MATERIALS AND METHODS -

- **Study Design** - Prospective study including laboratory investigations and observational analytical design.

- **Study Place** - Rama Medical College Hospital & Research centre, Kanpur, U.P.

- **Study Period** - The study will be conducted within a period of 12-18 months.

- **Study Population** - The non-repetitive samples will be collected from the different patients, sent to the microbiology laboratory for the culture identification and sensitivity testing will be included in the study.

- **Inclusion Criteria** – All clinical samples of both sexes will be included.

- **Exclusion Criteria** – Patients on antibiotic treatment.

- **Sample Size** - All clinical samples like sputum, urine, blood, pus, wound swab, aspirated fluids any tip/tube from patients during study period will be included for this study. For estimating proportion of patients having *Staphylococcal* infection attending Rama Hospital.

  **The following formula will be used**-

  \[
  n \geq \frac{Z_{1-\alpha/2}^2 \times p(1 - p)}{d^2}
  \]

  Alpha \((\alpha) = 0.05\)

  Estimated proportion \((p) = 0.174\)

  Estimation error \((d) = 0.051\)
Sample Size = 220

- **Statistical Analysis** - The assessment of association of different variables with clinical samples will be calculated and all the statistical analysis will be done using SPSS as per need.

- **Clinical Data** - The following information will be collected from the patient’s clinical records: Patient demographics
  - Date of isolation
  - Location of the patient at time of infection
  - Type of Wards, ICU, OPD
  - Durations of stay
  - Site of infection
  - Disease and Other complications.

- **Ethical consideration** - Ethical clearance will be taken from ethical committee of RMCH and RC, Kanpur.

- **METHODOLOGY** - Particulars of patient, relevant clinical history and reports of investigations will be recorded as per the proforma. The Molecular analyses will be done in the Research Laboratory.

- **Sample collection and processing**
  - All samples received in Microbiology section of Central Laboratory will be processed by routine microbiological methods.\(^{140}\)
  - The isolates will be first identified as *S. aureus* by standard microbiological technique and conventional methods (colony morphology, Gram stain, Catalase activity, and slide
and tube coagulase test, culture on blood agar, Deoxyribonuclease (Dnase Test), Phenolphthalein Phosphate Agar.\textsuperscript{140}

- Antibiotic susceptibility testing will be performed on Mueller Hinton Agar (Himedia) by modified Kirby Bauer’s Disc Diffusion technique recommended by CLSI, 2017.\textsuperscript{141} A suspension of each isolate with turbidity matching 0.5McFarland and then lawn culture will be done in MHA plate. Sterile commercially available antibiotic discs (Himedia) will be used and incubated at 37\(^\circ\)C overnight.\textsuperscript{141} A zone of inhibition will be measured in millimeters (mm) and the organism will be classified as sensitive, intermediate or resistant according to the zone size interpretation chart provided.\textsuperscript{141}

**Table: 1. Interpretation of the inhibition zone diameter (Himedia).**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Disc potency</th>
<th>Resistance (mm)</th>
<th>Intermediate (mm)</th>
<th>Sensitive (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin (GM)</td>
<td>10µg</td>
<td>&lt;12</td>
<td>13-14</td>
<td>&gt;15</td>
</tr>
<tr>
<td>Amikacin (AN)</td>
<td>30 µg</td>
<td>&lt;14</td>
<td>15-16</td>
<td>&gt;17</td>
</tr>
<tr>
<td>Vancomycin (VA)</td>
<td>5 µg</td>
<td>---</td>
<td>---</td>
<td>15</td>
</tr>
<tr>
<td>Teicoplanin (TEC)</td>
<td>30 µg</td>
<td>10</td>
<td>11-13</td>
<td>14</td>
</tr>
<tr>
<td>Tetracycline (TE)</td>
<td>30 µg</td>
<td>&lt;14</td>
<td>15-18</td>
<td>&gt;19</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>5 µg</td>
<td>&lt;15</td>
<td>16-20</td>
<td>&gt;21</td>
</tr>
<tr>
<td>Cefoxitin (FOX)</td>
<td>30 µg</td>
<td>&lt;21</td>
<td></td>
<td>&gt;22</td>
</tr>
<tr>
<td>Nitrofurantoin (F/M)</td>
<td>300 µg</td>
<td>&lt;14</td>
<td>15-16</td>
<td>&gt;17</td>
</tr>
<tr>
<td>Linezolid (LZD)</td>
<td>30µg</td>
<td>---</td>
<td>---</td>
<td>&gt;21</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Conc.</td>
<td>MIC</td>
<td>Zone</td>
<td>Disc Diameter</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------</td>
<td>------</td>
<td>------</td>
<td>---------------</td>
</tr>
<tr>
<td>Amoxicillin-Clavonic Acid (AMC)</td>
<td>20/10 µg</td>
<td>&lt;19</td>
<td>---</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Penicillin (P)</td>
<td>10 µg</td>
<td>&lt;28</td>
<td>---</td>
<td>&gt;29</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole (SXT)</td>
<td>1.25 µg</td>
<td>&lt;10</td>
<td>11-15</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Clindamicin (CL)</td>
<td>2 µg</td>
<td>&lt;21</td>
<td>---</td>
<td>&gt;21</td>
</tr>
<tr>
<td>Erythromycin (ER)</td>
<td>15 µg</td>
<td>&lt;13</td>
<td>---</td>
<td>&gt;13</td>
</tr>
</tbody>
</table>

**Detection of MRSA by Cefoxitin & Oxacillin Disc Diffusion Method**

Cefoxitin is a potent inducer of mecA regulatory system. Which is being widely used as a marker for detection of mecA gene mediated methicillin resistance. CLSI has recommended Cefoxitin disc method for the detection of MRSA. 0.5 McFarland standard suspension of isolates will be made and a lawn culture done on MHA plate. A 30 µg of Cefoxitin disc will be placed and plates are incubated at 37°C for 18 hours and diameter of zone of inhibition will be measured. Zone diameter < 21 mm will be considered as methicillin resistant and Zone diameter >22 mm as methicillin susceptible.

**Oxacillin Disc Diffusion Method** - Using the oxacillin, a plate containing 6 µg/ml of oxacillin in Mueller-Hinton agar supplemented with NaCl (4% w/v; 0.68 mol/L) at 30°C.
D test will be done by using Erythromycin (ER) & Clindamycin (CL) disc, by Kirby Bauer’s disc diffusion method on Mueller Hinton agar plates. D test will be done by using standard CLSI guidelines.\textsuperscript{142}

**Procedure and Interpretation:** - A 0.5 McFarland equivalent suspension of organisms will be inoculated into Mueller–Hinton agar (MHA) plate as described in the CLSI recommendations.\textsuperscript{142} CL (2 \(\mu\)g) and ER (15 \(\mu\)g) discs will be placed 15 mm apart from the centre on the MHA.\textsuperscript{125} Plates will be analyzed after 18 h of incubation at 35°C. Interpretation of the diameters of zones of inhibition will be done as follows:

- ER sensitive (ER-S) >23 mm,
- ER intermediate (ER-I) 14–22 mm,
- ER resistance (ER-R) <13 mm;
- CL sensitive (CL-S) >21 mm,
- CL intermediate (CL-I) 15–20 mm and
- CL resistance (CL-R) <14 mm.\textsuperscript{143}

The disk diffusion based on the D-test produced four phenotypes of Staphylococci, designated as D-positive, D-negative, resistant (R) and susceptible (S).

- D-shaped zone around the CL disk (D-positive phenotype), indicating an inducible CL resistance
- CL susceptible showing a circular shape zone around the CL disk (D-negative phenotype).
- Both CL and E resistant (R phenotype)
- Both CL and E susceptible (S phenotype).\textsuperscript{143}

**Genotypic detection of MRSA using PCR for meca & ermA gene**

* Mec A Forward primers AAAATCGATGGTAAAGGTTGGC 530bp
Mec A Reverse primers AGTTCTGCAGTACCGGATTTGC

ErmA Forward primers AAGCGGTAACCCCTCTGA 190bp

ErmA Reverse primers TTCGCAAATCCCTTCTCAAC

**Nucleotide Sequencing** – The PCR product of the isolates that carried the MecA and ErmA gene will be sent to Chromous Biotech Pvt. Ltd., Bangalore for gene sequencing. No 39, 3rd Floor, Metropolis Business Park, Industrial Area, 1st Main, Yelahanka New Town, Bangalore – 560064.

**Materials used in PCR**

**Equipments**

- PCR Thermo cycler
- Pipettes for 1µl to 1000 µl
- Electrophoresis unit
- Vortex
- Centrifuge Machine
- Water bath
- Eppendorf tubes
- Tips (filter) for pipettes 1 µl to 1000µl

**Reagents**

- Molecular Marker (Ladder 100 base pair)
- Ethidium bromide solution (1%)
- Loading Dye
- Electrophoresis Buffer (Tris Acetate EDTA and tris Borate EDTA)
- Agarose Gel
- Primer mecA forward
- Primer mecA reversed
- Magnesium chloride
- dNTP’s
- DNA Template

Peptone water will be inoculated with specimen from nutrient agar slant and incubated for 2 hours. The broth is used for PCR.

- The following steps will be followed for PCR
- DNA Extraction
- Prepared Master Mix
- Run on thermocycler (PCR)
- Agarose Gel Preparation
- Run on Electrophoresis
- Band observation