3.0 MATERIAL AND METHODS

3.1 General Methods

The study was conducted at Himalayan Institute of Medical Sciences, Jolly Grant, Dehradun over a period of three years from October 2013 to October 2016.

3.1.1. Samples - The study included all the clinical specimen referred for bacteriological culture from outdoor (OPD) as well as Indoor (IPD) patients of all age groups and both sexes from different wards of tertiary care hospital. The specimen comprised of urine, blood, sputum, pus, CSF, body fluids, swabs, endotracheal aspirate, tracheal tube aspirate etc.

3.1.2 Sample size - The worldwide prevalence of carbapenem resistance in Gram-negative bacilli varies from 4 to 60%, depending on the epidemiological setting; in India, the average prevalence is 12% (Gupta et al., 2006). Assuming a prevalence level of 10% in our hospital on the basis of a recent pilot study, 2000 isolates were estimated at 5% level of significance with 0.05 effect size. Procurement of fresh specimens was stopped once 200 carbapenem-resistant isolates were obtained, even if it means that fewer than 2000 isolates were screened.

3.2. Specimen processing

3.2.1. Isolation - Clinically significant specimens were inoculated on MacConkey II Agar (BBL^{TM}) and other media as appropriate, and incubated at 37^0C for 18 - 24 hours.

3.2.2. Gram-staining – All colonies isolated in pure culture or in predominant culture from clinically significant specimens were stained by Gram's Method. Gram-negative isolates were then further tested for our study.

3.2.4. Identification of isolates - Carbapenem-resistant colonies of Gram-negative bacilli, as determined by Gram-staining were then characterized biochemically and identified by standard methods (Lennette et al., 1985 & Forbes et al., 2007). After noting colony morphology and the presence or absence of swarming, isolates were tested for oxidase; Hugh-Leifson glucose; gas and H_2S production in triple sugar iron agar (TSI); fermentation of sucrose, cellobiose, arabinose, xylose, and adonitol; O-Nitrophenyl-β-D-Galactopyranoside (ONPG) hydrolysis; Methyl red (MR); Voges-proskauer (VP); indole production; citrate utilization; urea hydrolysis; phenylalanine deamination; lysine and ornithine decarboxylation; arginine dihydrolase activity; motility; and pigment production. Isolates that defied identification by these standard methods, were identified using the automated Vitek 2 system (bioMérieux).

3.2.3. Screening for carbapenem resistance – All Gram-negative isolates were tested for carbapenem resistance by the disc diffusion and results were interpreted according to the Clinical Laboratory Standard Institute (CLSI) guidelines (CLSI, 2013). Antibiotic discs were obtained from HiMedia®, Mumbai, India. The antimicrobials tested include ampicillin (10 mcg), amoxycillin-clavulanate (30 µg), sulbactam (10 µg), piperacillin-tazobactam (10 µg), cephalothin (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), aztreonam (30 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), cotrimoxazole (25 µg), tetracycline (30 µg), tigecycline (15 µg), chloramphenicol (30 µg), and colistin (10 µg).

Isolates which demonstrated a reduced susceptibility toward ertapenem, imipenem, or meropenem after upon preliminary testing with discs from HiMedia®, were retested with Neo-Sensitabs™ from Rosco Diagnostica, Denmark for confirmation.

3.2.4. Sub-culturing and Preservation of Isolates- Gram negative bacterial isolates with confirmed resistance to carbapenems were sub cultured and preserved for further studies. For short term storage of bacterial cultures, bacterial isolates were sub cultured on MacConkey II Agar (BBL™) and maintained at $+4^0$C and sub-cultured monthly every 10 days. For long term storage, the isolates were grown overnight on Nutrient agar, then suspended in Brain Heart Infusion broth (HiMedia®) with 15% glycerol (Sigma-Aldrich®) in micro-centrifuge tubes and kept at -20^0C

The resistant isolates were then characterized phenotypically and genotypically.

3.3. Characterization of carbapenemase resistance by phenotypic methods

3.3.1. Determination of minimum inhibitory concentration (MIC's) of carbapenem drugs

Isolates identified as carbapenem-resistant by the Kirby-Bauer method, were further tested by the Epsilometer Test to determine minimal inhibitory concentrations (MICs) of the carbapenems: imipenem, meropenem and ertapenem. Testing was done on BBL™ Mueller Hinton II Agar (cation adjusted) using Etest® strips (bioMérieux, France) with a range of 0.002 µg/ml to 32 µg/ml, according to the manufacturer's instructions. MIC results were interpreted according to the CLSI guidelines (CLSI, 2013).

Procedure

The stored isolates were revived. A bacterial suspension was prepared and standardised to a 0.5 McFarland. The bacterial inoculums were spread onto Mueller Hinton II agar plates and dried for 5 minutes. The Etest® strips were placed on the lawns using sterile forceps and the plates were incubated for 24 hours at 37^0 C.

Interpretation of Results

An elliptical zone of inhibition was formed and the MIC's were read directly from the graduated Etest strips at the point of intersection of the inhibition zone with the strip.

3.3.2. Modified Hodge test

Media used - BBL™ Mueller Hinton II Agar (Cation adjusted)

Antibiotic discs used - Neo-Sensitabs™ Ertapenem and Imipenem from Rosco Diagnostica, Denmark.

The test was performed as follows-

- 0.5 McFarland dilution of *Escherichia coli* ATCC®25922™ was prepared in 5 ml of saline.
- The above suspension was diluted 1:10 by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of MHB or saline.
- The 1:10 dilution of *E.coli* ATCC 25922 was swabbed on a Mueller Hinton II agar plate that was allowed to dry for five minutes.
- A 10 µg ertapenem for fermenters and 10 µg imipenem for non-fermenters susceptibility disc was placed in the centre of the test area.
- The test organism was streaked in a straight line from the edge of the disc to the edge of the plate. Not more than four organisms were tested on the same plate.
- Plates were incubated at 36°C in ambient air for 18 hours.

Interpretation of Results*:*

After 18 hours of incubation, the plates were examined for a clover-leaf shaped indentation of the zone of inhibition of *Escherichia coli* ATCC®25922™ around the streak of the test strain.

MHT Positive test has a clover leaf-like indentation of the *E. coli* 25922 growing along the test organism growth streak within the disc diffusion zone.

MHT Negative test has no growth of the *E. coli* 25922 along the test organism growth streak within the disc diffusion.

3.3.3. Carbapenemase detection and identification based on inhibitor profile.

The KPC and MBL Confirm Kit from Rosco Diagnostica, Denmark was used for detection of *Klebsiella pneumoniae* carbapenemase (KPC), metallo-β-lactamases (MBL) and AmpC in Enterobacteriaceae. The test was performed according to the manufacturer instructions. Briefly:

- Bacterial suspension of 0.5 McFarland was lawn cultured on Mueller Hinton II agar (BBL^{TM}) medium by sterile swab stick.
- Then different tablets namely tablet A meropenem, tablet B meropenem and dipicolinic acid (MBL inhibitor), tablet C meropenem and cloxacillin (AmpC inhibitor), and tablet D meropenem and boronic acid (KPC inhibitor) were placed on the surface of the medium.
- The plates were then incubated at 35^0C for 16-24 hours.

Interpretation of Results:

The zone of inhibition of tablet A was compared to the zones of inhibition of each of the carbapenem-plus-inhibitor tablets (B, C, and D).

- If tablet B shows a zone difference of ≥5 mm from tablet A, the organism was recorded as demonstrating MBL activity.

- If tablet D shows a zone difference of ≥5 mm from tablet A, the organism was recorded as demonstrating KPC activity.
- If tablets C and D both show a zone difference of ≥5mm from tablet A, the organism was recorded as demonstrating AmpC activity coupled with porin loss. (impermeability)

3.3.4. Rapid CARB Blue Kit

The above kit from Rosco Diagnostica, Denmark was used for the carbapenemase detection in Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* spp. isolates

The test was performed according to the manufacturer instructions. Briefly-

- Bacterial suspension of McFarland standard 2 was prepared from Mueller Hinton II Agar (BBL^{TM}) plates.
- Fifty µL of this suspension was mixed in 100 µL of 0.9 % saline solution at pH 5 in two separate tubes.
- The suspension was vortexed for 1 minute and then maintained at room temperature for 30 minutes.
- The Imipenem(x2) +bromothymol blue and negative control tablet were then put into these solutions separately.
- The tubes were incubated at 37 °C for 2 hours.

Interpretation of Results

- Green colour of the test suspension and blue colour of negative control indicated a positive reaction.

- Yellow colour of the test suspension and green colour of the negative control also indicated a positive reaction
- A light yellow colour of the negative control indicated an invalid test and an uninterpretable result.
- Blue or green colour of both the test suspension and the negative control indicated a negative result.

3.3.5. Neo-Rapid CARB Kit

This kit from Rosco Diagnostica, Denmark was used for the detection of carbapenemase in Enterobacteriaceae and *Pseudomonas* spp. isolates

This test was performed according to the manufacturer instructions. Briefly-

- Bacterial suspension of McFarland standard 4 was prepared from Mueller Hinton II Agar (BBL^{TM}) plates.
- Fifty µL of this suspension was mixed in 100 µL of 0.9 % saline solution at pH 7.5 in two separate tubes.
- The suspension was vortexed for 1 minute and then maintained at room temperature for 30 minutes.
- The imipenem (x2) +indicator (CARB) and negative control tablet were then put into these solutions separately.
- The tubes were incubated at 37 °C for 2 hours.

Interpretation of Results

- A change in the colour of the test suspension from red to orange or some shade of yellow indicated a positive reaction, provided the negative control remained red.
- Red colour of both test and negative control tubes indicated a negative reaction.

3.3.6 Carba NP test

This test was used for the detection of carbapenemases in Enterobacteriaceae and *Pseudomonas* spp (Nordmann et al., 2012 d)

The test was performed as follows-

- One calibrated loop (10 µL) of the tested strain directly recovered from the antibiogram was resuspended in a 100 µl lysis buffer (PUReGeNe™).
- The above suspension was vortexed for 1 minute and further incubated at room temperature for 30 minutes.
- 50 µL of the suspension, corresponding to the enzymatic bacterial suspension, was mixed in a 96-well tray with 100 µL of solution made of 6 mg imipenem monohydrate (Sigma-Aldrich®) pH 7.8, phenol red solution, and 0.1 mmol/L ZnSO4.
- The phenol red solution was prepared by mixing 2 mL of a phenol red (Sigma-Aldrich®) solution 0.5% (wt/vol) with 16.6 mL of distilled water. The pH value was then adjusted to 7.8 by adding drops of 1 N sodium hydroxide (NaOH).
- A microtiter plate with mixture of the phenol red solution and the enzymatic suspension being tested was incubated at 37°C for a maximum of 2 hours.

Interpretation of Results

(I) If the colour of the well containing phenol red plus imipenem plus $ZnSO_4$ turned from red to yellow-orange but the well containing phenol red plus $ZnSO_4$ plus remained red, the strain produced a carbapenemase.

(II) If the colour of both the wells remained red, the strain was not a carbapenemase producer; and

(III) If both the well colour turned from red to yellow-orange, the test was considered as not interpretable.

3.3.7. CarbAcineto NP Test .

The CarbAcineto NP test was adapted from the updated version of the Carba NP test used for the detection of carbapenemase-producing Enterobacteriaceae and *Pseudomonas* spp. (Dortet et al., 2014) in order to use the test for *Acinetobacter* spp.

In this updated version, the lysis buffer was replaced by a 5 M sodium chloride solution, avoiding any buffer effect, and the bacterial inoculum was doubled from one-third to one-half of a calibrated loop (10 µl) to a full calibrated loop in order to increase the enzyme quantity.

- Briefly, a full calibrated loop (10 µl) of the tested strain was recovered from MHA plates and resuspended in two 1.5-ml Eppendorf tubes/microtiter plates (A and B) containing 100 µl of 5 M NaCl.
- 50 µL of the bacterial suspension was mixed in two separate well of a microtiter plate with one well containing 100 µL of phenol red plus 0.1 mmol/L ZnSO4 solution and other well containing 6 mg imipenem monohydrate (Sigma-Aldrich®) plus phenol red plus 0.1 mmol/L ZnSO4. The microtiter plate was then incubated at 37°C for a maximum of 2 hours.
- Optical reading of the colour change of each well was performed. The carbapenemase activity was detected by a colour change of phenol red solution (red to yellow/orange) resulting from the hydrolysis of imipenem into a carboxylic derivative, leading to a decrease of the pH value.

Interpretation of Results

(i) If the colour of the well containing phenol red plus imipenem plus $ZnSO_4$ turned from red to yellow-orange but the well containing phenol red plus $ZnSO_4$ plus remained red, the strain produced a carbapenemase.

(ii) If the colour of both the wells remained red, the strain was not a carbapenemase producer; and

(iii) If both the well colour turned from red to yellow-orange, the test was considered as not interpretable

The phenol red revealing solution was prepared as previously described (Nordmann et al., 2012 d). The 5 M NaCl solution was prepared from a dilution of NaCl powder (HiMedia™) in distilled water

3.3.8. Blue Carba Test

This test was used for the detection of carbapenemases in Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* isolates (Pires et al., 2013).

The test was performed as follows-

- The test solution consisted of an aqueous solution of bromothymol blue (Fluka®) at 0.04% adjusted to pH 6.0, 0.1 mmol/liter ZnSO4, and 6 mg/ml of imipenem, with a final pH of 7.0.
- A negative-control solution (0.04% bromothymol blue solution, pH 7.0) was prepared to control the influence of bacterial components or products in the pH of the solution.
- A loop (approximately 5 µl) of a pure bacterial culture recovered from Mueller-Hinton II agar (BBL™) was directly suspended in 100 µl of both test and negative-

control solutions in a 96-well microtiter plate and incubated at 37°C with agitation at 150 rpm for 2 hours.

Interpretation of Results

- If the colour of the well containing bromothymol blue and $ZnSO_4$ plus imipenem turned from blue to green or yellow, but the well containing bromothymol blue and $ZnSO_4$ plus remained blue, the strain produced a carbapenemase.
- If the colour of the well containing bromothymol blue and $ZnSO_4$ plus imipenem turned from green to yellow, but the well containing bromothymol blue and $ZnSO_4$ plus remained green, the strain produced a carbapenemase.
- If both the wells remained the same shade of blue or green colour, the strain produced no carbapenemase.
- If both wells changed colour from blue to green or from green to yellow, it indicated an invalid test and an uninterpretable test result.

Modifications tested

In addition, 'in-house' variations, all the tests were carried out in the following ways in an attempt to improve their performance, in the form of either better sensitivity or lower costs.

1. Bacterial biomass for the tests was taken from two different sites on the same antimicrobial sensitivity testing plate: one near the carbapenem disc and other away from the disc.
2. Imipenem of two different grades were used: a more expensive analytical grade chemical from Sigma-Aldrich™, and a less expensive pharmaceutical grade powder imipenem-cilastatin from Lupinem®.

3.3.9. Carbapenem Inactivation Method (CIM)

This method was used for the detection of carbapenemases in Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* spp. isolates (Zwaluw et al., 2015) as follows:

- A suspension was prepared by suspending loopful of bacterial culture from the Mueller Hinton II agar (BBL™) or blood agar plate in 400 µl of sterile distilled water.
- Subsequently, a meropenem (BD BBL™ Sensi-Disc™) was immersed in the suspension.
- The above suspension was incubated for minimum of two hours at 35^0 C.
- After incubation, the disc was removed from the suspension using an inoculation loop.
- The discs were placed on a Mueller-Hinton agar plate inoculated with a susceptible *Escherichia coli* ATCC®25922™ indicator strain.
- The above plates were then incubated overnight at 35^0 C.

Interpretation of Results

- If the bacterial isolate produced carbapenemase, the meropenem in the susceptibility disc was inactivated allowing uninhibited growth of the susceptible indicator strain.
- Discs incubated in suspensions that did not contain carbapenemases yielded a clear inhibition zone in the susceptible indicator strain.

3.4. Control Strains

Following strains were used for quality control

- *Escherichia coli* ATCC ® 25922™ (Pan-sensitive)
- *Pseudomonas aeruginosa* ATCC ® 27853 ™ (Pan-sensitive)
- *Klebsiella pneumoniae* ATCC® BAA-1705 ™ (KPC positive)
- *Klebsiella pneumoniae* ATCC® BAA-2146 ™ (MBL positive)
- *Pseudomonas aeruginosa* ATCC® 10145 ™ (MBL positive)
- *Klebsiella pneumoniae* ATCC ® 700603 ™ (ESBL positive)

List of Abbreviations

ATCC	=	American Type Culture Collection
KPC	=	*Klebsiella pneumoniae* carbapenemase
MBL	=	Metallo-β-lactamases
ESBL	=	Extended spectrum β-lactamases

3.5. Molecular Methods

3.5.1. DNA Extraction

- 1.5 ml of an overnight bacterial culture in Luria-Bertani broth was centrifuged at 6000 rpm for 10 minutes and the supernatant discarded.
- 180 µl of lysis buffer I and 20µl of Proteinase K was added to the pellet and mixed thoroughly by vortexing. The above was incubated at 55º C for 3 hours.
- After first incubation, 200 µl of lysis buffer II was added and mixed thoroughly by vortexing and incubated at 70^0 C for 20 minutes.
- After the second incubation, the above suspension was centrifuged at 10,000 rpm for 10 minutes and the resulting supernatant was collected in a 1.5 ml vial.
- 4 µl of RNase (100 mg/ml) was added to the supernatant, mixed by vortexing and incubated at room temperature for 5-10 minutes.
- After this 200 µl of absolute ethanol was added and mixed thoroughly by vortexing.
- The GeneiPure™ column was then placed in a 2 ml collection tube and sample ethanol mixture was added to the column.
- The above was centrifuged at 11,000 rpm for 5 minutes. The collection tube was discarded with the flow through.
- The GeneiPure™ column was placed in fresh 2 ml collection tube and diluted with 500 µl of wash buffer I (Prepared by diluting 1 volume of wash buffer I with 3 volumes of absolute ethanol.). It was centrifuged at 11,000 rpm for 1 minute. The collection tube was discarded with the flow through.

- The GeneiPure^{TM} column was then placed in a fresh 2 ml collection tube and this time diluted with 500 µl of wash buffer II (Prepared by diluting 1 volume of wash buffer II with 3 volume of absolute ethanol) and then centrifuged at 11,000 rpm for 3 minutes.
- The wash fraction was discarded and the collection tube was retained for the next step.
- The empty GeneiPure^{TM} column was then centrifuged at 11000 rpm for 2 minutes. After this the collection tube was discarded.
- 200 µl of elution buffer was placed in a fresh sterile 1.5 ml vial and was pre warmed at 70^0 C for 5 minutes.
- At the same time, the GeneiPure^{TM} column was placed in a sterile 1.5 ml vial and 200 µl of pre-warmed elution buffer was added to the centre of this column.
- The above was incubated at room temperature for 5 minutes and then centrifuged at 11,000 rpm for 2 minutes to elute the DNA
- The eluted DNA was stored at -20^0 C.

3.5.2. Primer sets

The primers used in this study were synthesized at Eurofins Genomics, India. The detailed information of the nucleotide sequences of primer set used is shown in Table 3.

Table 3: Nucleotide sequences of primers for Polymerase chain reaction (PCR)

Target	Primer name	Sequence (5'- 3')	Amplicon size (bp)	References
blaKPC type	KPC-F KPC-R	TCGCTAAACTCGAACAGG TTACTGCCCGTTGACGCCCAATCC	785	Monteiro et al., 2009
blaNDM-1	NDM-F NDM-R	TTGGCCTTGCTGTCCTTG ACACCAGTGACAATATCACCG	82	Monteiro et al., 2012
blaOXA-48	OXA-48-F OXA-48-R	TGTTTTTGGTGGCATCGAT GTAAMRATGCTTGGTTCGC	177	Monteiro et al., 2012
blaVIM type	VIM-F VIM-R	GTTTGGTCGCATATCGCAAC AATGCGCAGCACCAGGATAG	382	Mendes et al., 2007

3.5.3. Polymerase chain reaction (PCR) mixture

For the detection of each of the four carbapenemase genes (KPC, NDM-1, VIM and OXA-48) primers were dissolved in nuclease-free PCR water to form stock solutions of 100µg/µL and stored at -20^{0}C. The working solution was prepared by adding 1µl of each primer (the forward and reverse primers), 12.5 µl of master mix (Thermo Scientific), and 8.5 µL of the free DNAse/RNase water. Each PCR reaction was carried out using 2µl DNA and 23 µL of the previously prepared master mix in a final volume of 25 µL.

3.5.4. PCR amplification of Carbapenems resistance genes

PCR amplification was carried out using thermal cycler Veriti®96-Well (Applied Biosystems®, USA). PCR conditions were as follows for each individual primer set.

Initial denaturation	95^0 C for 5 minutes
Denaturation	95^0 C for 45 seconds
Annealing	60^0 C for 45 seconds
Extension	72^0 C for 1 minute
Final extension	72^0 C for 8 minutes

3.5.5. Agarose gel electrophoresis

The PCR products were analyzed by electrophoresis in 2.0% agarose gel to detect specific amplified product by comparing with standard molecular weight markers of 100 and 50 base pair (DNA ladder). Two percent agarose gel was prepared by suspending 2.0 gm agarose in 200 ml of 50x Tris acetate EDTA (TAE) buffer, boiling to dissolve, and cooling to about 50ºC. Ethidium bromide at a working concentration of 0.5 µg/ml was added, and the solution was poured into gel trays with combs in place.

3.5.6. Loading and electrophoresis of the sample

On electrophoresis, the gel was placed in a horizontal electrophoresis apparatus containing 50X TAE buffer. Five µL of amplified product was mixed with 2.0 µL of loading dye, mixed, loaded into the well. Electrophoresis was carried out at 90 volts for 45 minutes.

3.5.7. Visualization of the gel

The amplified products of the study samples were visualized by trans-illuminator, photographed by a digital camera and transferred to computer data for labelling and storage.

3.6. Data analysis

Data were analyzed using the Microsoft Excel. Qualitative data were expressed in terms of number and percent.

3.7. Efflux pump mechanism

The efflux pump in the isolates was tested by the following two methods

3.9.1. Ethidium bromide cartwheel method

3.9.2. Using Reserpine as efflux pump inhibitor

Ethidium bromide cartwheel method

- Trypticase soy agar (Merck Biosciences) plates were prepared with ethidium bromide (GeNei^{TM}).
- The prepared plates were divided into sectors by radial lines (Cartwheel pattern)
- 0.5 McFarland standard of multidrug-resistant Gram-negative isolates were prepared and then swabbed on to the EtBr-TSA plates from the centre of the plate to the margin.
- The plates were then incubated at 37^0 C for 16 hours.
- After incubation, the plates were examined under UV light.
- The concentration of ethidium bromide that produces fluorescence of bacterial mass was recorded.

Using Reserpine as efflux inhibitor

- Minimum inhibitory concentration (MIC) assays by agar dilution method for meropenem were performed with Mueller Hinton II agar (BBL™) plates with or without 25 µg/ml and 50 µg/ml Reserpine (Fluka®).
- Differences in the MIC s of the strains (decrease in the MIC s of the control plates with Reserpine) suggested a putative efflux mechanism.