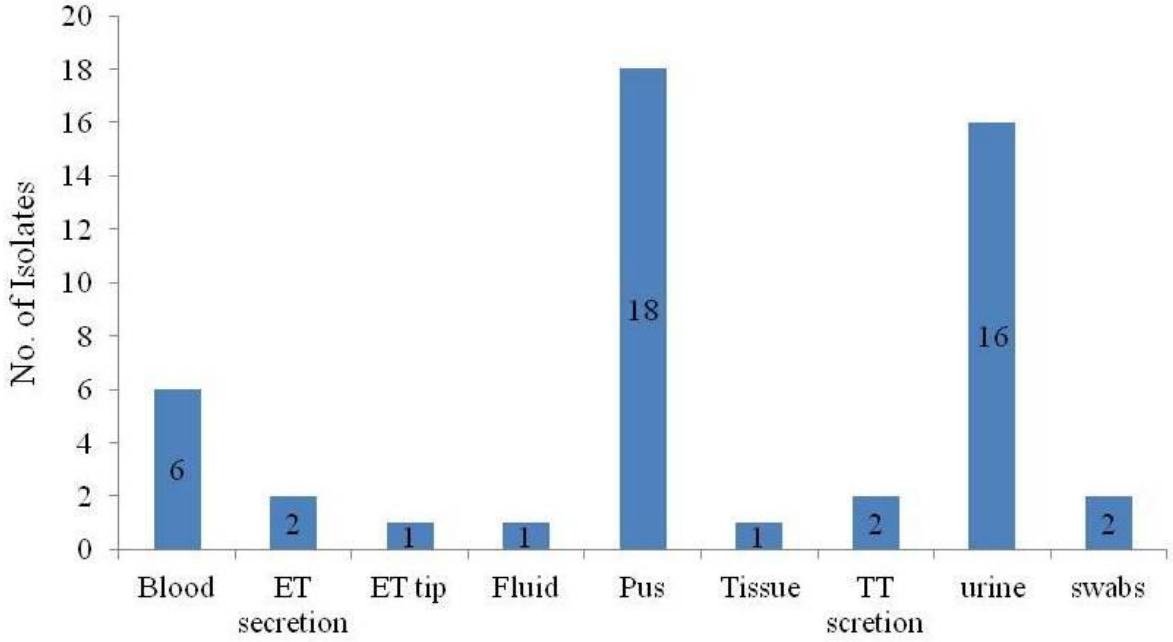


Figure 6: Types of specimens positive for CRE

4.3.2. Carbapenem-resistant Enterobacteriaceae: Ward-wise distribution

Of the 49 carbapenem-resistant Enterobacteriaceae isolates, the majority (11/49; 21%) were from the adult ICU, followed by surgical wards. Among surgical wards, the neurosurgical (6/49; 12.2%), orthopaedics (6/49; 12.2 %) and male surgical (5/49; 10.2 %) wards contributed the largest numbers, as shown in Figure 7.

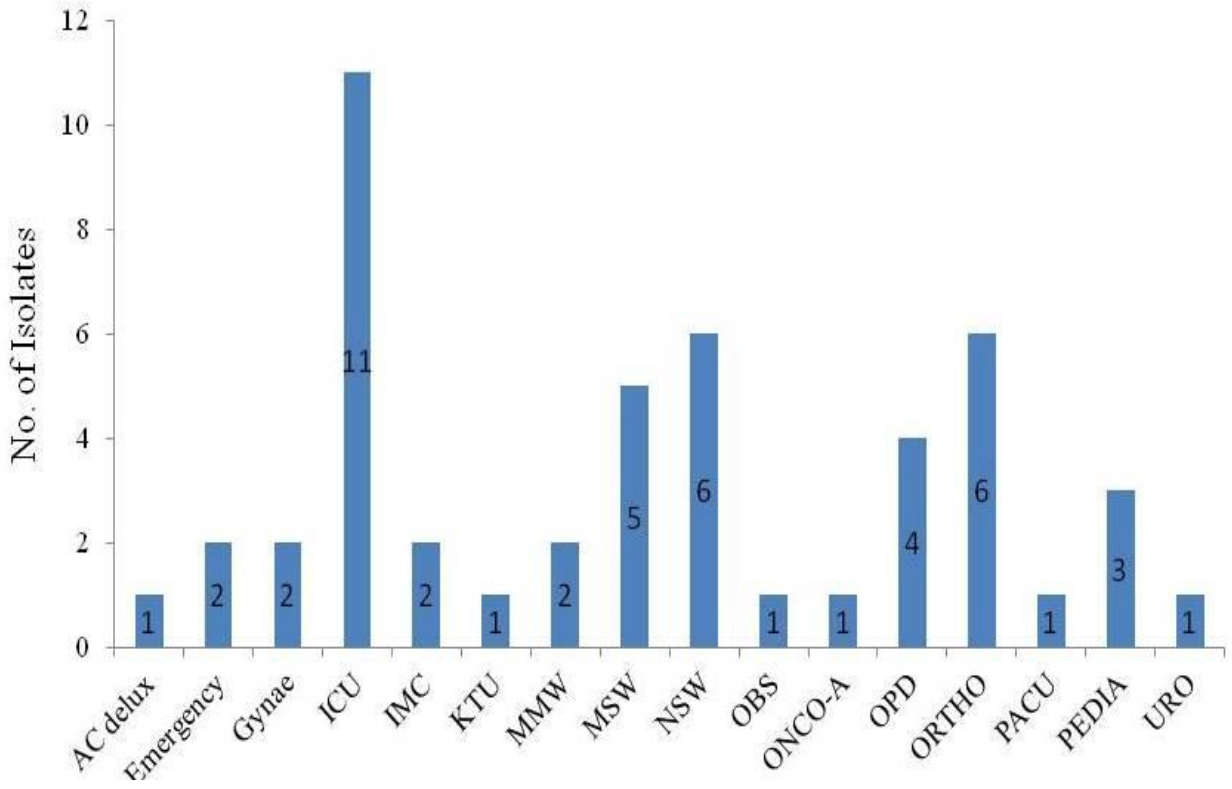


Figure 7: Ward-wise source of CRE

Abbreviations		
AC delux=Deluxe AC Ward	Gynae=Gynaecology Ward	ICU=Intensive Care Unit
IMC=Intensive Medical Care Unit	KTU=Kidney Transplant Unit	MMW=Male Medical Ward
MSW=Male Surgical Ward	NSW=Neurosurgery Ward	OBS=Observation Ward
Onco-A=Oncology A ward	OPD=Outpatient Department	Ortho=Orthopaedics
PACU=Post-Anaesthesia Care Unit	Pedia=Paediatrics Ward	Uro=Urology Ward

4.3.3. Enterobacteriaceae: Resistance to other antimicrobials

Most carbapenem-resistant isolates were simultaneously resistant to the majority of other antimicrobials tested. Tigecycline (87.7%) and colistin (85.7%) were the only antimicrobials against which resistance rates were below 15%, as shown in Figure 8.

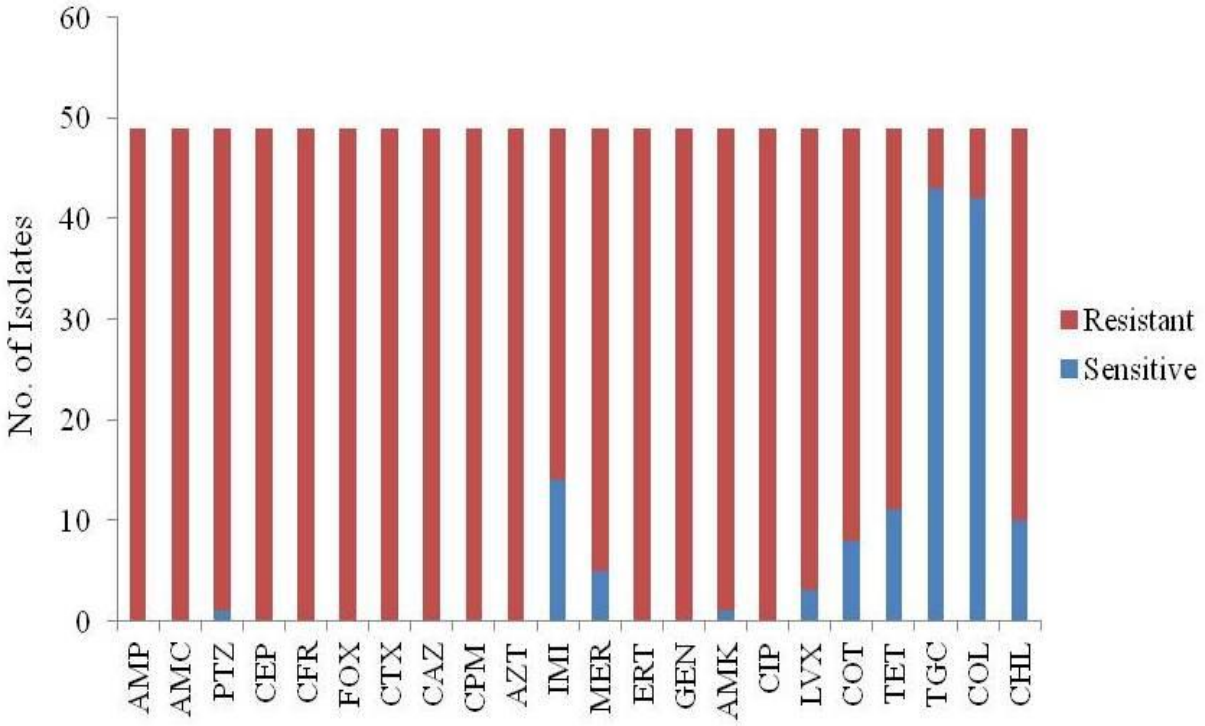


Figure 8: Antibiotic sensitivity profile of CRE

Abbreviations		
AMP=Ampicillin	AMC=Amoxycillin-clavulanate	PTZ=Piperacillin-Tazobactam
CEP=Cephalothin (class-representative 1st-gen ceph)	CFR=Cefuroxime (2nd-gen ceph)	FOX=Cefoxitin (cephamycin)
CTX=Cefotaxime (class-representative non-pseudomonal 3rd-gen ceph)	CAZ=Ceftazidime (anti-pseudomonal 3rd-gen ceph)	
CPM=Cefepime (4th-gen ceph)		AZT=Aztreonam
IMI=Imipenem	MER=Meropenem	ERT=Ertapenem
GEN=Gentamicin	AMK=Amikacin	CIP=Ciprofloxacin
LVX=Levofloxacin	COT=Cotrimoxazole	TET=Tetracycline
TGC=Tigecycline	COL=Colistin	CHL=Chloramphenicol

4.3.4. Carbapenem resistant Enterobacteriaceae: Determination of carbapenem minimum inhibitory concentration (MIC)

Carbapenem MIC was measured by the Epsilometer Test using Etest strips from bioMérieux (France). The majority of carbapenem-resistant isolates had MICs ≥32 µg/ml for all three carbapenem drugs. Few CRE had carbapenem MICs <32 µg/ml.

MIC distribution of imipenem, meropenem and ertapenem among CRE isolates is shown below (Figures 9, 10, 11).

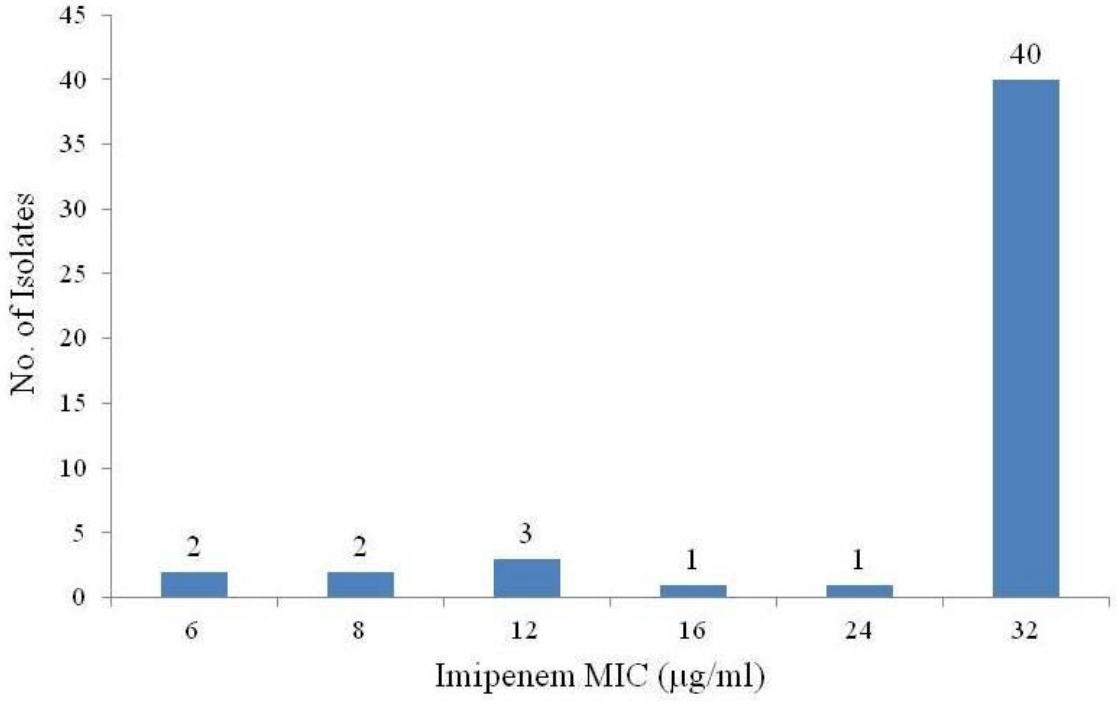


Figure 9: Imipenem MIC in Carbapenem-resistant Enterobacteriaceae

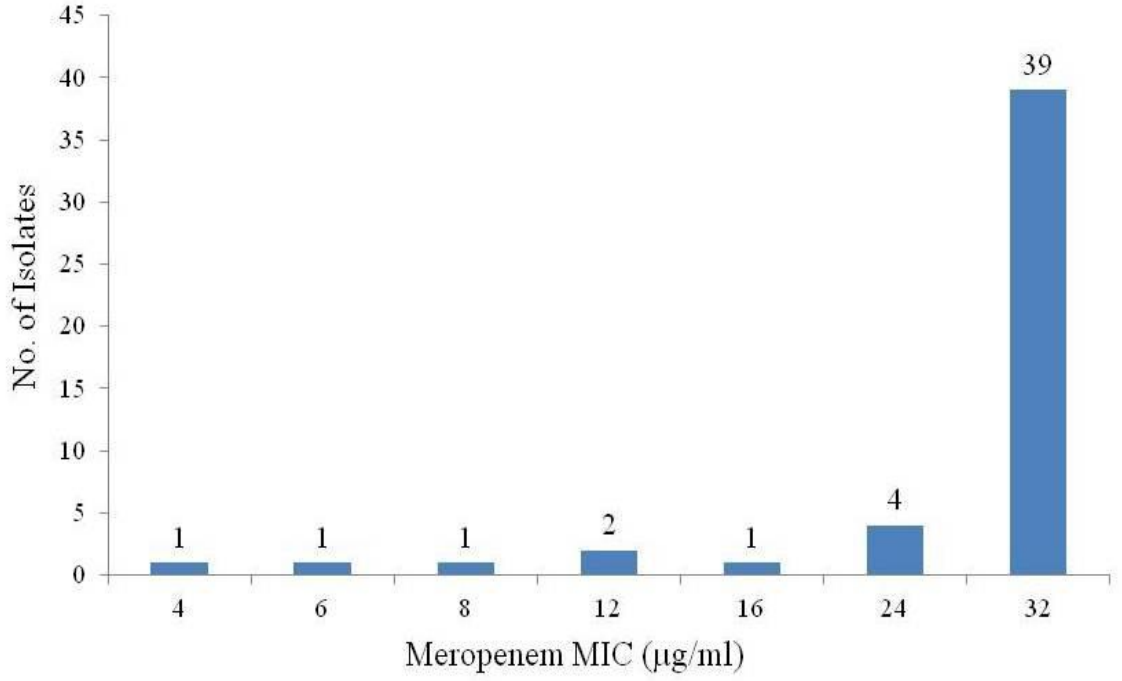


Figure 10: Meropenem MIC in Carbapenem-resistantEnterobacteriaceae

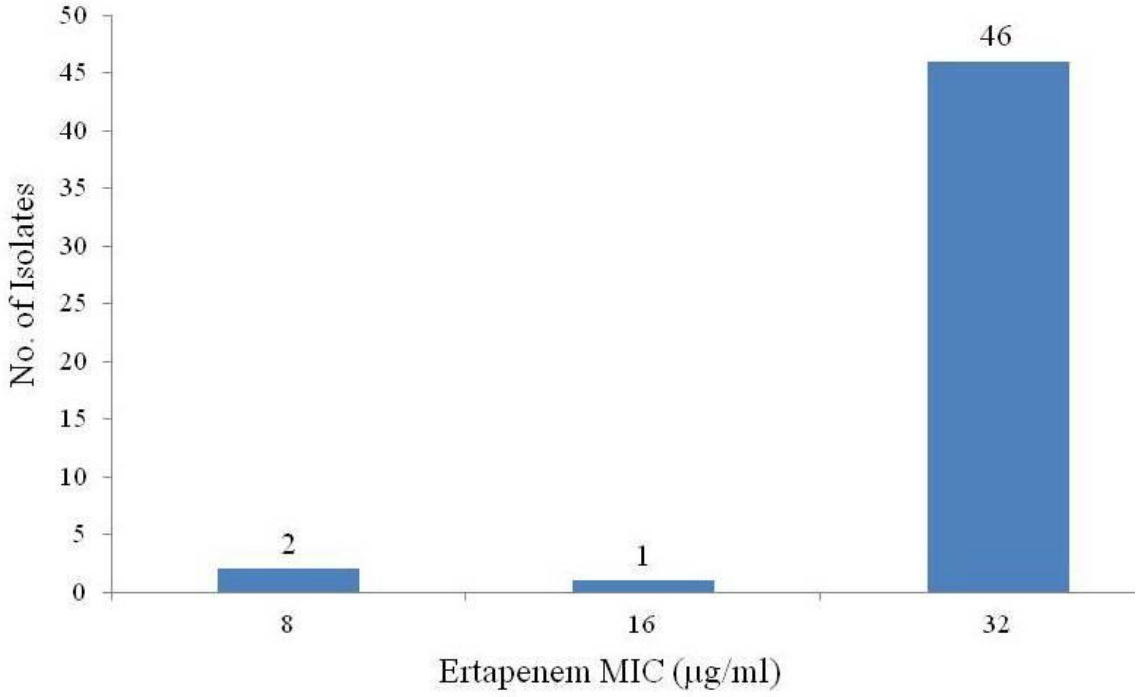


Figure 11: Ertapenem MIC in Carbapenem-resistant Enterobacteriaceae

4.4 *Acinetobacter calcoaceticus-baumannii* complex (n=104)

Most isolates were derived from endotracheal tube aspirates (41/104; 39.4%), pus (22/104; 21.1%) and tracheal tube aspirates (11/104; 10.5%), as shown in Figure 12.

The majority of carbapenem-resistant ACBC isolates were from the adult ICU (55/104; 52.8%), followed by the neurosurgery (14/104; 13.4%) and orthopaedics (10/104; 9.6%) wards, as shown in Figure 13.

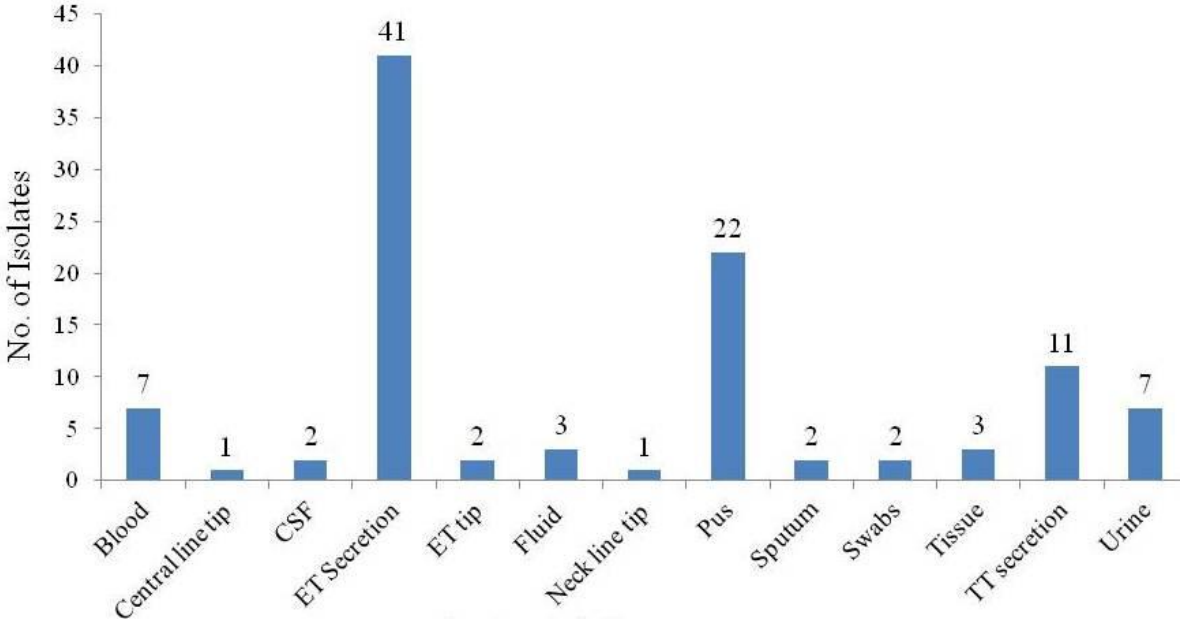


Figure 12: Specimen types positive for carbapenem-resistant ACBC

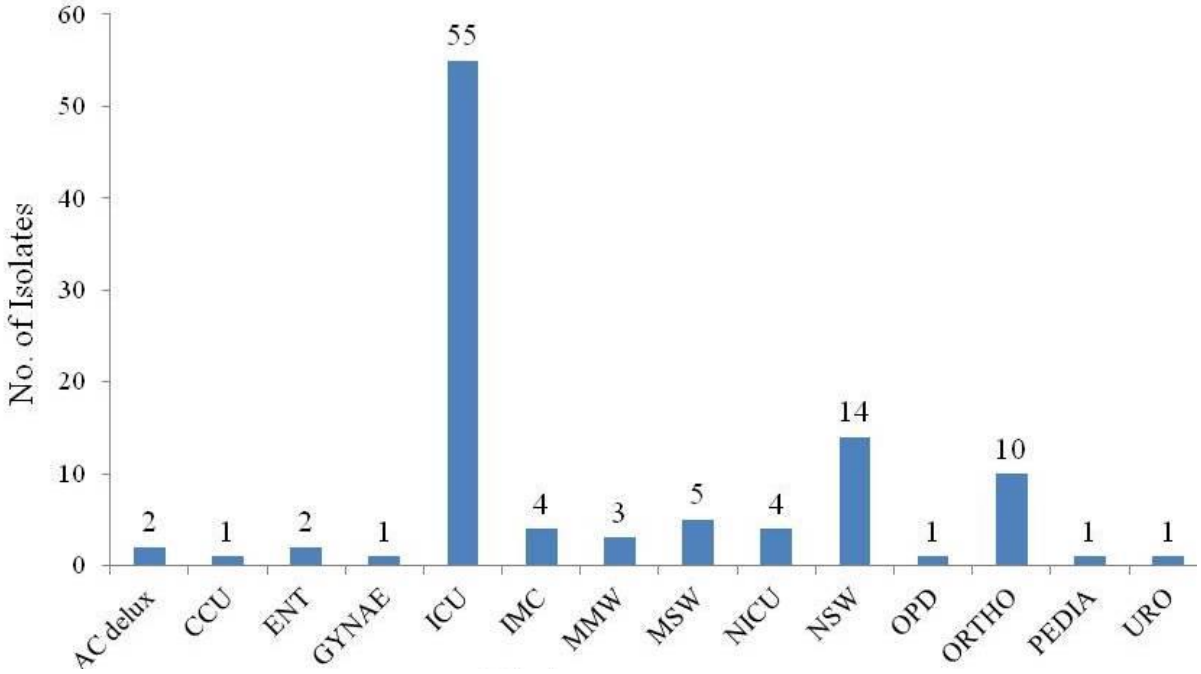


Figure 13: Ward-wise source of carbapenem-resistant ACBC

Abbreviations		
AC delux=Deluxe AC Ward	Gynae=Gynaecology Ward	ICU=Intensive Care Unit
IMC=Intensive Medical Care Unit	KTU=Kidney Transplant Unit	MMW=Male Medical Ward
MSW=Male Surgical Ward	NSW=Neurosurgery Ward	OBS=Observation Ward
Onco-A=Oncology A ward	OPD=Outpatient Department	Ortho=Orthopaedics
PACU=Post-Anaesthesia Care Unit	Pedia=Paediatrics Ward	Uro=Urology Ward

4.4.1. ACBC: Antimicrobial susceptibility testing and MIC distribution

All isolates were resistant to most other antimicrobials tested. The only antimicrobials that were effective in ≥90% of ACBC isolates were tigecycline and colistin. Susceptibility to tigecycline was 95.1% and 90.3% to colistin, as shown in Figure 14.

Carbapenem MICs of all ACBC isolates were very high at ≥12 µg/ml. Imipenem MIC ranged from 12 µg/ml to 32 µg/ml; meropenem MICs were even higher, ranging between 12 µg/ml and 32 µg/ml, as shown in Figure 15 and 16.

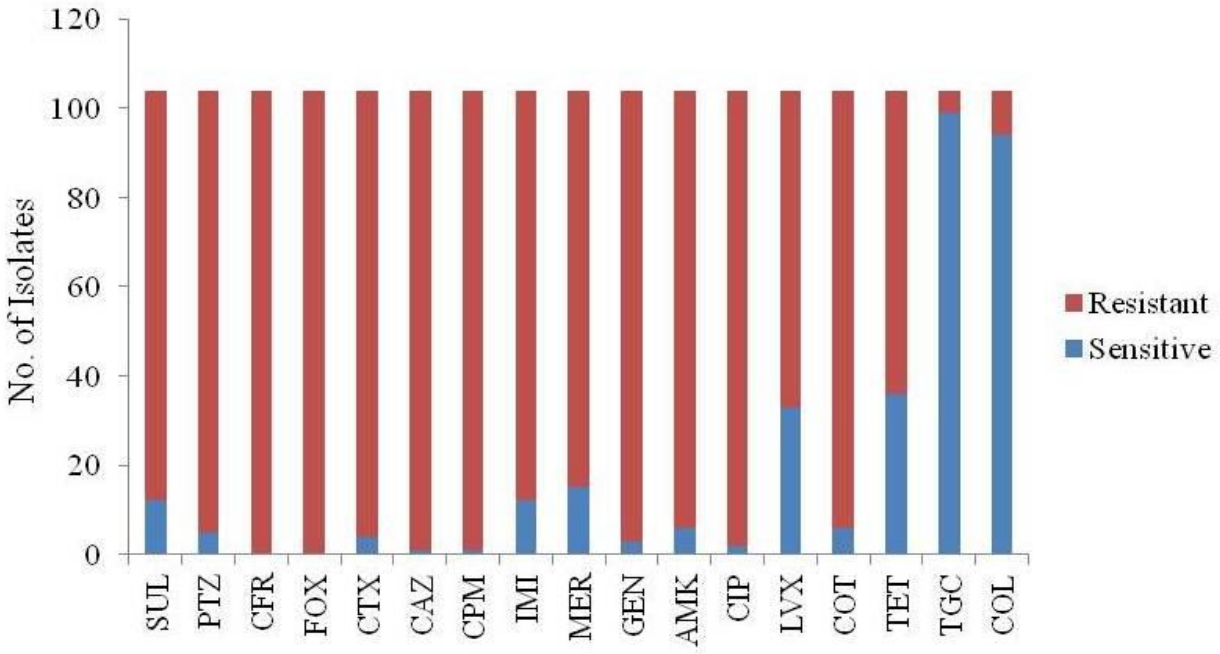


Figure 14: Antibiotic sensitivity profile of carbapenem-resistant ACBC

Abbreviations		
SUL=Sulbactam	PTZ=Piperacillin-Tazobactam	CFR=Cefuroxime (2nd-gen ceph)
FOX=Cefoxitin (cephamycin)	CTX=Cefotaxime (class-representative non-pseudomonal 3rd-gen ceph)	CAZ=Ceftazidime (anti-pseudomonal 3rd-gen ceph)
CPM=Cefepime (4th-gen ceph)	IMI=Imipenem	MER=Meropenem
GEN=Gentamicin	AMK=Amikacin	CIP=Ciprofloxacin
LVX=Levofloxacin	COT=Cotrimoxazole	TET=Tetracycline
TGC=Tigecycline	COL=Colistin	

Other antibiotics AMC, AZT AMP, CEP, ERT and CHL were not tested since ACBC is intrinsically resistant to these drugs

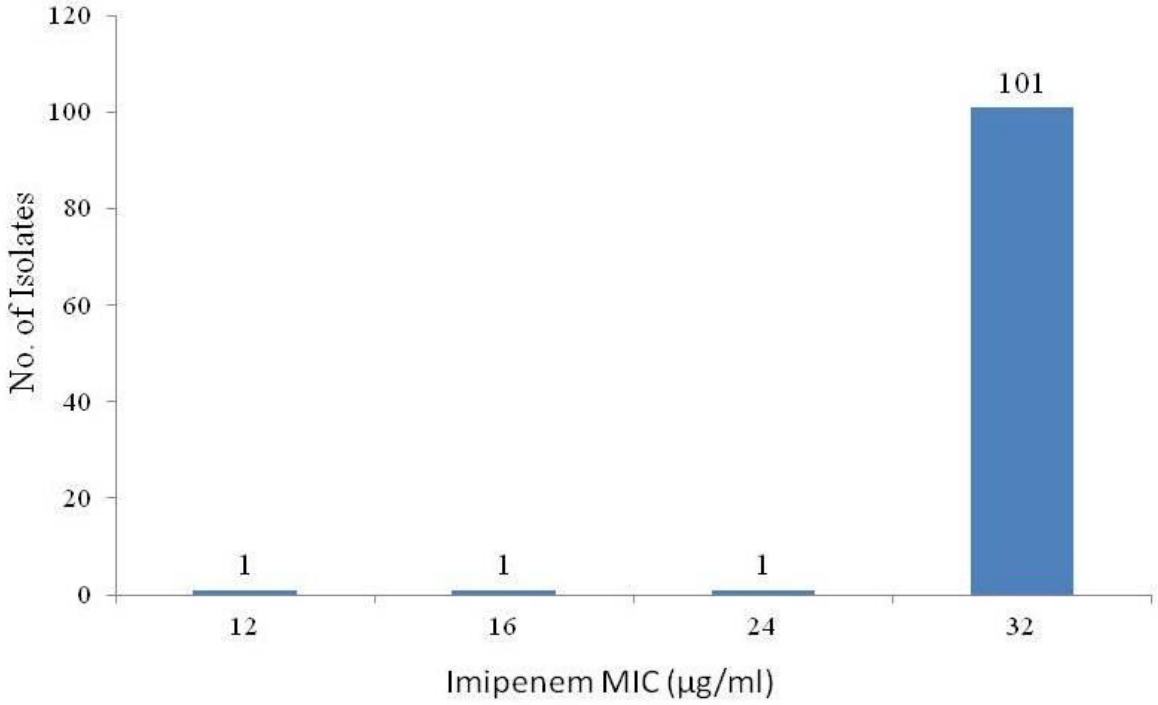


Figure 15: Imipenem MIC in Carbapenem-resistant ACBC

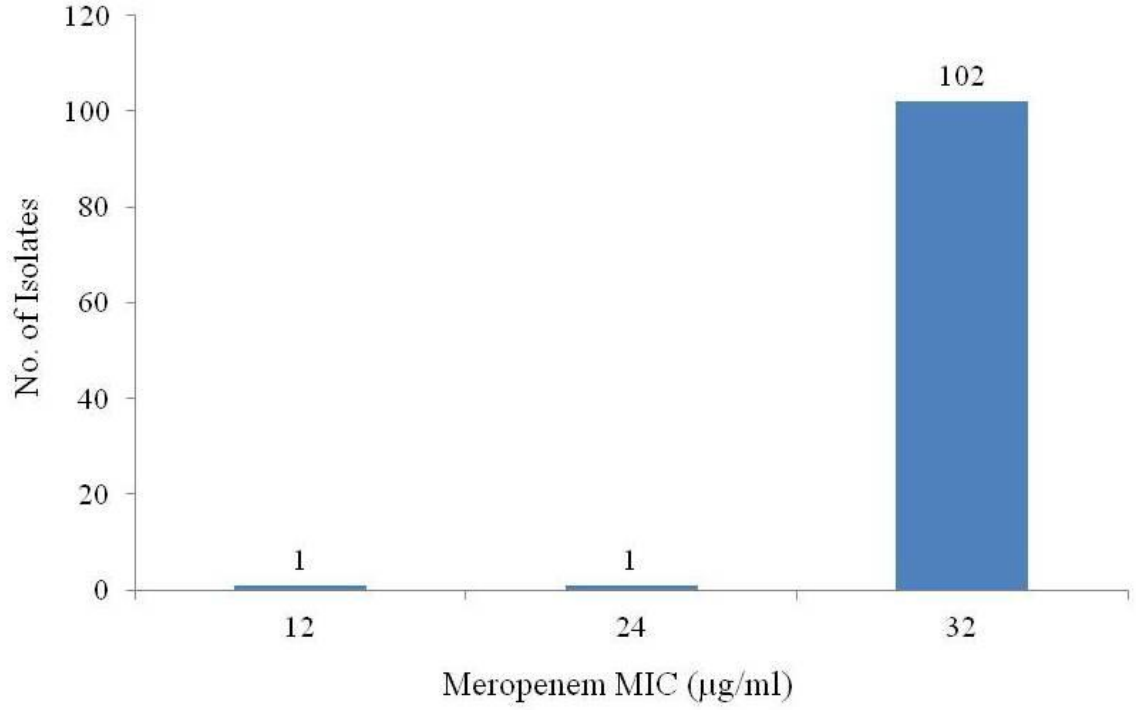


Figure 16: Meropenem MIC in Carbapenem-resistant ACBC

4.5. *Pseudomonas aeruginosa* (n=31)

The second commonest carbapenem-resistant NFGNB in our study was *P. aeruginosa*. The majority of these were isolated from urine (10/31; 32.2%), followed by TT secretion (7/31; 22.5%) and ET secretion (6/31; 19.3 %), as shown in Figure 17. Ward-wise, the majority of isolates were from ICU (14/31; 45%) followed by neurosurgery (6/31; 19.3%) and other wards, as shown in Figure 18.

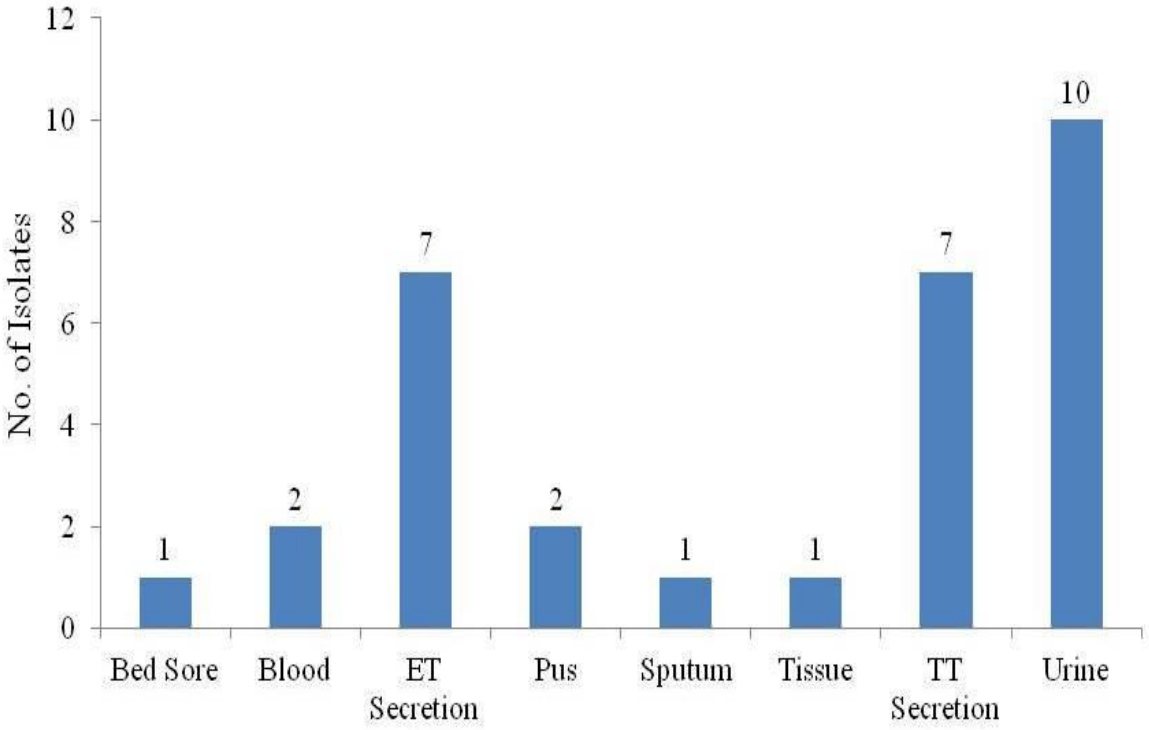


Figure 17: Specimen types positive for carbapenem-resistant *P. aeruginosa*

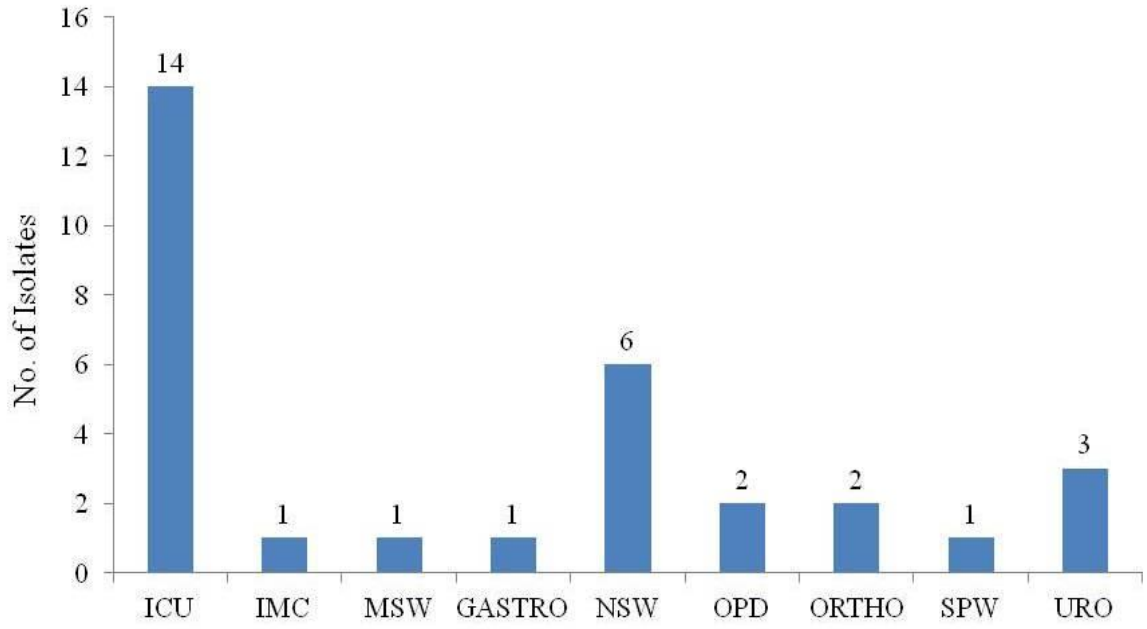


Figure 18: Ward-wise distribution of carbapenem-resistant *P. aeruginosa*

Abbreviations		
ICU=Intensive Care Unit	IMC=Intensive Medical Care Unit	MSW=Male Surgical Ward
GASTRO=Gastroenterology	NSW=Neurosurgery Ward	OPD=Outpatient Department
Ortho=Orthopaedics	SPW=Semi-private ward	Uro=Urology Ward

4.5.1. Antimicrobial susceptibility testing and MIC determination

The same pattern of resistance was observed towards different tested antibiotics. Colistin was only drug of choice with 90.3% susceptibility as shown in Figure 19.

Imipenem MIC was ≥32 µg/ml for all *P. aeruginosa* isolates. Meropenem MIC ranged between 12 and ≥32 µg/ml, as shown in Figure 20.

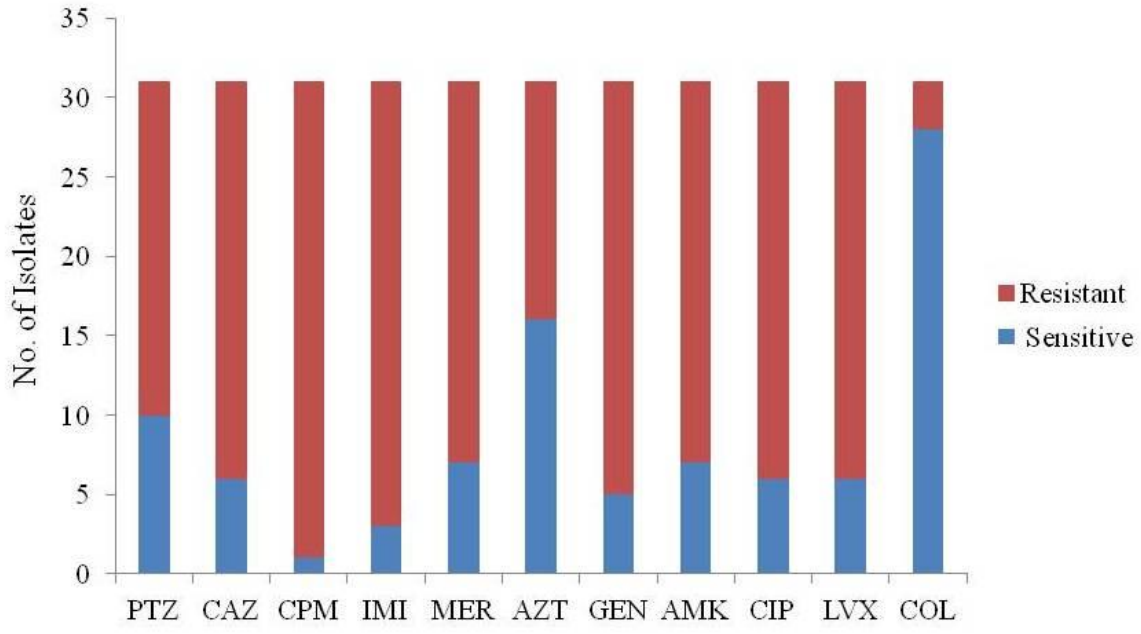


Figure 19: Antibiotic sensitivity of carbapenem-resistant *P. aeruginosa*

Expanded forms of Abbreviated Antimicrobial Names		
PTZ=Piperacillin-Tazobactam	CAZ=Ceftazidime (class-representative anti-pseudomonal 3rd-gen cephalosporin)	CPM=Cefepime (class-representative 4th-generation cephalosporin)
IMI=Imipenem	MER=Meropenem	AZT=Aztreonam
GEN=Gentamicin	AMK=Amikacin	CIP=Ciprofloxacin
LVX=Levofloxacin	COL=Colistin/Polymyxin B	

Other antibiotics such as AMP, CEP, CFR, FOX, CTX, COT, TET, ERT and CHL were not tested, since *P. aeruginosa* is intrinsically resistant to these drugs.

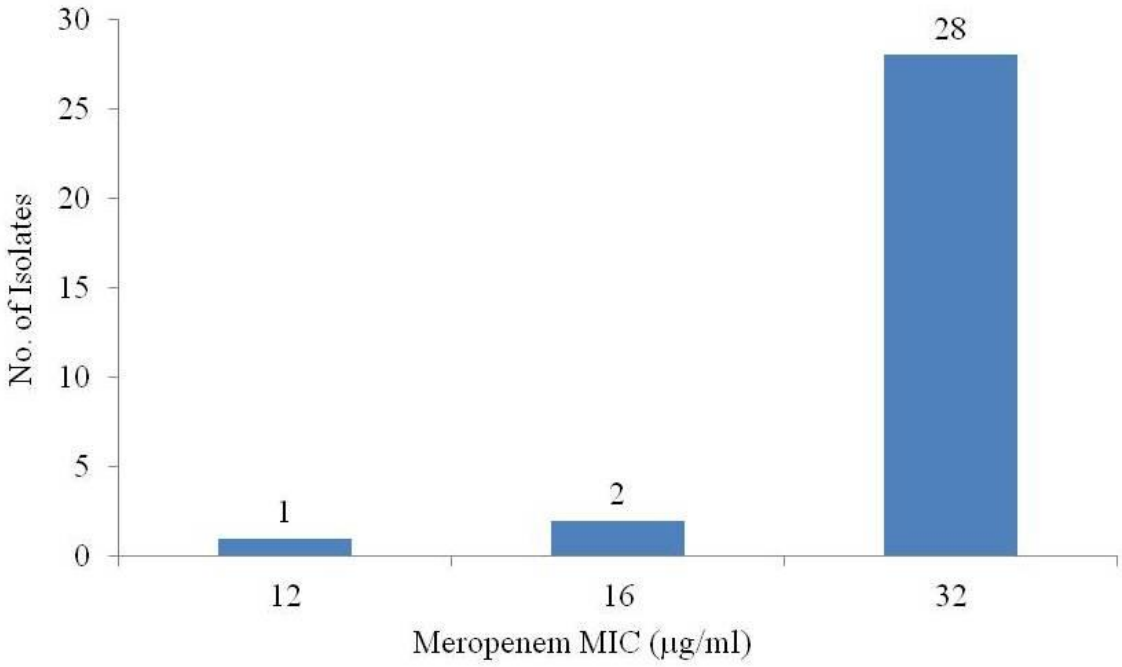


Figure 20: Meropenem MIC in Carbapenem-resistant *P. aeruginosa*

4.6. Testing Carbapenem MIC with Etest Strips

The appearance of inhibition zones of carbapenem-sensitive quality-control strains and tests strains is shown in Figure 21 and 22.

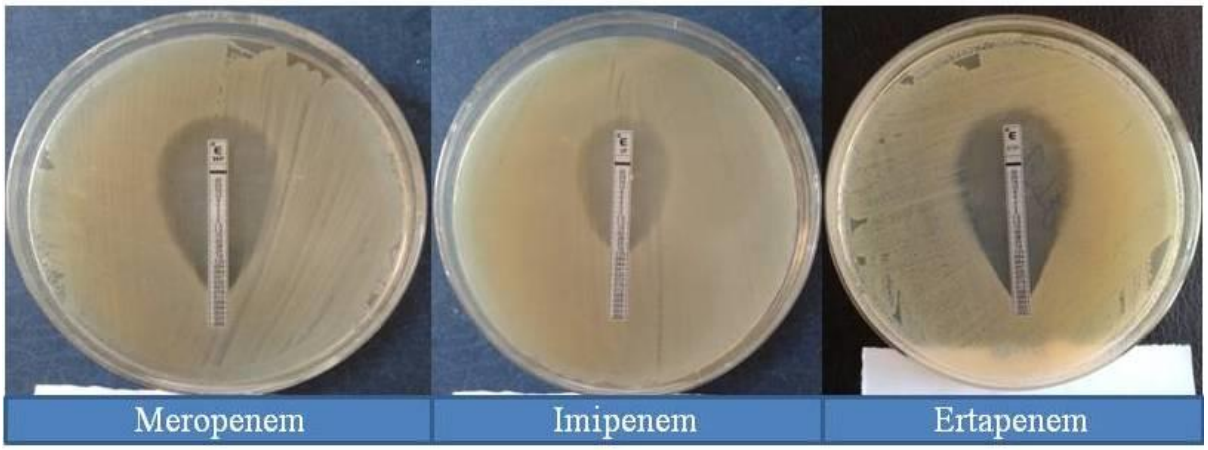


Figure 21: Results of Etest with the quality-control strains

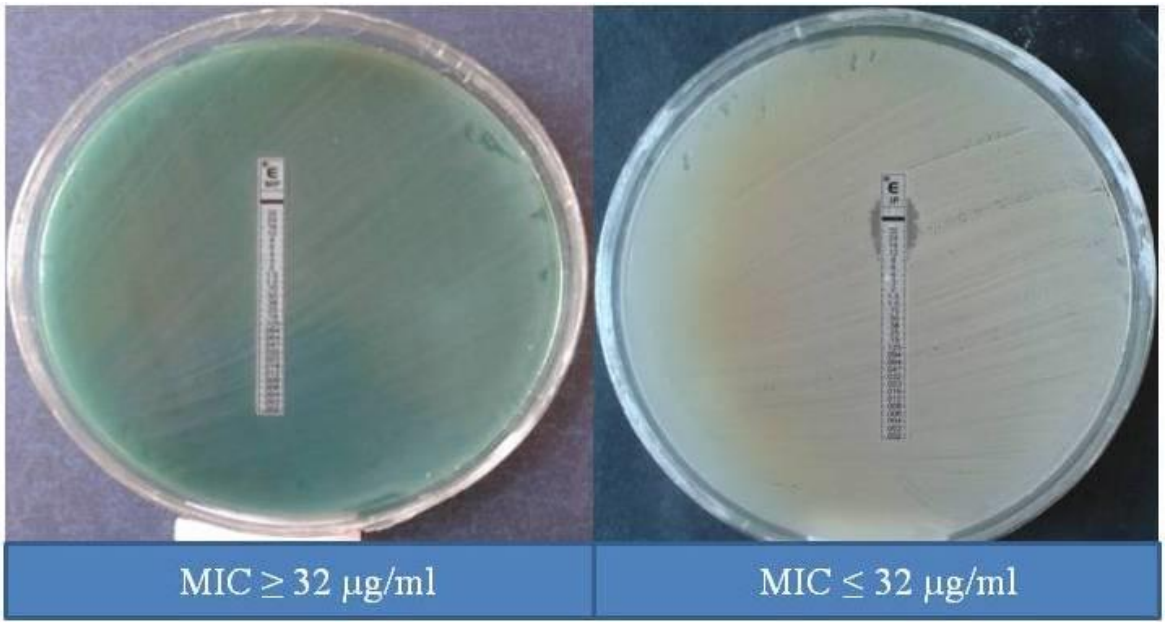


Figure 22: Results of Etest with test strains

4.7. Carbapenemase Detection Method

4.7.1. Modified Hodge Test (MHT)

MHT was positive in only 22 of the 184 isolates tested (Figure 23, 24 and 25).

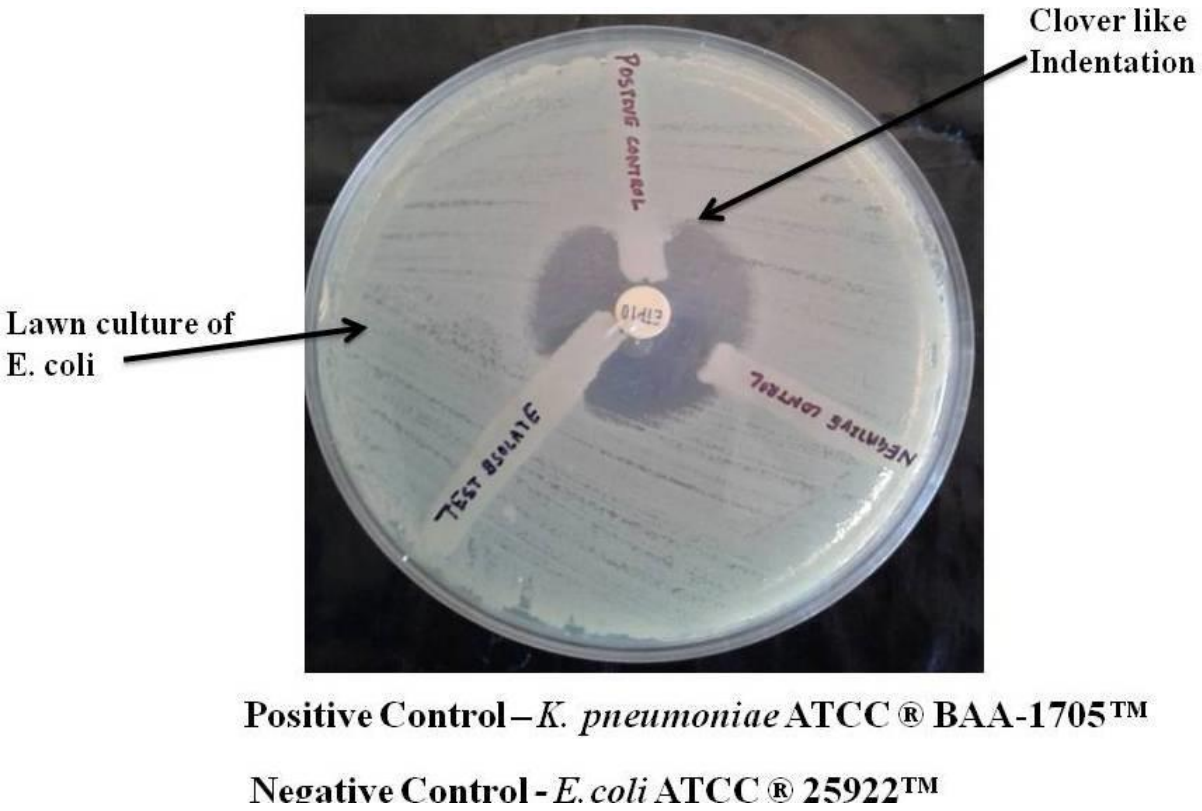
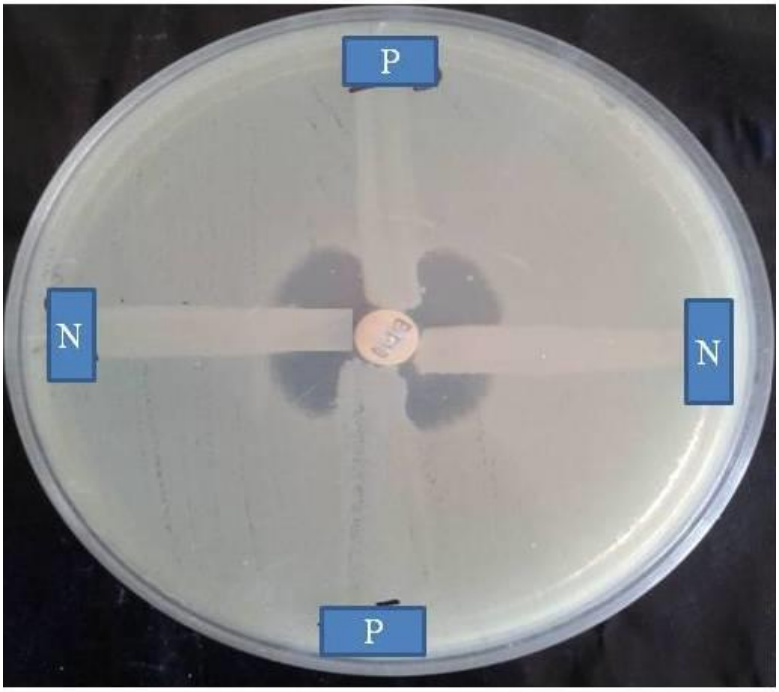


Figure 23: Results of MHT with the control strain



P = MHT Positive
N = MHT Negative

Figure 24: Results of MHT with the test strains

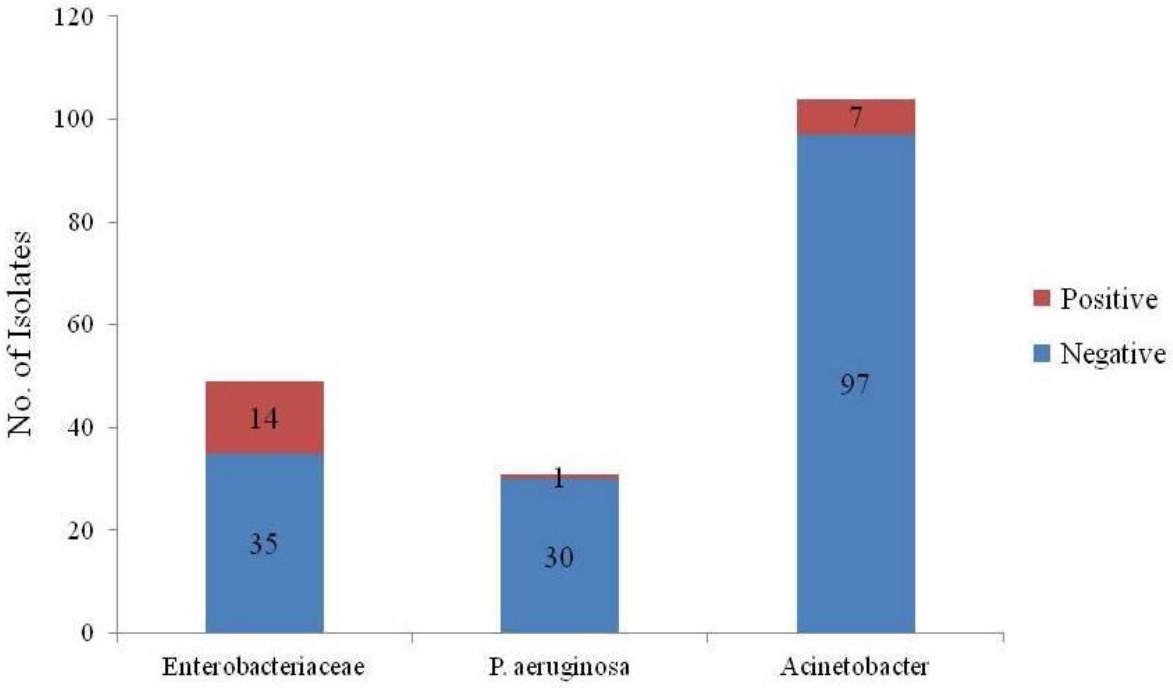
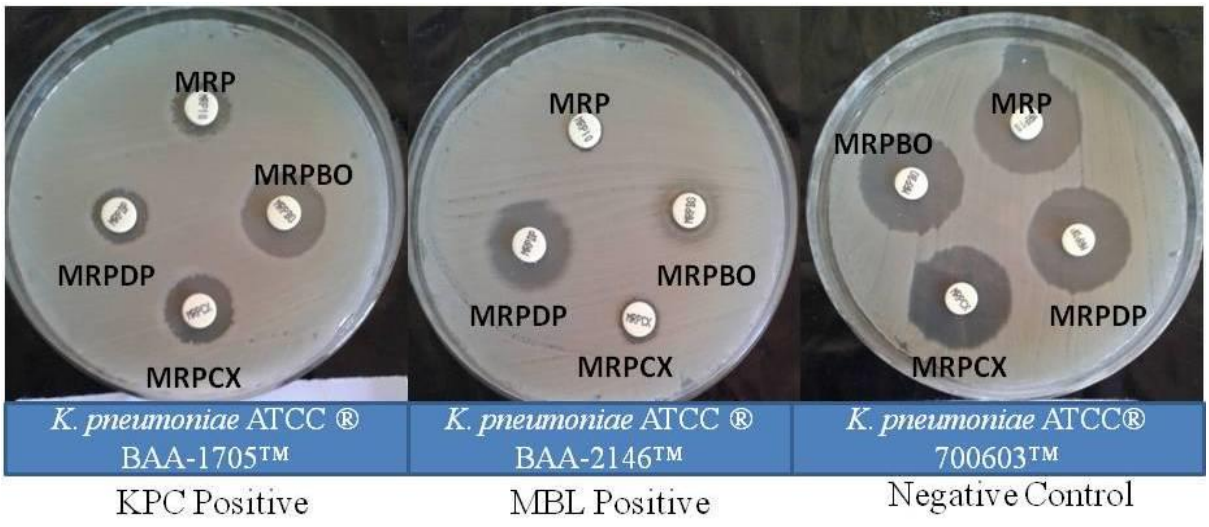


Figure 25: Modified Hodge Test Results

4.7.2. KPC+MBL Confirm ID kit (for Enterobacteriaceae)

Among the carbapenem-resistant isolates, 40 were positive for MBL production and two also tested positive for KPC. The remaining did not show the presence of any of these enzymes; images of representative cases have been shown in Figures 26, 27 and 28.



MRP = Meropenem, MRPDP =Meropenem+Dipicolinic acid,
MRPBO = Meropenem+Phenylboronic acid,
MRPCX = Meropenem+Cloxacillin

Figure 26: Results of KPC/MBL Kit with the control strains

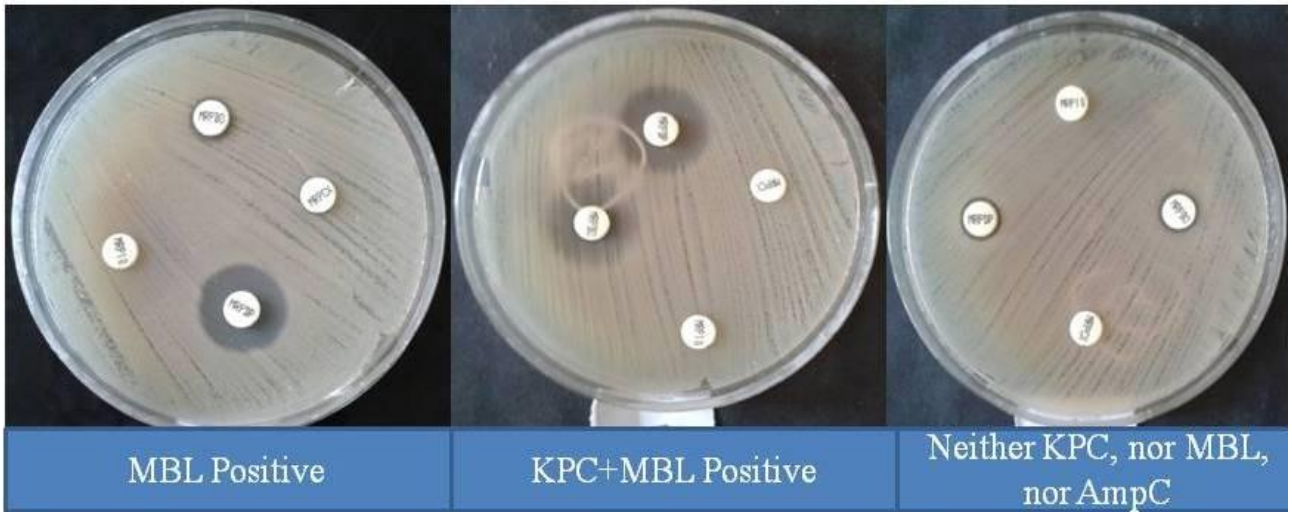


Figure 27: Results of KPC/MBL Kit with the test strains

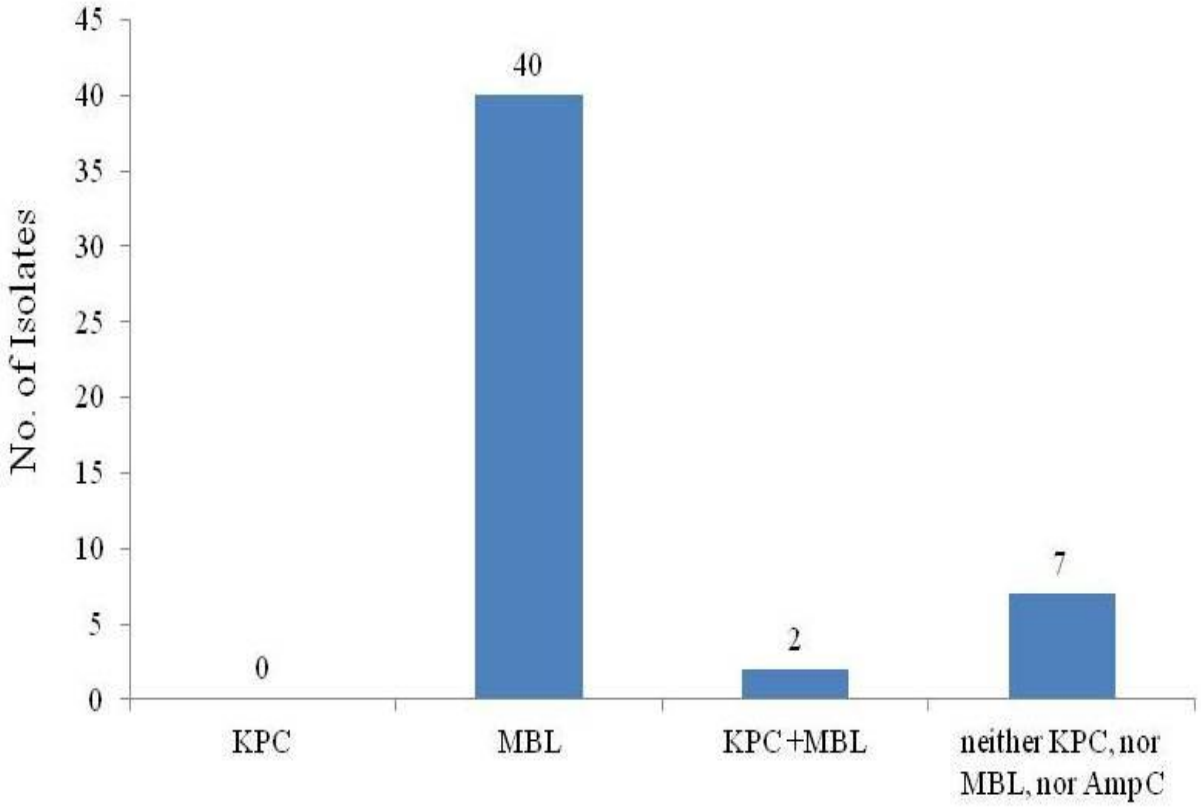


Figure 28: Distribution of KPC and MBL by KPC/MBL Kit

4.7.3. Rapid CARB Blue test Kit (for *Acinetobacter, P. aeruginosa* and Enterobacteriaceae)

A total of 105 isolates were tested positive for carbapenemase production by this kit. Among the positive, 43 were ACBC, 13 were *P. aeruginosa* and 39 belong to Enterobacteriaceae family (Figure 29, 30 and 31).

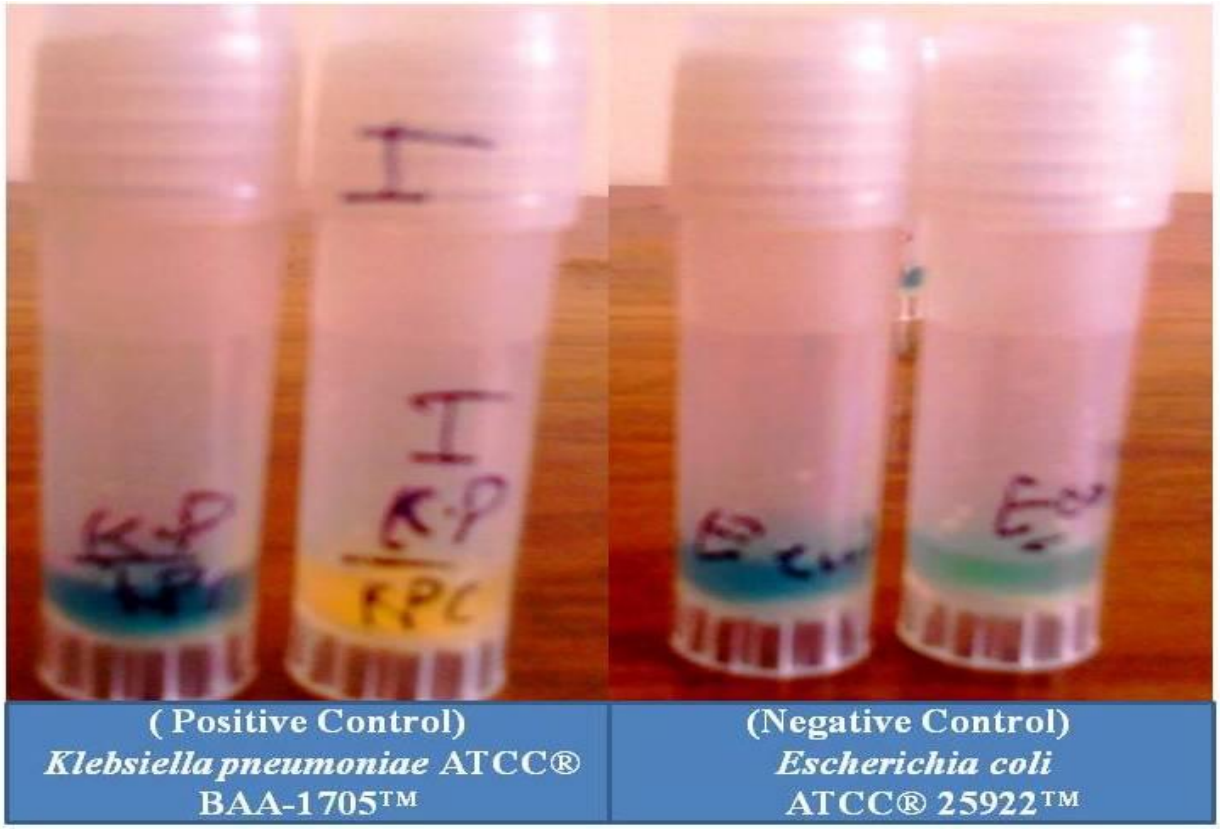


Figure 29: Results of Rapid CARB Blue Test with control strains

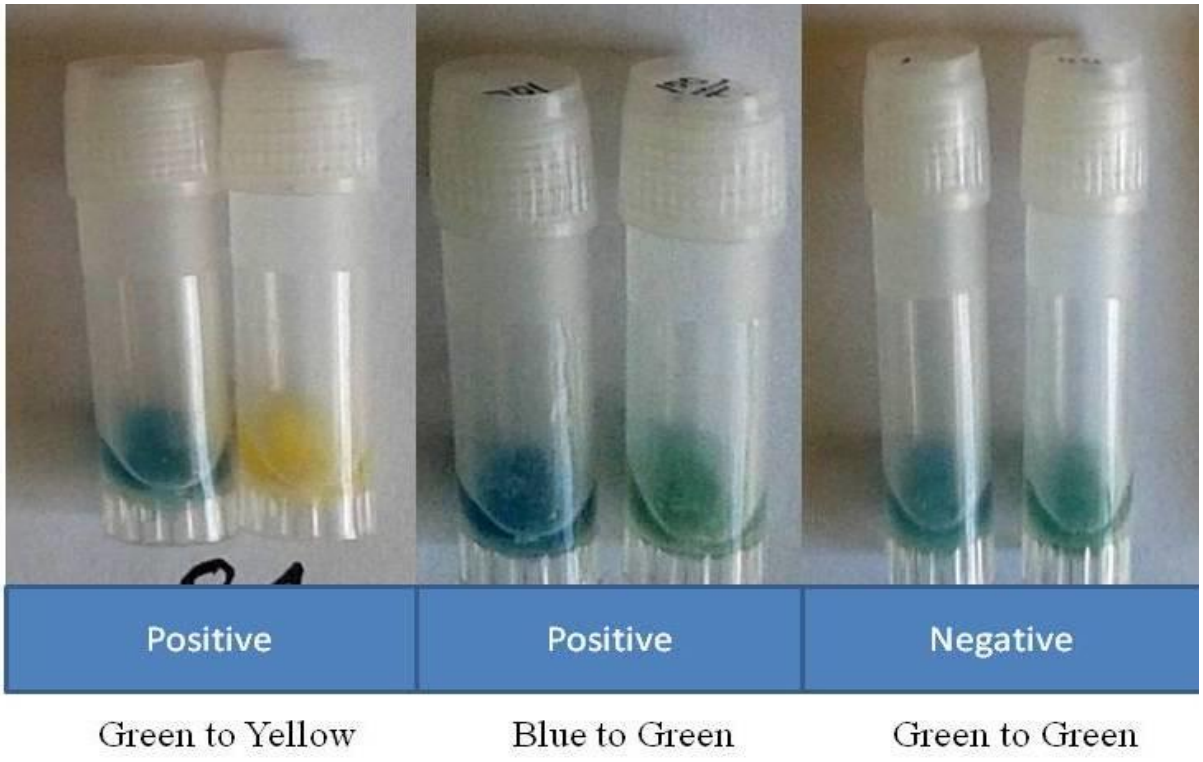


Figure 30: Results of Rapid CARB Blue Test with test strains

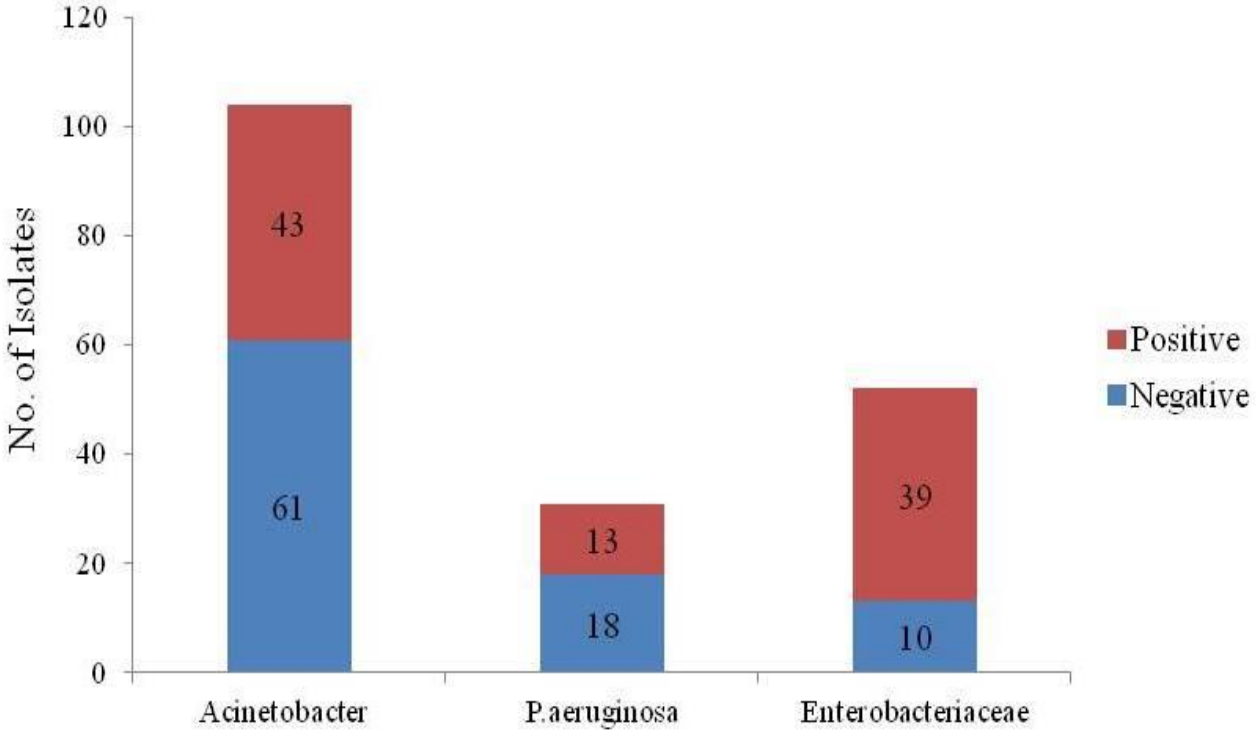


Figure 31: Distribution of results by Rapid CARB Blue Kit

4.7.4. Neo-Rapid CARB kit (For Enterobacteriaceae and *P. aeruginosa*)

40 (81.6%) of the 49 Enterobacteriaceae isolates and 16 (51.6%) of the 31 *P. aeruginosa* isolates tested positive by this kit (Figure 32, 33, 34).

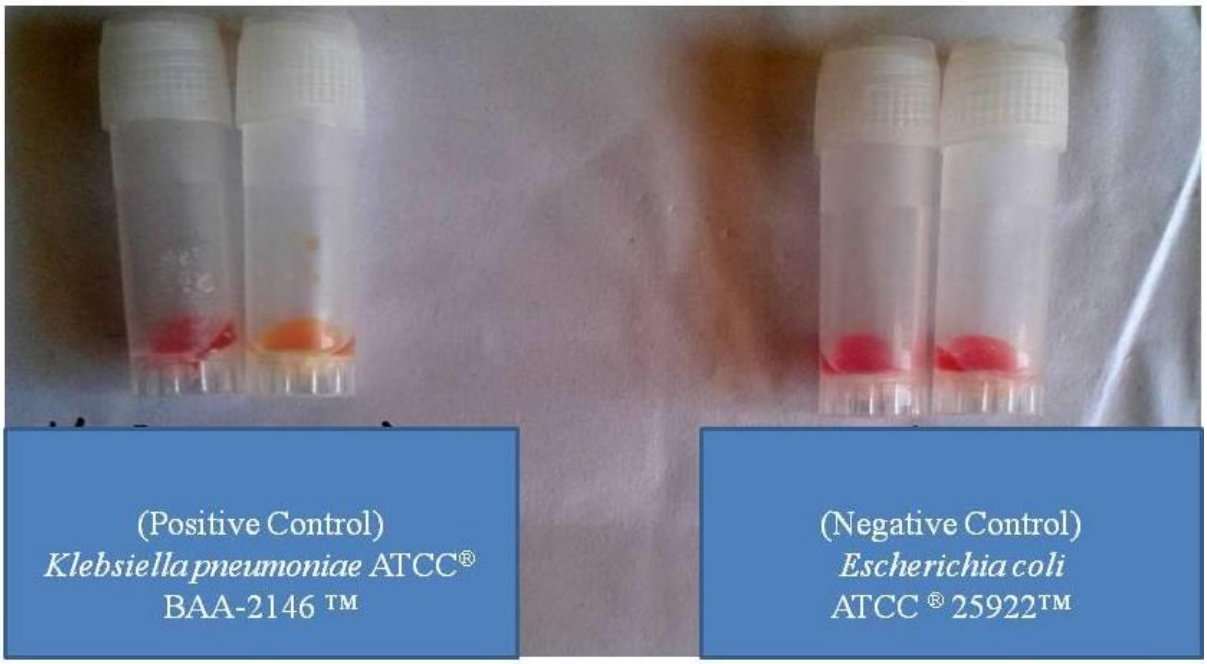


Figure 32: Results of Neo-Rapid CARB Test with control strains

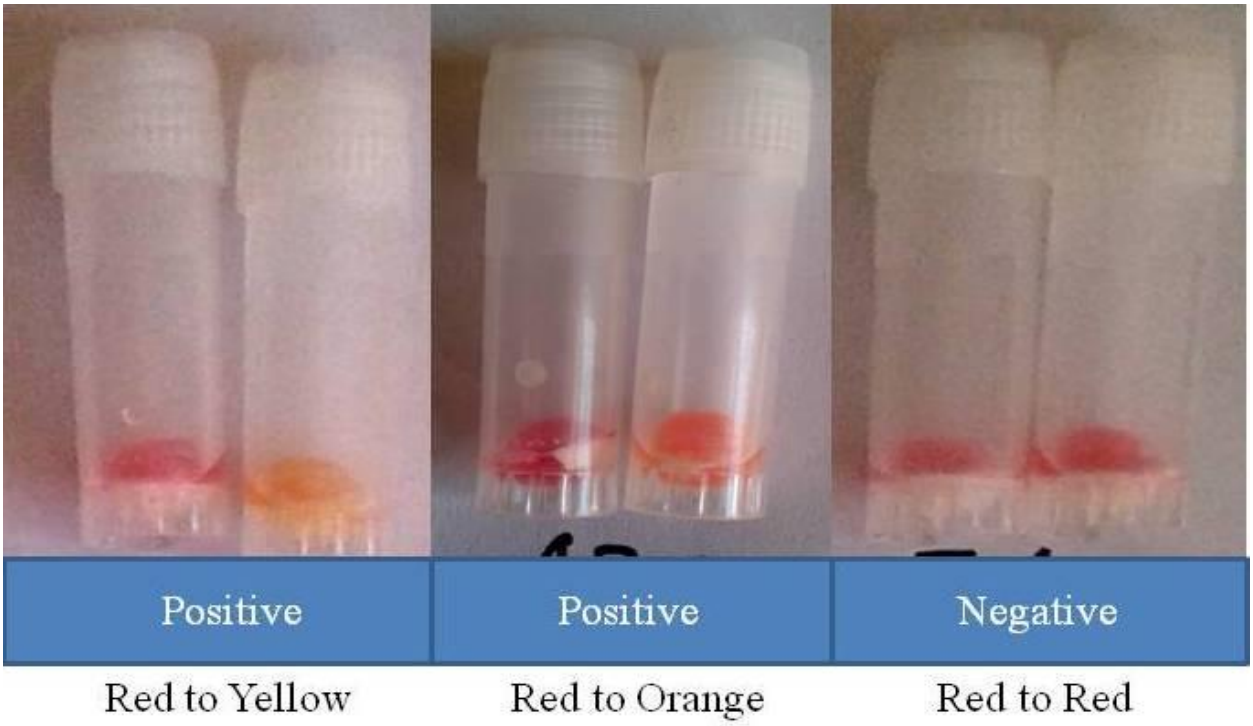


Figure 33: Results of Neo-Rapid CARB Test with test strains

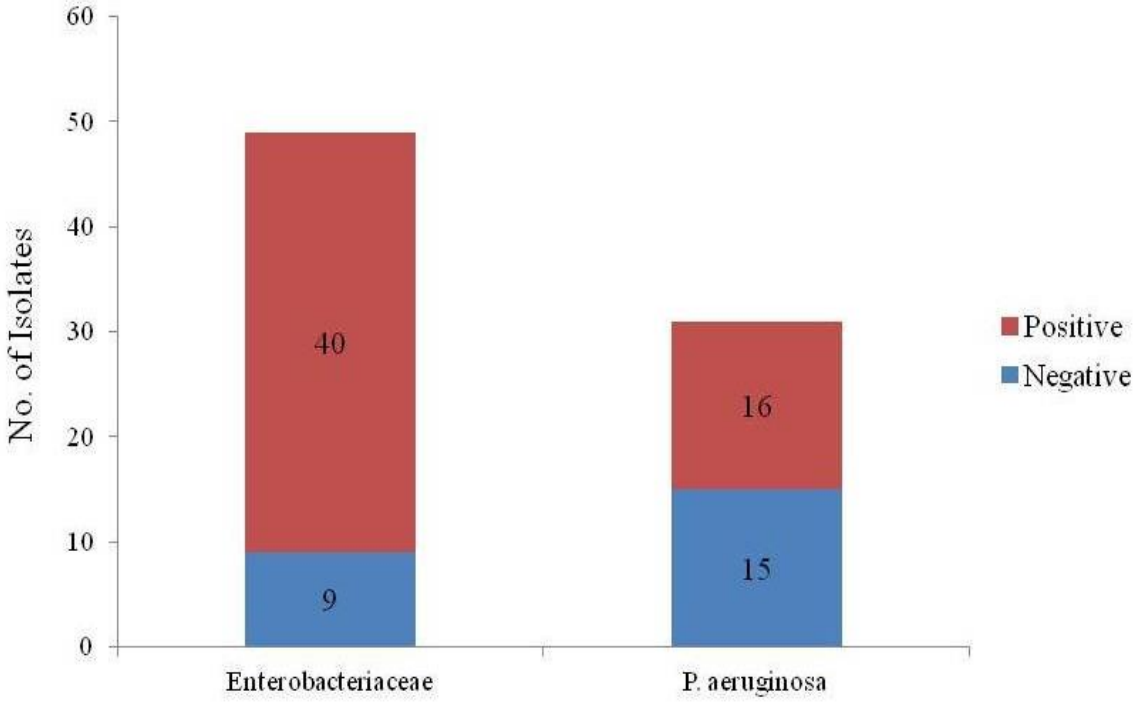


Figure 34: Distribution of results by Neo Rapid CARB Kit

4.7.5. Carba NP, CarbAcineto NP, and Blue Carba tests, along with their modifications

The Carba NP and Blue Carba tests had an identical sensitivity of 91.8% (45/49) for Enterobacteriaceae and 61.2% (19/31) for *P. aeruginosa* isolates for detecting carbapenemase enzymes, yielding positive results in the same strains in all cases.

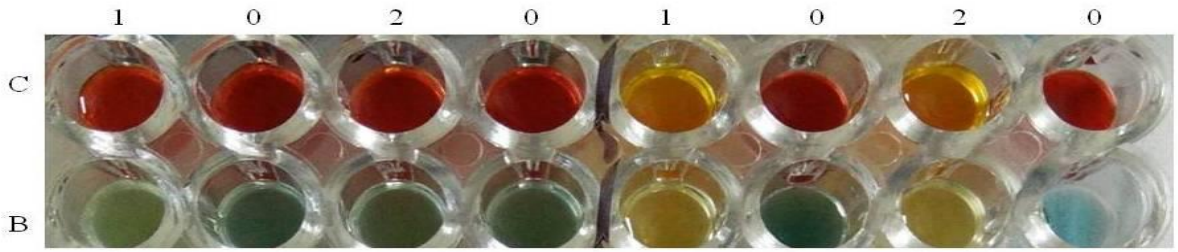
Similarly, the CarbAcineto NP and Blue Carba tests had an identical sensitivity of 84.6% (88/104) for detecting carbapenemase enzymes in ACBC strains, yielding positive results in the same strains in all cases.

Modifications:

i) Substituting analytical reagent grade imipenem monohydrate with pharmaceutical grade imipenem-cilastatin, made no difference to results, as long as the absolute quantity of imipenem was kept the same by doubling the amount of imipenem-cilastatin.

ii) Induction of carbapenemases by prior exposure to carbapenem through proximity to carbapenem test discs. Results obtained with bacteria growing adjacent to carbapenem discs, were compared with those obtained with bacteria growing in other parts of the plate. In case of Enterobacteriaceae, three (6.25%) strains gave a positive result only when bacterial biomass was taken from near the imipenem sensitivity testing disc, suggesting that carbapenemase production was induced by imipenem in these strains. In case of ACBC and *P. aeruginosa,* the site from where bacterial biomass was collected relative to the position of the imipenem sensitivity testing disc, made no difference to results.

The result of the various tests and their variations on some isolates is shown in Figure 35 and 36.



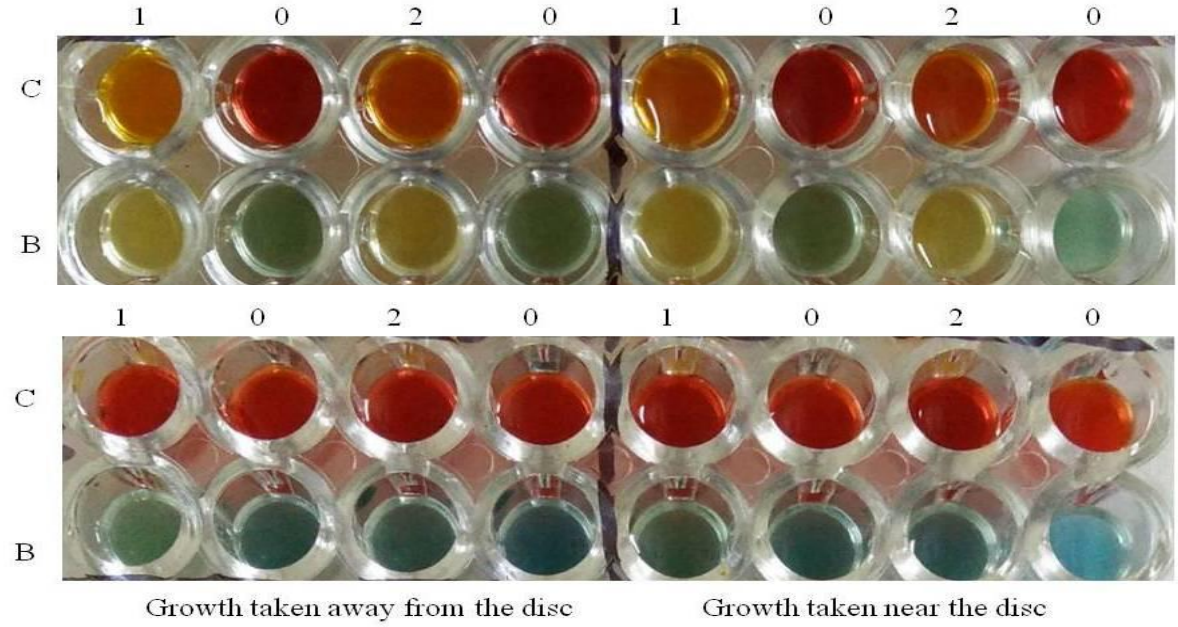
Abbreviations

0=Drug free control, 1=Pharmaceutical grade imipenem-cilastatin

2=Analytical reagent grade imipenem monohydrate

B=Blue Carba, C=Carba NP

Figure 35: Enhancement of sensitivity of carbapenemase detection in Enterobacteriaceae caused by enzyme induction in bacteria growing adjacent to imipenem disc



Abbreviations

0=Drug free control, 1=Pharmaceutical grade imipenem-cilastatin

2=Analytical reagent grade imipenem monohydrate

B=Blue Carba, C=Carba NP

Figure 36: No induction of carbapenemase in Acinetobacter growing adjacent to imipenem disc, with no enhancement of test sensitivity for carbapenemase detection

4.7.6. Carbapenem Inactivation Method for *Acinetobacter, P. aeruginosa* and Enterobacteriaceae

Of all carbapenem-resistant isolates, 73 (70.1%) *Acinetobacter*, 15 (48.3%) *P. aeruginosa* and 38 (77.5%) Enterobacteriaceae isolates tested positive by this test, as shown in Figures 37 and 38.

Figure 37: Inhibition zones on carbapenem-inactivation testing

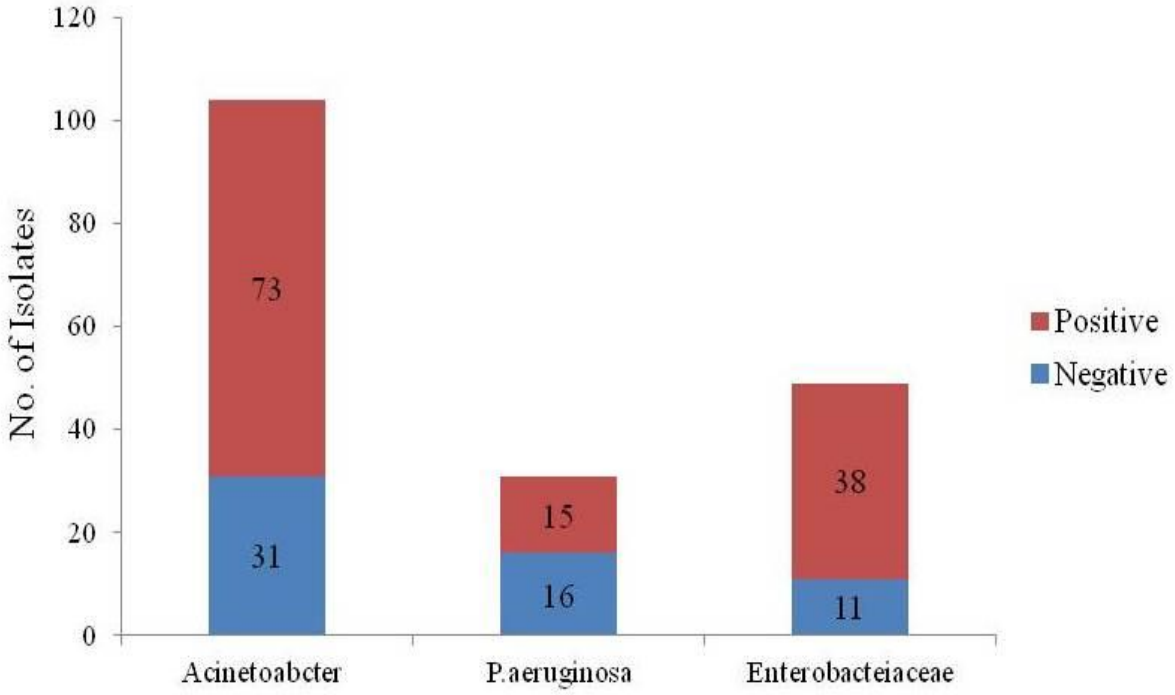


Figure 38: Distribution of results on carbapenem inactivation testing

4.8. Genotypic Methods

Multiplex PCR

The gel showed an extra, unexpected band under the targeted 82-bp band as shown in Figure 39. This was a non-specific, primer dimer band arising from the presence of many primers in a multiplex PCR mix.

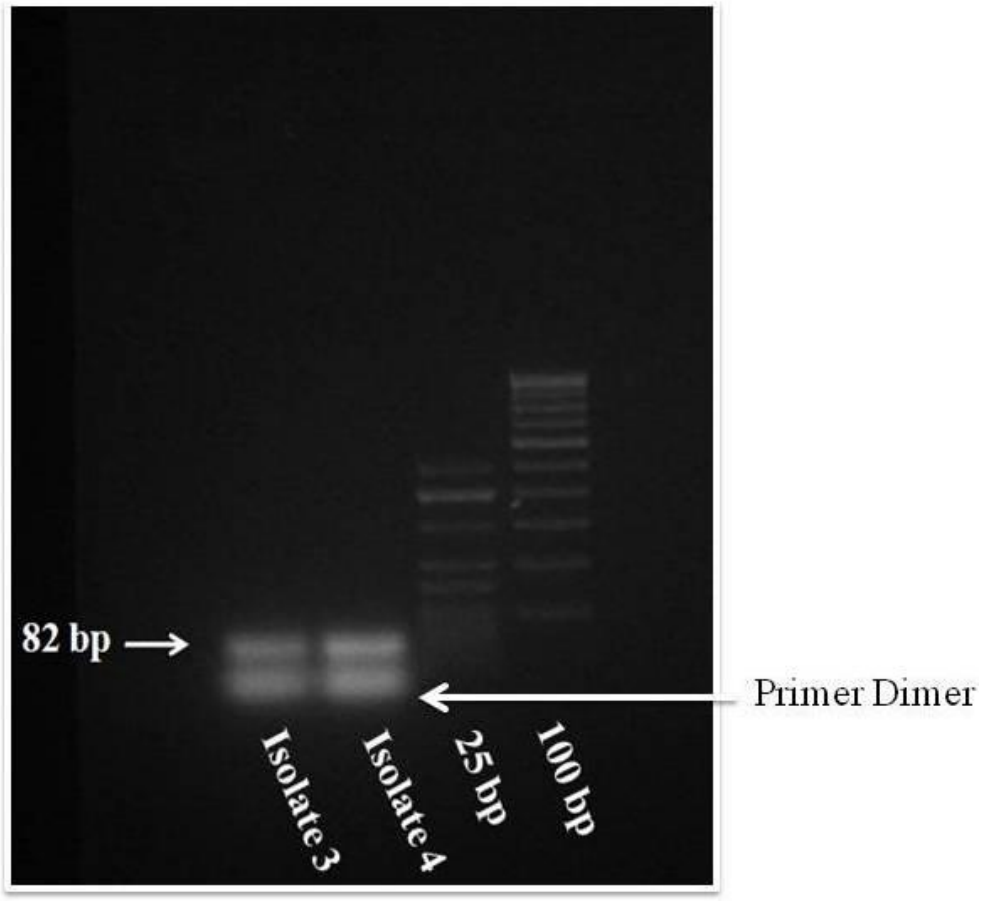


Figure 39: Gel showing primer dimer

This problem was overcome with simplex PCR, which was designed after recalculating the annealing temperature of each primer with the **NCBI-BLAST** software

After that a **Gradient PCR** was set up to optimize the annealing temperature. After amplification, the gel showed a single band of 82-bp indicating the presence of the NDM-1 gene as shown in Figure 40.

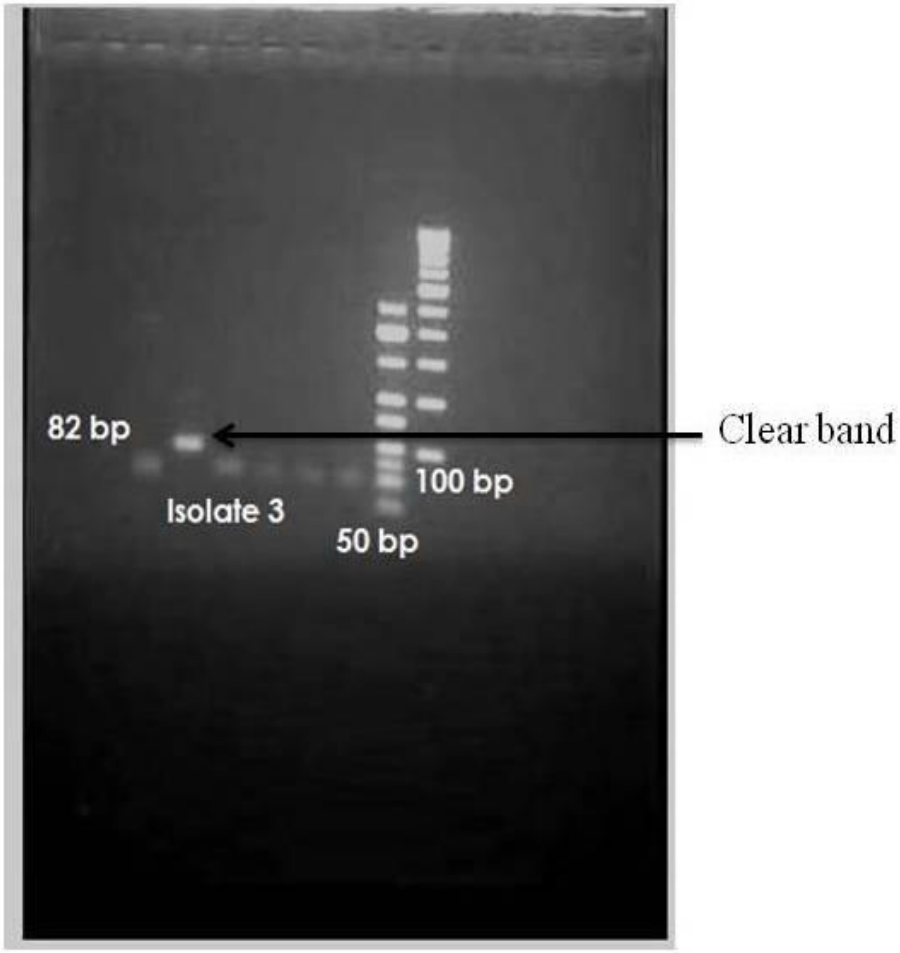


Figure 40: Gel showing single band

Simplex PCR was done for NDM-1, VIM, KPC and OXA-48 in all resistant isolates.

4.8.1. Enterobacteriaceae: PCR for carbapenemase genes

blaNDM-1: All resistant Enterobacteriaceae isolates were positive.

blaOXA-48: Detected in 8 Enterobacteriaceae isolates.

blaVIM: One isolate positive.

blaKPC: Not detected in any Enterobacteriaceae isolate

4.8.2. *Acinetobacter calcoaceticus-baumannii* complex: PCR for carbapenemase genes

blaNDM-1: All ACBC isolates were found to be positive for the blaNDM-1gene.

blaVIM: Three isolates co-produced blaVIM gene.

blaOXA-48: Not detected in any *Acinetobacter* isolate

blaKPC: Not detected in any of the study isolates.

4.8.3. *P. aeruginosa*: PCR for carbapenemase genes

blaNDM-1: blaNDM -1 was present in all carbapenem-resistant isolates

blaVIM : Twenty isolates co-produced the blaVIM gene.

blaOXA-48: Not detected in any *P. aeruginosa* isolate

blaKPC: Not detected in any of the study isolates

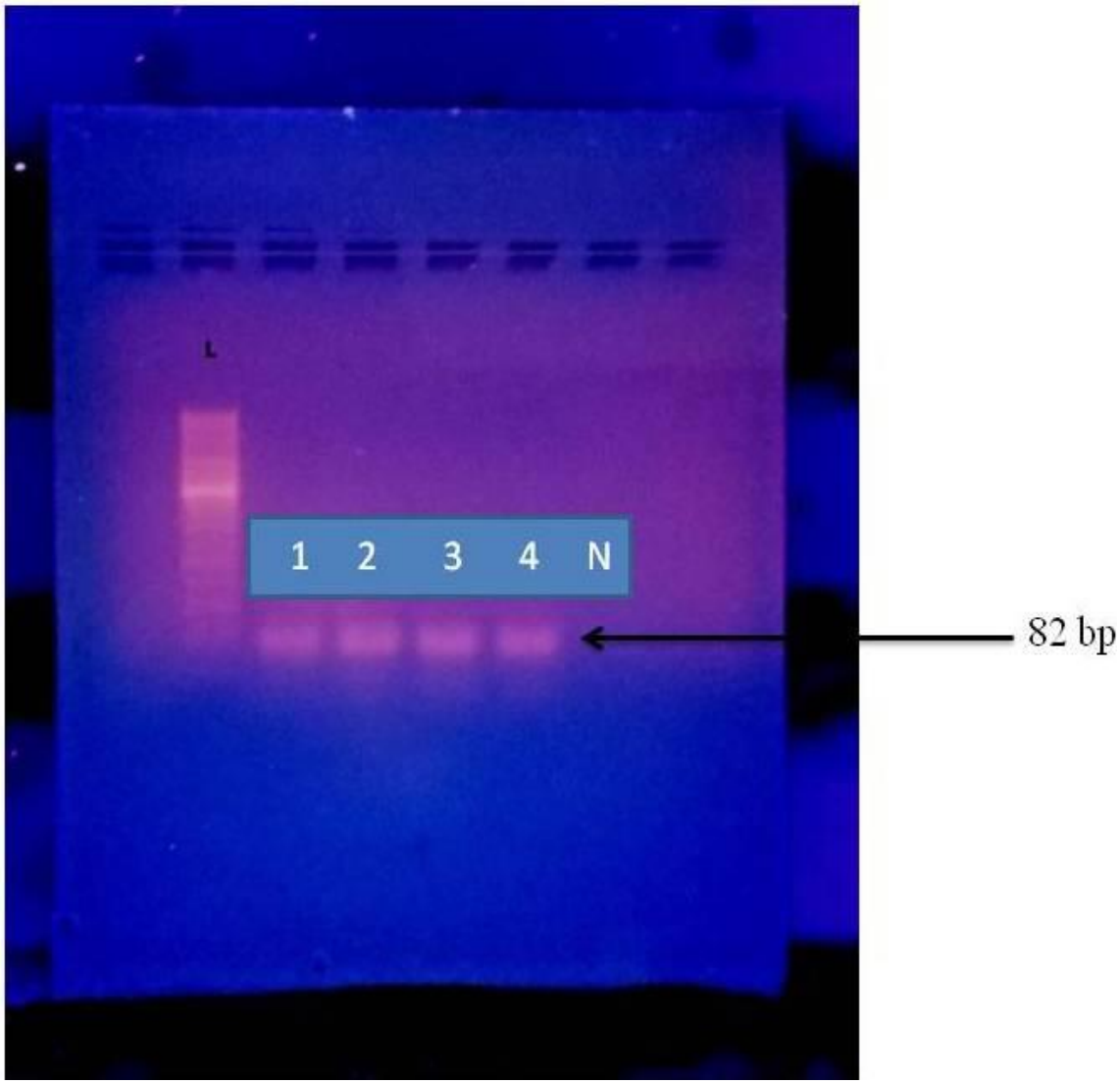


Figure 41: Gel showing the presence of amplicons of the blaNDM-1gene

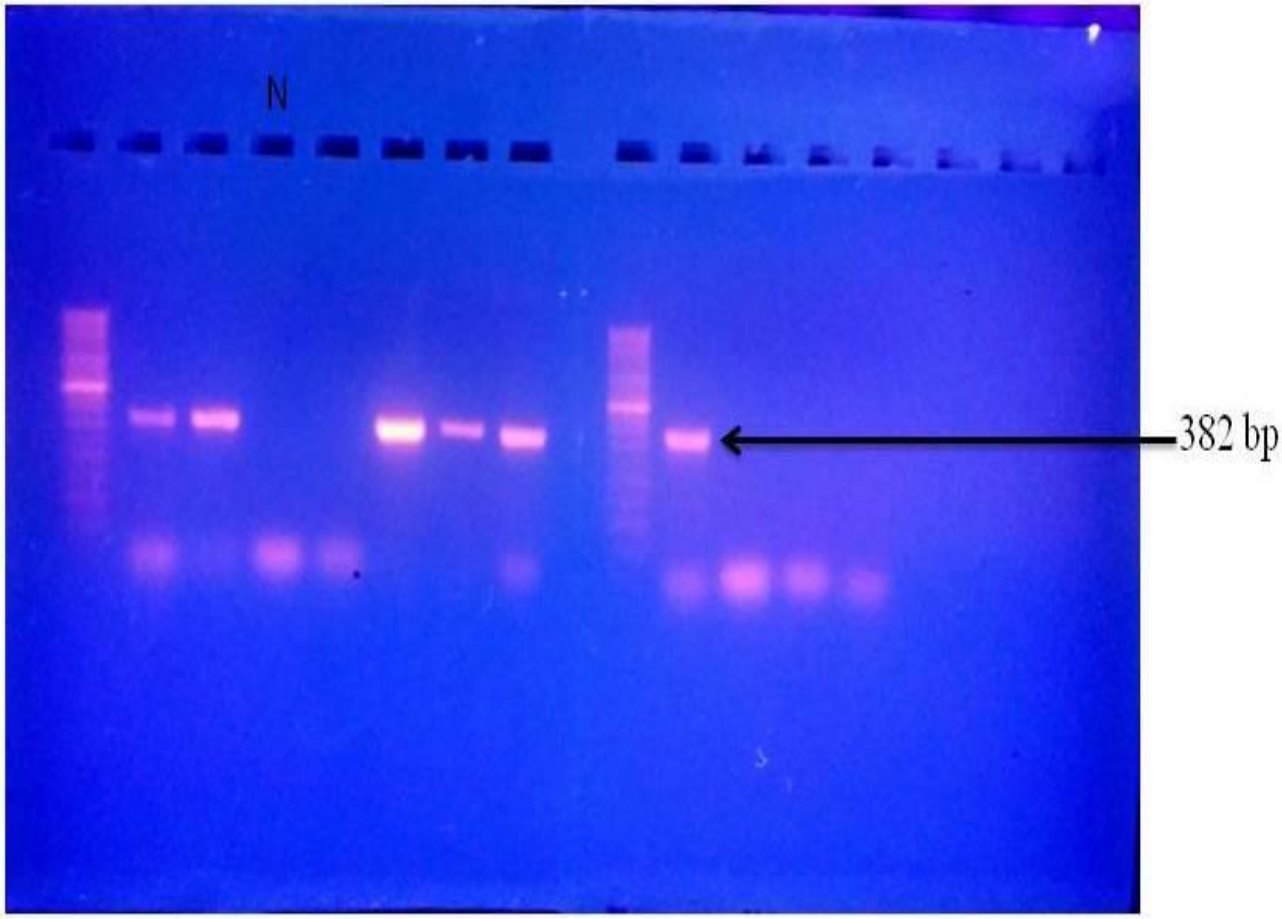


Figure 42: Gel showing the presence of amplicons of the blaVIM gene

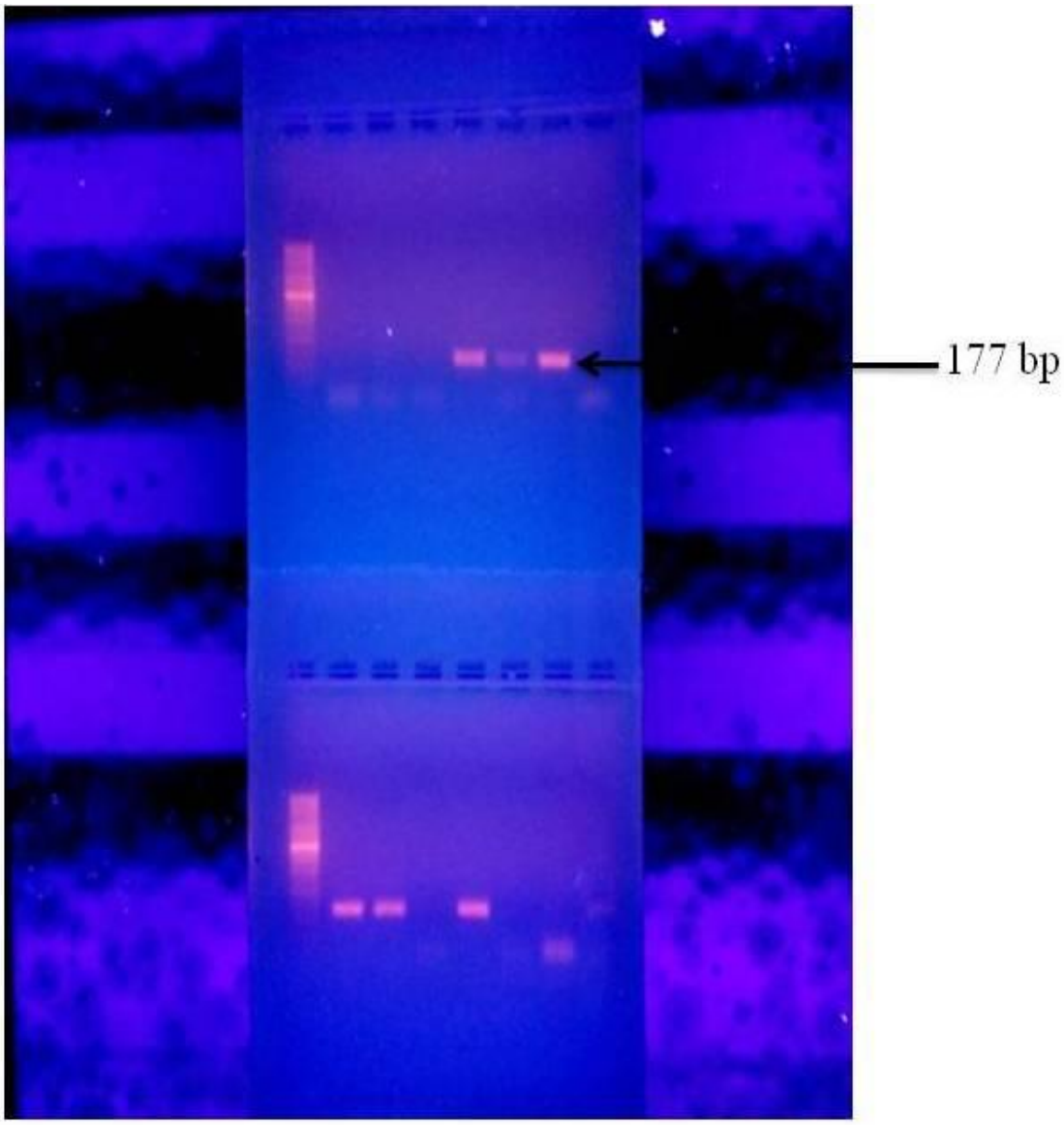


Figure 43: Gel showing the presence of amplicons of the blaOXA-48 gene

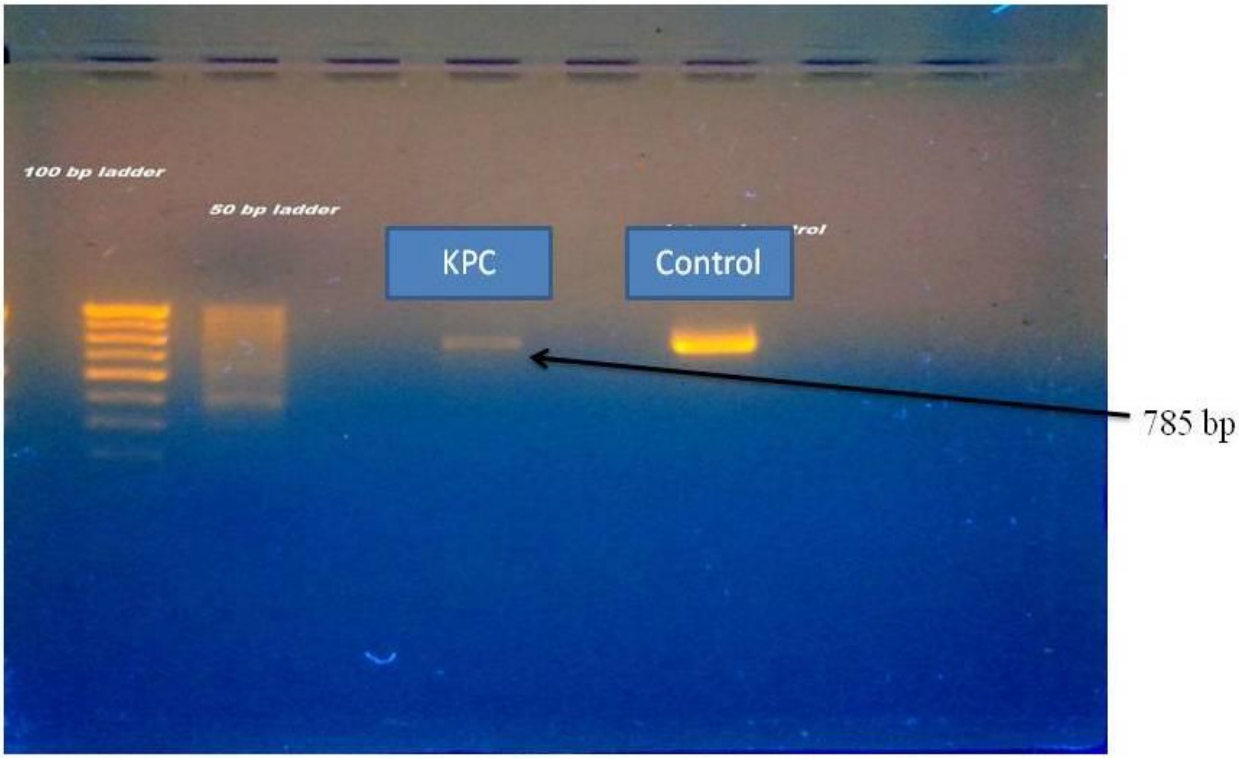


Figure 44: Gel showing the presence of amplicon of blaKPC gene from a quality-control strain, along with kit control

Carbapenemases	*Acinetobacter*	*P. aeruginosa*	Enterobacteriaceae
NDM-1	104	31	49
VIM	03	20	01
OXA-48	0	0	08
KPC	0	0	0

Table 4: Distribution of carbapenemase genes

4.9. Efflux pump detection

The Ethidium bromide concentration test was positive in 27 isolates, indicated by the lack of fluorescence under UV light, as shown in Figure 44.

The Reserpine Inhibition method showed a positive result in 20 isolates. A positive result was indicated by a decrease in carbapenem MIC upon addition of reserpine in the testing medium. All isolates positive by the reserpine-inhibition test were also positive by the ethidium bromide concentration test.

Species distribution of reserpine-inhibition positivity has been shown in Table 3.

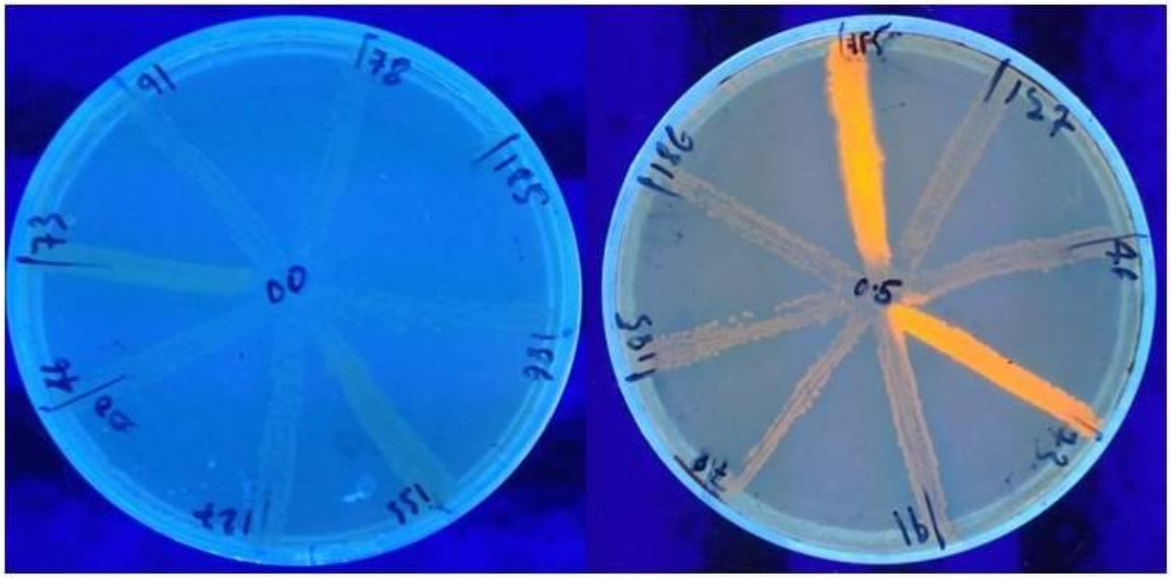


Figure 45: Demonstration of Efflux Pump Activity by the Ethidium bromide Concentration Method

Efflux Pump Detection Methods	*E. coli*	*P. aeruginosa*	*Acinetobacter*	*C. freundii*	*E. cloacae*	*K. pneumoniae*	*E. aerogenes*
Ethidium bromide cartwheel	07	06	07	02	02	02	01
Reserpine Inhibition	04	06	05	00	02	02	01

Table 5: Species distribution of efflux-positive isolates