Material 
& 
Methods
Material and Methods

This study was conducted in the Department of Pediatrics, M.L.B. Medical College, Jhansi, over a period of one year in active collaboration with Department of Microbiology, in 50 infants of upto six months of age with features of lower respiratory tract infection, of atleast one week duration.

Inclusion Criteria –
1. Infants upto six months of age.
2. Cough and tachypnoea with or without chest wall retraction for more than one week duration despite conventional treatment for pneumonia. Tachypnoea was defined as respiratory rate more than sixty per minute in a child less than two months, or more than fifty per minute in an infant more than two months.

Exclusion criteria –
Infants with defects known to be associated with prolonged lower respiratory tract infection such as congenital heart disease, congestive heart failure, bronchopulmonary dysplasia, or a history consistent with tracheo-esophageal fistula were excluded from study.

Sample size –
Fifty cases were taken in this study to find out the prevalence of chlamydia trachomatis associated pneumonitis in
patients with lower respiratory tract infection in the first six months of life.

Detailed study protocol –

The WHO definition of lower respiratory tract infection was used to select patients, which is presence of cough and tachypnea with or without respiratory distress and retractions. Tachypnea was defined as respiratory rate more than sixty per minute in infants less than two months and more than fifty per minute in infants greater than two months. Chest wall indrawing was defined as a definite inward movement of the lower chest wall on breathing.

The respiratory physical findings were also recorded in each patient, but they were not used as a part of the minimum diagnostic criteria for pneumonia.

During the first clinical evaluation the demographic details including age, sex, any significant perinatal history along with obstetric and gynecologic history of the mother were noted. The infants were categorized into one of the three age categories, each interval being of two months duration.

Clinical history included a history of cough and its duration, type of cough and its severity, wheezing and its duration, cyanosis, history of nasal, eye or ear discharge, their character and duration were noted. Refusal to feed, vomiting and fever were recorded.

Examination findings involved sensorial changes, temperature, pulse rate, respiratory rate and examination of conjunctiva. Child was carefully evaluated for tachypnea,
respiratory rate was counted for one minute, and presence of intercostal and subcostal reccessions were observed. Mild suprasternal reccessions were ignored as it is a normal feature in this age group.

In infants less than two months respiratory rate was monitored twice for one minute each time, ten minutes apart. Only persistent fast breathing was considered to be significant. Chest findings included type of breath sounds, presence of rhonchi and creptitations.

The following investigations were done in each patient:
2. Chest X-ray.
3. IgM ELISA kit test for Chlamydia trachomatis.

Hemoglobin percentage estimation was done using Sahli’s hemoglobinimeter. Blood cell counts were done by standard methods and absolute eosinophil values were calculated by multiplying the total leucocyte count and percentage of eosinophils in a Wright’s stained smear.

**IgM ELISA Kit Test**

**Principle of the assay :-**

The qualitative immunoenzymatic determination of IgM – class antibodies against chlamydia trachomatis is based on ELISA technique.

Microtitre strip wells are precoated with chlamydia trachomatis antigen to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample
material horse radish peroxidase (HRP) labelled anti-human IgM conjugate was added. This conjugate binds to the captured chlamydia specific antibodies. This immune complex formed by the bound conjugate was visualized by adding Tetramethylbenzidine (TMB) substrate which gave a blue reaction product.

The intensity of this product was proportional to the amount of chlamydia specific IgM antibodies in the specimen. Sulfuric acid was added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450nm is read using an ELISA microwell plate reader.

**Required Testing Material : -**

1. Chlamydia trachomatis coated wells (IgM).
2. IgM sample diluent.
3. Stop solution containing sulfuric acid.
4. Washing solution.
5. Chlamydia trachomatis anti-IgM conjugate containing peroxidase labelled rabbit antibody to human IgM.
6. TMB substrate solution.
7. Chlamydia trachomatis IgM positive control.
8. Chlamydia trachomatis IgM negative control.
9. ELISA Microwell plate reader.
10. Microtitre plate washers.
Specimen Collection and Storage: -

Serum was used and the usual precautions for venipuncture were observed. No sample pretreatment was done. The specimen was stored at 2 – 8 °C for upto 24 hours or was stored deep frozen (-20 °C) for longer intervals. Repeated freeze thawing was avoided. Thawed samples were inverted several times prior to testing. Grossly hemolysed, icteric or grossly lipemic specimens was not used.

Assay Method: -

Sample Addition -

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100 μl ready to use negative control in two wells.
100 μl ready to use positive control in one wells.
100 μl of each diluted patient samples into remaining wells.
Leave well for substrate blank.
Incubate for 1 hour at 37°C.
Aspirate off contents of wells and wash them three times with 300 μl of working washing solution.

Conjugate Addition -

Dispense 100 μl chlamydia trachomatis anti-IgM conjugate into all wells except for the blank well.
Incubate for 15 Minutes at room temperature in the dark (20 – 25 °C).

Repeat washing procedure.

Substrate Addition -
Dispense 100 µl of ready to use TMB solution into all wells. Incubate for exactly 15 minutes at room temperature (20° – 25 °C) in the dark.
Dispense 100 µl of stopping solution into all wells.

Measurements :-
Adjust the ELISA Microwell plate reader to zero using the substrate blank.
Measure the absorbance of all wells at 450 nm and record the absorbance values for each control and patient sample.

Results :-
The cut-off was calculated by addition of 0.30 absorbance units to the measured absorption of the mean value of the 2 negative control determinations.
Cutoff = Absorbance mean value of the Negative control + 0.30.

Interpretation :-
Samples were considered positive if the absorbance value was higher than 10% over the cut off.
Samples with an absorbance value of 10% above or below the cut off were not considered as clearly positive or negative.
Grey Zone.

The test was repeated again 2 – 4 weeks later with a fresh sample. If the results in the second test were again in the gray zone the sample were considered negative. Samples were considered negative if the absorbance value was lower than 10% below the cut off.