Materials and Methods
MATERIALS AND METHODS

The study was conducted with specific aims and objectives in three different phases involving three groups of population from different settings. Informed consent was taken from the patients or their parents before enrolling them into the study. This study was cleared by the Ethical Committee of JIPMER.

Phase I: Pharyngitis among school children

Study in the first group involved isolation, characterisation and antibody levels to beta hemolytic Group A Streptococci in cases of pharyngitis, in children of some rural and urban schools in Pondicherry during a one year period.

STUDY POPULATION:

This included 322 rural and 295 urban school children from six schools under the care of rural and urban health centres of JIPMER, Pondicherry at Ramanathapuram and Kuruchikuppan, respectively.

Rural schools included- (1) Ramanathapuram Primary School, (2) Thondamanatham Government Primary School and (3) Pillaiarkuppam Middle School. Urban schools included- (1) Calve Primary School, (2) St. Francis Assie High School, Vaithikuppan and (3) Kuruchikuppam Government Primary School. Table I shows the sample size from each school children aged 5 to 15 years who were screened for
### TABLE - I
Sample size from different Schools in Urban and Rural Pondicherry

<table>
<thead>
<tr>
<th>NAME OF THE SCHOOL</th>
<th>SAMPLE SIZE</th>
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<tbody>
<tr>
<td><strong>URBAN:</strong></td>
<td></td>
</tr>
<tr>
<td>Jvalve Primary school</td>
<td>61</td>
</tr>
<tr>
<td>St. Francis Assiss High School</td>
<td>136</td>
</tr>
<tr>
<td>Juruchikuppam Govt. High School</td>
<td>98</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>295</strong></td>
</tr>
<tr>
<td><strong>RURAL:</strong></td>
<td></td>
</tr>
<tr>
<td>Jramanathapuram Primary School</td>
<td>54</td>
</tr>
<tr>
<td>Jthondamanatham Govt. Primary School</td>
<td>135</td>
</tr>
<tr>
<td>Jpilliarkuppam Middle School</td>
<td>133</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>322</strong></td>
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the presence of pharyngitis and tonsillitis, with or without submandibular lymphnode enlargement.

**Specimen collection:**

All children from each class were examined by a pediatrician. Two throat swabs were collected from all suspected cases of pharyngitis and or tonsillitis (Photograph No.1).

**Swab No.1:** Cotton tipped swabs were collected by effecting tight pressure over both the tonsils and across the posterior pharyngeal wall. Exudate, when present was also swabbed. The swab was immediately rubbed on to a sterile Whatman No.1 filter paper strip (2 x 6 cm) wrapped with butter paper and enclosed in aluminium foil¹ (Manual of reference procedures in Streptococcal bacteriology and serology, WHO Regional Office for South-East Asia, New Delhi)²⁶². Each foil was labelled with necessary information.

**Swab No.2:** Dacron tipped swab (Himedia) were collected simultaneously for direct antigen detection. They were transported to the laboratory in sterile tubes appropriately labelled.

Filter paper strips were removed from the aluminium foil and butter paper wrapper using sterile forceps and placed on gentamicin sheep blood agar
specimen surface firmly in contact with the surface of the medium. Plates were incubated aerobically for four hours, following which the filter papers were removed and placed on an adjacent area on the medium. These plates were then incubated in 5-10% CO₂ at 37°C overnight. Plates were examined after removal of the filter paper, for the presence of betahemolytic colonies. A Gram stain was done, followed by presumptive identification using bacitracin test. Colonies were inoculated on sheep blood agar to give a confluent growth. Bacitracin disc containing 0.02 units were placed on the inoculum. Readings were taken following overnight incubation at 37°C. Development of clear zone of inhibition measuring ≥ 14 mm in diameter was taken as presumptive identification of Group A Streptococci.

All strains presumptively identified as GABHS were confirmed by serogrouping, using specific antisera.

**Serogrouping of Streptococcal isolates:**

Commercially available reagent a rapid Latex slide agglutination test kit for Streptococcal grouping was used for serogrouping of the isolates (Appendix II).

**Preparation of antigens:**

Micronitrous acid extraction method was used for antigen extraction in the following manner. One to 3 colonies of betahemolytic Streptococci were emulsified in 20 μl of 2M sodium nitrite solution placed in small test tube. To this 3 μl of glacial
acetic acid was added and incubated at room temperature for 15 minutes. Approximately 16 to 24 mg of sodium bicarbonate was added to the above suspension. Final volume was brought to 60 μl with distilled water. This was used as the antigen extract.

**Test procedure:**

One drop of the antigen suspension was placed in a clean glass slide, and to that 1 drop of reagent (after thorough shaking of the reagent bottle) was added. The antigen and reagent were mixed with an applicator stick. The slide was then rocked to and fro for 1 minute. The reaction was observed by placing the slide on a black paper. All positive reaction agglutinated by one minute. Equivocal reactions were repeated, following fresh extraction.

**Antibiotic susceptibility testing:**

All isolates of Group A Streptococci were subjected to antibiotic susceptibility testing on Mueller Hinton-sheep blood agar plate (Appendix I). Antibiotic against which they were tested were Penicillin (10 unit), Erythromycin (15 ug), Tetracycline (30 ug), Clindamycin (15 ug) and Trimethoprim sulphamethaxozole (25 ug) (tested on ox blood agar). Kirby-Bauer method was followed according to standard procedure\textsuperscript{253} (Baily and Scott's Diagnostic Microbiology). Five to six colonies were suspended in sterile saline of 5 ml quantity and adjusted to McFarland's 0.5 tube. A cotton tipped swab was dipped into the suspension, pressed against the side to
remove excess fluid and swabbed on to the agar surface. The plates were allowed to dry, before putting the antibiotic discs, following which they were incubated at 37°C overnight. Standard strain of Staphylococcus (S. aureus ATCC 29213) was also inoculated simultaneously to ensure quality control. Inhibition zones were recorded the following day using calipers (Photograph No.2).

**Direct antigen detection from throat swab:**

A solid phase, enzyme-linked immunosorbent assay for qualitative detection of Group A Streptococcal antigen directly from throat swabs was done. Visuwell® Strep A11 Kit from ADI diagnostics was used for the test. Procedure according to the manufacturers was followed. Antigens were extracted from the specimen on the swabs by extraction reagents and incubating for three minutes at room temperature. Precoated microwells were marked for each specimen and one drop of the antigen extract with the control were added into the wells. One drop of the conjugate (polyclonal antibody specific for Group A conjugated with urease) was added to each well. The microwell in the holder was gently tapped and held at room temperature for 5 minutes. The wells were then emptied by shaking out the contents and washed five times in saline provided by the manufacturers. Following the final wash, the microwells were inverted and tapped dry. Two free falling drops of the substrate (urea) were added to each well and incubated for five minutes at room temperature. Reading was taken immediately by measuring absorbance at 620 nm in the ELISA reader. Absorbance at 620 for positive control \( \geq 0.40 \) and negative control \( \leq 0.08 \).
All readings $>0.04$ were taken as positive. Visual reading was also taken by placing the microwells on a white reading platform and noting the change in colour. Yellow denoted a negative reaction and green to blue denoted a positive test. All readings were recorded (Photograph No.3).

**Anti-streptolysin O test:**

Following a positive throat culture for GABHS or direct antigen detection from throat swab an attempt was made to determine the ASLO level. A repeat visit was made to the school and 5 ml of blood was collected in sterile containers. The child was advised to visit the health centre for treatment and followup as well as a cardiology checkup to rule out rheumatic activity.

A rapid latex agglutination test was used for qualitative and semiquantitative determination of antistreptolysin O in serum. Following separation of serum, all reagents (ASLO Kit$^\text{R}$ Rashmi diagnostics Pvt. Ltd.) were brought to room temperature. One drop of serum was added onto the test slide. ASO liquid reagent was mixed before dispensing one drop to the slide. The reagents were mixed using a stirrer over the entire surface of the ringged area on the slide. Following rocking the card to and fro for 3 minutes, readings were taken. Agglutination with clearing of the backdrop was taken as positive. Any uniform turbidy was recorded as negative. All positive sera, by the qualitative test, were further subjected to semiquantitative method. Doubling dilution of the sera were prepared in physiological saline and the
test was repeated using the same procedure as for the qualitative test. According to the manufacturers instructions and interpretative table, positive and negative values were recorded (Photograph No4).

All the results obtained from various tests were informed to the concerned school incharge, with a request to direct the child to the health centre for specific treatment and followup.

**Phase II: Patient Group from Primary Health Centres**:

Second set of materials were collected from children below 15 years of age with ALRI who attended various rural and urban, primary health centres, community health centres and the General Hospital of Pondicherry. To give an uniform distribution, the study area was selected, based on the 1994 census of Department of Health and Family Welfare, Government of Pondicherry. Urban Primary Health Centres included Lawspet, Mettupalayam and Reddiarpalayam. Rural Primary Health Centres included Ariankuppam, Bahoor, Kalapet, Katterikuppam, Thirubhuvanei. Community Health Centres in the rural sector included Karikalampakkam Subsidiary Centres belonged to Abhishekapakka, Nettapakkam and Sorapet. Table II shows the population and sample size from each collection site. Map of the Union Territory of Pondicherry with the health centres visited is shown in Figure 1.
TABLE - II

Population covered by; and Sample size from Primary Health Centres and Government General Hospital, Pondicherry

<table>
<thead>
<tr>
<th></th>
<th>POPULATION</th>
<th>SAMPLE SIZE</th>
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<tr>
<td>URBAN:</td>
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<td></td>
</tr>
<tr>
<td>Lawspet</td>
<td>64780</td>
<td>125</td>
</tr>
<tr>
<td>Mettupalayam</td>
<td>111331</td>
<td>118</td>
</tr>
<tr>
<td>Reddiarpalayam</td>
<td>87813</td>
<td>32</td>
</tr>
<tr>
<td>General Hospital</td>
<td>1128715</td>
<td>182</td>
</tr>
</tbody>
</table>

|                  |            |             |
| RURAL:           |            |             |
| Ariankuppam      | 162964     | 62          |
| Bahoor           | 130961     | 52          |
| Kalapet          | 146729     | 68          |
| Katterikuppam    | 43140      | 50          |
| Thirubhuvanei    | 70781      | 73          |
| Karikalampakkam  | 156298     | 127         |
| Abhishekhapakkam | 34469      | 80          |
| Nettapakkam      | 85661      | 53          |
| Sorapet          | 42764      | 37          |
| Total            |            | 1059        |

Samples were collected over a period of 10 months from October 1993 to August 1994.

Selection of Cases:

Children with acute respiratory tract infections were screened by the attending Medical Officer of the Health Centres and a prescribed proforma (Appendix III) was filled in for each child enrolled.

URI:

Upper respiratory infection included running nose, sore throat with or without fever. Presence of tonsillar enlargement pharyngitis and lymphnode enlargement were noted.

LRI:

Lower respiratory tract infection included children with cough, fever and respiratory rate of $\geq 50/min$ with or without chest indrawing.

Specimen Collection:

Throat swabs and nasopharyngeal swabs were collected using rayon tipped swabs (Ray swabs) (Photograph No.5). Specimens were transported to laboratory in Stuarts transport media and inoculated on various media the same day.
Laboratory Procedure:

Throat swabs were rolled over one-fourth of a double layered blood agar plate and streaked with a loop to obtain isolated colonies. The plates were incubated anaerobically (using a Gas Pak system obtained from BBL Gas Pak) at 37°C overnight.

Nasopharyngeal swabs were inoculated on to (1) Double layered blood and (2) Chocolate agar plates (Appendix I) which were similarly streaked to get isolated colonies. An Optochin (oxoid) disc was placed on the primary inoculum site on chocolate agar plate before incubation to provide rapid indication of the presence of Streptococcus pneumoniae. Chocolate agar was incubated in 5% (V/V) CO₂ at 37°C overnight. Blood agar was incubated anaerobically at 37°C overnight (Photograph No.6).

Beta-hemolytic Streptococci colonies were identified as 5 mm diameter, transparent colonies with a well defined zone of complete hemolysis. Confirmation of the colonies was done using Bacitracin 0.02 unit discs and Nucleate disc-(Fluka). Nucleate disc was prepared according to the manufacturer's instructions (Appendix I). An enhanced haemolysis around Nucleate disc and a zone diameter of ≥ 14 mm around Bacitracin disc was considered to confirm GABHS²⁵³.(Photograph No.7).
Sensitivity testing:

Four to 6 colonies were suspended in 5 ml of sterile saline and adjusted to McFarlands tube 0.5. Test was done as described earlier, using Tetracycline (15 μg), Erythromycin (15 μg), Clindamycin (15 μg) and Cotrimoxazole (25 μg) discs. All resistant strains were preserved in sterile calf serum at -70°C for MIC testing.

Haemophilus influenzae:

Large flat colonies, colourless to gray opaque on chocolate agar were processed for confirmation. Suspected colonies were streaked on Nutrient agar plates and XV, X and V factor discs (Oxoid) were placed on three different places. Plates were incubated at 37°C in 5% CO₂ overnight (Photograph No.8). Satellitism was also done as another confirmatory test. Suspected colonies of Haemophilus were streaked on blood agar plate, and thereafter S.aureus colonies were streaked on the middle of the plate, which was incubated in 5% CO₂ at 37°C.

Detection of Beta-lactamase enzyme production:

A chromogenic test using Nitrocefin (chromogenic cephalosporin) solution (Oxoid) was used to detect beta-lactamase producing strains. A filter paper moistened with a small amount of Nitrocefin solution was prepared just before detection. A suspected colony was applied to the impregnated filter paper. A pink to red colour within 15 seconds indicated beta-lactamase production (Photograph No.9).
Sensitivity testing:

Similar procedure was adopted for sensitivity testing using standardised inoculum. Medium used was H1-Sensitest medium (Appendix I). Antibiotics tested were Penicillin V (10 µg), Ampicillin (10 µg), Erythromycin (15 µg), Chloramphenicol (30 µg), Trimethoprim-Sulphamethoxazole SXT (25 µg), Cefalexin (30 µg) and Cefotaxime (30 µg) (Oxoid discs). Plates were incubated at 37°C in 5% CO₂ overnight. Readings were taken the next day using calipere and zone diameters were recorded (Photograph No.10). All the strains were preserved in fetal calf serum in 1 ml quantities at -70°C for detecting the minimum inhibitory concentration at a later date.

Serotyping:

Fresh isolates from chocolate agar were serotyped using Bacto reagent (Difco Laboratories, Detroit, Michigan) *H.influenzae* serotype specific antisera. A saline suspension of the organism was made on a clean glass slide and a drop of the antisera was added. A clumping of the suspension was taken as positive. All results were recorded (Photograph No.11).

Streptococcus pneumoniae:

Round translucent colonies with bleaching on chocolate agar were confirmed by subculturing on blood agar and placing an optochin disc. After overnight
incubation at 37°C in 5% V/V CO₂ all strains showing a zone of inhibition of ≥ 10 mm around the disc were identified as *Streptococcus pneumoniae*.

Sensitivity testing was done on Isosensitest Blood agar (Appendix I) using Oxacillin (1 ugm), Erythromycin, Chloramphenicol, Trimethoprim sulphamethoxazole, Cephalexin and Cefotaxime discs (as described earlier) (Photographs No.12,13,14).

All resistant strains were preserved in fetal calf serum at -70°C for MIC determination.

*Moraxella catarrhalis:*

Small gray white colonies on chocolate agar which could be easily swept across the plate with a loop, without losing its colony morphology was suggestive of *Moraxella catarrhalis*. A positive oxidase test with freshly prepared reagent on a moist filter paper, of gram negative diplococci on Gram stain were further identifying features of Moraxella. Negative carbohydrate fermentation test using glucose, sucrose, maltose and lactose confirmed the isolates²⁵³, according to the method by Facklam RA in Manual of Clinical Microbiology.

Sensitivity to antibiotics was done using Haemophilus sensitest medium and standard procedure described earlier. Antibiotics used were Penicillin V, Ampicillin, Chloramphenicol, Cotrimoxazole, Cefalexin and Cefotaxime.
Beta-lactamase production by penicillin resistant strains was tested by using Nitrocefin discs. Strains were stored at -70°C in fetal calf serum for MIC determination.

**Determination of Minimum Inhibitory Concentration (MIC):**

MIC of various antibiotics to the resistant strains was determined according the procedure described by D.F. Sahm and J.A. Washington. Agar dilution method was used for the MIC determination. Antibiotic dilutions were made using standard dilution method to include the break points of each antibiotic for the organisms.

Antibiotic substances in powder form were obtained from various manufacturers (Appendix II) as pure ingredients. They were dissolved in the various solvents as mentioned by the manufacturer. Stock solution of the antibiotics were made in phosphate buffer pH 7.2. Using standard protocol of Daniel F. Sahm (Manual of Clinical Microbiology), Isosensitest agar (for *H.influenzae*) and Mueller Hinton lysed blood agar (for *S.pneumoniae, M.catarrhalis* and GABHS) were incorporated with different dilutions of antibiotic solution in the ratio of 1:10 (antibiotic solution : medium). Plates were appropriately labelled and set aside for inoculation.
**Preparation of Inoculum:**

Four to five colonies were picked up from an overnight growth on agar-based medium and inoculated into 5 ml of brain heart infusion broth, and incubated at 37°C until turbid. This was then adjusted to McFarlands 0.5 standard (10 CFU/ml). Using a calibrated loop to deliver 0.002 ml of the broth, a loopful of the broth of each isolate was delivered on to the agar plate. Plates without any antibiotics were used to check for the viability of the organisms. Standard ATCC strains of Staphylococci (ATCC 29213) were incorporated in each batch of test. Plates were allowed to stand until inocula were completely absorbed by the medium, then they were incubated at 37°C for 16 to 20 hours. End point of 90% inhibition of growth was taken as MIC\textsubscript{90} and results were recorded. MIC interpretative standards recommended by NCCLS\textsuperscript{255} (National Committee for Clinical Laboratory Standards) were used to determine the susceptible intermediate and resistant values for each of the organisms tested (Photographs No.15,16).

**Phase III: Hospitalized patient group:**

Third part of the study involved ALRI in children who required hospitalization. This was carried out over a period of 27 months from February 1994 to May 1996.

Study population included 329 children below 5 years of age who were admitted to the paediatric wards of JIPMER with clinical and radiological diagnosis of acute lobar pneumonia, bronchopneumonia or empyema. All children selected had
a duration of illness of less than 4 weeks. Criteria for admission were tachypnoea, inability to feed, respiratory distress and evidence of toxaemia. Children with upper respiratory infections or croup were excluded.

**Controls:** Three hundred and fourteen age and sex-matched controls (from the well baby clinic, or healthy children from other wards admitted for surgical procedures were selected.

Detailed history and clinical examination was carried out by attending paediatricians. History of prior antibiotic was also sought and noted. Respiratory distress and other signs of toxaemia were noted and recorded in a proforma (Appendix III).

**Investigations:**

Haemoglobin, total and differential counts and chest X-ray at admission were done for all cases.

**Bacteriological Investigation:**

Relevant bacteriological investigations included the following:

1. *Blood culture*: Using aseptic precautions 3-5 ml of blood was collected by venipuncture and inoculated into 50 ml of brain heart infusion broth (BHI broth) at the bedside by the attending medical officer or resident doctor. The sample was
transported to the Department of Microbiology where it was immediately incubated in 5% CO₂ jar (caps were slightly loosened) at 37°C overnight. A twenty-four and forty-eight hours, subcultures were done from the BHI broth on to chocolate, blood and McConkey agars. Plates were incubated in 5% V/V CO₂ at 37°C for first 24 hours and a further 48 hours if there was no growth.

Organisms grown were identified by standard procedures (described earlier). Aerobic spore bearers, micrococci and *Staphylococcus epidermidis* and mixed growth of gram negatives in less than 2 media were considered to be contaminants and were not analysed. All isolates were subjected to antibiotic sensitivity testing using prescribed procedures. All *Haemophilus influenzae* isolates were serotyped using specific antisera (Bacto reagents from Difco Laboratories).

2. *Serum for antigens detection and bactericidal activity:* 2-3 ml of blood was collected during venipuncture for blood culture. Serum was separated in the laboratory and stored at -70°C till use.

3. *Nasopharyngeal swabs:* Rayon tipped flexible nasopharyngeal swabs (Ray swabs®) were introduced through the nostrils and posterior nasopharynx was swabbed.
4. Throat swabs were collected from the posterior pharyngeal and tonsillar areas from all the enrolled children. Throat and nasopharyngeal swabs were also collected from healthy controls.

**Laboratory procedure:**

Swabs were inoculated on to chocolate, blood and MacConkey agar plates with use of a four quadrant streaking technique and incubated at 37°C in 5% CO₂ for 24 hours.

All growth of *Streptococcus pneumoniae* and *Haemophilus influenzae* were taken to be significant if colonies were found in pure form or up to the last streak line in the presence of mixed flora. GABHS, Staphylococci (in pure form) and Gram negative organisms were taken as significant and processed further to confirm identity. *Streptococcus pneumoniae* was confirmed by Optochin sensitivity testing. *Haemophilus influenzae* was confirmed by its requirement of XV factor for growth, and absence of growth only in the presence of X or V factor alone. Presence of satellitism was also a positive indicator of *H.influenzae*. All isolates of *H.influenzae* were serotyped using standard antisera obtained from Difco Laboratories. GABHS was confirmed by inhibition to 0.02 unit of bacitracin.

*Staphylococcus aureus* was confirmed by the coagulase test according to the standard procedure (Bailey and Scott's Diagnostic Microbiology). One drop of 1:10
dilution of rabbit plasma was added to 1 ml of overnight broth culture. Presence of a coagulum was noted after 4 hours. In the absence of coagulum in the tube, bound coagulase was demonstrated by the slide test.

Gram negative isolates were identified by carbohydrate fermentation tests by standard technique according to Cowan and Steel's Manual of Medical Microbiology.²⁵⁶

Antibiotic sensitivity testing was done for all significant isolates using appropriate media with quality checks as described earlier. MIC of various antibiotics were tested for all the resistant S.pneumoniae and H.influenzae isolates from cases and controls.

Antigen detection in serum samples:
Two methods of antigen detection techniques were used. Commercially available Latex agglutination kit (Directigen) was compared with co-agglutination reagent prepared in the laboratory (for detection of H.influenzae antigen). Coagglutination test was used to detect S.pneumoniae antigen in the serum.

Staphylococcal Co-agglutination Method for Antigen Detection:
Method followed was as described by Lalitha et al.²⁵⁷ Cowan 1 strain of Staphylococcus aureus was grown in Todd Hewitt broth overnight and harvested by
centrifugation at 3000 RPM for 30 minutes. The sediment was washed five times in PBS 7.2. Bacteria were then suspended in 0.5% formaldehyde buffer (Appendix II) and kept at room temperature for 3 hours. Following this the supernatant was discarded and the sediment washed once with PBS (7.2). The resultant suspension was heated at 50°C in a water bath for 1 hour. The suspension was then centrifuged and resuspended to get a 10% Cowan 1 suspension.

**Colouring reagent:** To every 1 ml of 10% suspension 0.1 ml of 1% methylene blue and Grams iodine was added. This was washed 5 times in PBS.

**Preparation of typing reagent:** To 1 ml of 10% suspension 0.1 ml of typing sera were added. Typing sera used were - a) Pneumococcal Omniserum (Statens Serum Institute Copenhagen Denmark) and b) Haemophilus type b antiserum (Difco Laboratories) (0.5 ml of suspension was added to every 50 ul of antiserum). The suspension was left for 30 minutes at room temperature with intermittent shaking. It was then centrifuged at 1500 RPM for 15 minutes. Supernatant was discarded and sediment re-suspended in 5 ml PBS. They were distributed in 1 ml vials and stored at +4°C till use (Photograph No.20).

**Test procedure:**

Serum was processed as per the instructions of the manufacturer for LA test (Beckton Dikinson Kit: Directigen), and used for both the tests. Serum specimens
were diluted 1:1 in buffer PBS and mixed. This was heated at 100°C in a water bath for 5 minutes, and cooled. Any resultant clot was broken up and vortexed vigorously for 5 seconds. Specimen was then centrifuged at 1400 x g for 15 minutes. The supernatant was tested.

One drop of the supernatant each was added to one drop of

a) Co-A reagent for *Streptococcus pneumoniae* (Pneumococcal Omniserum coated Cowan 1 strain) (Photograph No.17)

b) Co-A reagent for *Haemophilus influenzae* type b (Anti *H.influenzae* type b rabbit antibody coated Cowan 1 strain) (Photograph No.18)

c) Directigen kit for *Haemophilus influenzae* type b (Anti *H.influenzae* type b rabbit antibody coated Latex suspension) (Photograph No.19)

in a ringed glass slide. The slide was then placed on a rotator adjusted to 100 rpm for 10 minutes. A moistened cover was placed over the slides to prevent evaporation. Results were read macroscopically in transmitted light. With each test positive and negative controls were added.

**Serum Bactericidal Test:**

Serum bactericidal activity was done on 100 sera (Quantity of sera was too less in others for the test). A modified method of Arthur L.Bany and L.D.Sabath was followed. 0.5 ml of serum (inactivated at 56°C for 1/2 hour to remove nonspecific serum factors) was taken in a tube and diluted with 0.5 ml of pooled
normal human serum. 20 µl of *Staphylococcus aureus* ATCC 295213 strain adjusted to contain $10^5$/CFU/ml (using a McFarland standard) of viable cells diluted in BHI broth was added to the serum and incubated overnight. Control tubes of serum alone and broth alone were also included. Subcultures were made from each tube on to BA plates to detect the growth of organism. Absence of growth denoted bactericidal activity of the serum. Findings were correlated with a history of antibiotic therapy within last 48 hours prior to admission and collection of samples. All the data were analysed using a statistical package (Photograph No.21).

**Statistical Analysis:**

Univariate analysis of the variables were done with the use of $\chi^2$ test, and Fisher's exact test. Difference between variables were assessed by means of Standard error of proportions. A 'P' value of $\leq 0.01$ was considered statistically significant. EPI INFO6 (Version 6.03 - 1994), a word processing database and statistical programme and SPSS. Statistical package were used for analysing the data.
Photograph No.1: Throat swab collection from school children.

Photograph No.3: Direct GABHS antigen detection from throat swabs by enzyme immuno assay using Visuwell Strep A Kit.
Antigen positive - Violet
Antigen negative - Yellow

Photograph No.4: Antistreptolysin O antibody detection from serum of pharyngitic children by latex agglutination.
Positive: Central ring
Negative: Left and right rings
Photograph No.5: Nasopharyngeal and throat swabs used for sample collection from children with acute respiratory infections.

Photograph No.6: Screening of samples for S.pneumoniae using optochin disc in the primary inoculum.
Photograph No. 7: Screening of GABHS using Bacitracin and Nucleate discs, showing inhibition by Bacitracin and enhanced haemolysis by Nucleate discs.

Photograph No. 8: Confirmation of *H. influenzae* on Nutrient agar using XV, X and V factor discs.
Photograph No.9: Betalactamase production by *H. influenzae* detected by Nitrocefin, a chromogenic cephalosporin turning pink.

Photograph No.10: Antibiotic susceptibility of *H. influenzae* using Iso-Sensitest Agar. A multidrug resistant strain showing resistance to Cotrimoxazole, Ampicillin and Erythromycin discs.
Photograph No.11: Typing of \textit{H.influenzae} strain using \textit{Hib} antisera (Difco laboratories)

Photograph No.12: Antibiotic susceptibility of \textit{S.pneumoniae} using Mueller-Hinton lysed sheep blood agar. Strain showing resistance to Oxacillin (1 ugm) disc.
Photograph No.13: Antibiotic susceptibility of *S. pneumoniae* using Mueller-Hinton lysed sheep blood agar showing resistance to Cotrimoxazole.

Photograph No.14: Antibiotic susceptibility plate of *S. pneumoniae* under transmitted light, showing a sensitive strain.
Photograph No.15: Minimum inhibitory concentration using agar dilution technique. *S.pneumoniae* on plate with 4 ug/ml of penicillin, in Mueller-Hinton lysed sheep blood agar.

Photograph No.16: Minimum inhibitory concentration using agar dilution on Iso-sensitest agar medium showing growth of two strains of *H.influenzae* in the presence of 16 ug/ml of ampicillin.
Photograph No. 17: Detection of pneumococcal antigen in serum using Staphylococcal co-agglutination.

Photograph No. 18: Detection of *H. influenzae* type b antigen in serum using Staphylococcal coagglutination.
Photograph No.19: Hib antigen detection in serum using Directigen antigen detection system.

Photograph No.20: Antigen detection reagents.
Photograph No.21: Bactericidal activity of serum in tubes; and growth on blood agar following subculture.