

**THE PREVALENCE OF**

**CHLAMYDIAE IN**

**LATE ONSET NEONATAL**

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**PNEUMONIA IN THE NEWBORN UNIT -**

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*A dissertation presented in part  
fulfilment for the degree of Master of  
Medicine (Paediatrics)  
At the University of Nairobi*

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**BY:**

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**2001**

## ***DEDICATION***

**This work is dedicated to my:-**

❖ **Son Matthew**

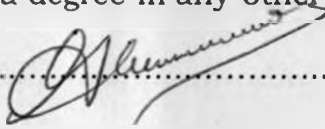
❖ **Husband Horace**

❖ **Parents Tereza and Thomas**

## DECLARATION

I declare that this dissertation is my original work and has not been presented for a degree in any other university.

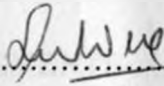
Signed

  
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DR. FRIDAH AFANDI GOVEDI

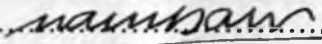
This dissertation has been submitted for the examination with our approval as university supervisors.

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## **LIST OF ABBREVIATIONS AND EXPLANATIONS**

1.	C/section	-	Caesarian Section
2.	CXR	-	Chest x-ray
3.	Chl. pneumoniae	-	Chlamydia pneumoniae
4.	Chl. trachomatis	-	Chlamydia trachomatis
5.	DNA	-	Deoxyribonucleic acid
6.	EB	-	Elementary body
7.	ELISA	-	Enzyme linked immunosorbent Assay
8.	IgG	-	Immunoglobulin G
9.	IgM	-	Immunoglobulin M
10.	LPS	-	Lipopolysaccharide
11.	LBW	-	Low birth weight
12.	MIF	-	Microimmunofluorescence
13.	N	-	Nucleus
14.	NBU	-	Newborn Unit
15.	NPA's	-	Nasopharyngeal aspirates
16.	RB	-	Reticulate body
17.	RNA	-	Ribonucleic acid
18.	SVD	-	Spontaneous Vertex Delivery
19.	WHO	-	World Health Organisation

For the purpose of this study, atypical pneumonia was defined by the presence of interstitial pneumonitis on chest radiographs and evidence of colonization with Chlamydiae in nasopharyngeal aspirates by direct antigen detection using immunofluorescence technique.

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## **ACKNOWLEDGEMENT**

I wish to extend my sincere heartfelt appreciation and thanks, to all those who in one way or another, contributed to the successful completion of this work.

I am greatly indebted to my supervisors Dr. F. Were, Dr. J. S. Wambani, Dr. G. Revathi for their valuable guidance and patience to the end of the study. I will always remember their selfless devotion and personal attachment to my work by timely advice, constructive criticism, facilitating acquisition of various items for this study, even financial assistance, taking valuable time off their busy schedule both at work or privately, through the rigours of proposal, dissertation writing and finally thesis completion. God bless you all.

I also extend my gratitude to senior colleagues, technical staff in the Departments of Paediatrics, Microbiology, Immunology for the support and encouragement extended to me during this period that seemed such a nightmare to me.

My sincere thanks to Mr. Mwinyi Bakari, senior medical laboratory technologist and Mr. Bernard A. W. Mutua, medical laboratory technician, Immunology section for their tireless dedication, personal attention, excellent laboratory work given to this study even during public holidays.

My gratitude to Mr. Nyabola, Department of community health, Mr. Munyiu, Kemri for their statistical input during data analysis, Mr. Francis Shikhubale of Studio Unique for the excellent processing of the medical illustrations.

My special thanks to Mrs. Patel of the Aga Khan Hospital for unhesistantly having made it possible for me to document the microscopic findings.

A big thanks to Abbot Laboratories for their assistance and encouragement during this study.

To my sister Anne, I say thank you very much for the quick secretarial work.

To my family members, thank you very much for the constant encouragement and prayers.

To my colleagues and especially members of staff of newborn unit, thank you very much for your prayers, encouragement and assistance accorded me during my stay in the newborn unit.

Last but not the least, I wish to remember my patients both the ones alive and those dead for being there for me to learn and for the dead ones, I say "May the Almighty God rest your souls in eternal peace". Asante sana.

I wish to acknowledge Kenyatta National Hospital for allowing me to use their facilities during my training programme.

It is not possible to thank all of you individually, but to all I say, ASANTE SANA.

**May the Almighty God bless you all!**

## **SUMMARY**

Fifty-two neonates who presented with late onset pneumonia were sequentially recruited for this study, whose study objective was:- to determine the prevalence of Chlamydiae colonization of the upper respiratory tract in neonates at Kenyatta National Hospital and to estimate the proportion of late onset neonatal pneumonia that may be associated with Chlamydiae in these neonates.

Chest radiographs were used to describe interstitial pneumonitis and Chlamydiae antigen detection in nasopharyngeal aspirates by direct immunofluorescence technique was conducted in all these neonates.

Probable contributory factors to Chlamydiae colonization and probable causation of atypical pneumonia such as mode of delivery, birth weight, and gestation were also evaluated.

Thirty-three patients had Chlamydiae isolated in their nasopharyngeal aspirates. This gave a colonization rate of 63.5%.

Twenty-four out of forty-seven patients had a combination of Chlamydiae colonization and radiological evidence of interstitial pneumonitis. This fulfilled the case definition of atypical pneumonia. This gave a prevalence of atypical pneumonia of 51%.

The positive predictive value of chest radiographs alone for diagnosis of atypical pneumonia was 0.73 with a sensitivity of 80% and specificity of 50%.

Atypical pneumonia did not have any association with mode of delivery, maturity, birth weight, sex, and postnatal age.

### **Conclusion**

It was concluded that there is a high carrier rate of Chlamydiae often associated with actual pneumonia in our centre. As mode of delivery did not alter this carriage rates, nosocomial sources were speculated.

**“THE PREVALENCE OF CHLAMYDIAE IN LATE ONSET  
NEONATAL PNEUMONIA IN THE NEWBORN UNIT -  
KENYATTA NATIONAL HOSPITAL**

**INTRODUCTION AND LITERATURE REVIEW**

In the delicate neonatal life, with the neonate undergoing a lot of physiological adjustments to independent extra-uterine life, lower respiratory illnesses especially pneumonia remain a significant cause of hospitalisation, morbidity and mortality.

Late onset neonatal pneumonia has been noted especially in neonates who have had a prolonged stay in the newborn unit. Prematurity and ventilatory assistance necessitates the use of various invasive procedures for diagnostic and therapeutic purposes. These include indwelling catheters, vascular catheters, ventricular shunts, endotracheal tubes. The later is particularly accredited with increased colonization by atypical microbes. These procedures lead to alteration in skin and mucous membrane host defense barriers, providing a portal of entry by pathogenic organisms, culminating in the frequent use of broadspectrum antibiotics in this group of patients.

Late onset neonatal infections are acquired after the perinatal period within the first twenty-eight days of life. However, similar infections may be seen in infants particularly preterms during the first sixty days of life.<sup>1</sup> The etiological agents may be transmitted from a variety of human sources, such as the mother, family contacts, hospital personnel or other vehicles like; contaminated respiratory equipment, intravenous fluids, medications and even disinfectants.

Atypical pneumonia or “afebrile pneumonia syndrome” has been attributed mainly to the intermediate organisms that colonize the maternal vaginal tract and the infants but occasionally cause invasive disease. These include the following organisms:-<sup>3,4</sup>

1. Chlamydia trachomatis / pneumoniae
2. Ureaplasma urealyticum / Mycoplasma hominis
3. Cytomegalovirus
4. Herpes Simplex virus – type II
5. Respiratory syncytial virus
6. Adenovirus
7. Candida albicans
8. Aspergillus species
9. Others
  - Gardinella vaginalis
  - Polymicrobial anerobic flora

This type of pneumonia presents with a slowly progressive respiratory syndrome characterized by persistent cough, respiratory distress, hypoxia, rales, apneic spells but usually without fever. Chest radiographs show perihilar and interstitial infiltrates. These are initially subtle but may later progress to reticular infiltrates from the hilum of the lung. The infiltrates worsen with disease progression and may finally form a “white out” lung picture. Peripheral blood leucocyte counts are generally not helpful though significant eosinophilia may be present. Tracheal aspirates and nasopharyngeal swabs for Chlamydial culture or antigen detection by ELISA are the mainstay of microbiological diagnosis.

The uniqueness of neonatal infections is as a result of a number of factors.

These include:-<sup>1</sup>

1. Varied modes of transmission of infection to the baby including; transplacental, aspiration of infected amniotic fluid and birth canal secretions and postnatal nosocomial acquisition.
2. Immaturity of the newborn immune system, especially the reduction of the functional integrity of polymorphonuclear leucocytes, cytokines, antibodies and the cell mediated immunity.
3. Comorbidity in the newborn often making the diagnosis and management difficult. For example hyaline membrane disease often co-exists with bacterial pneumonia.
4. Vague clinical presentation of neonatal infections.

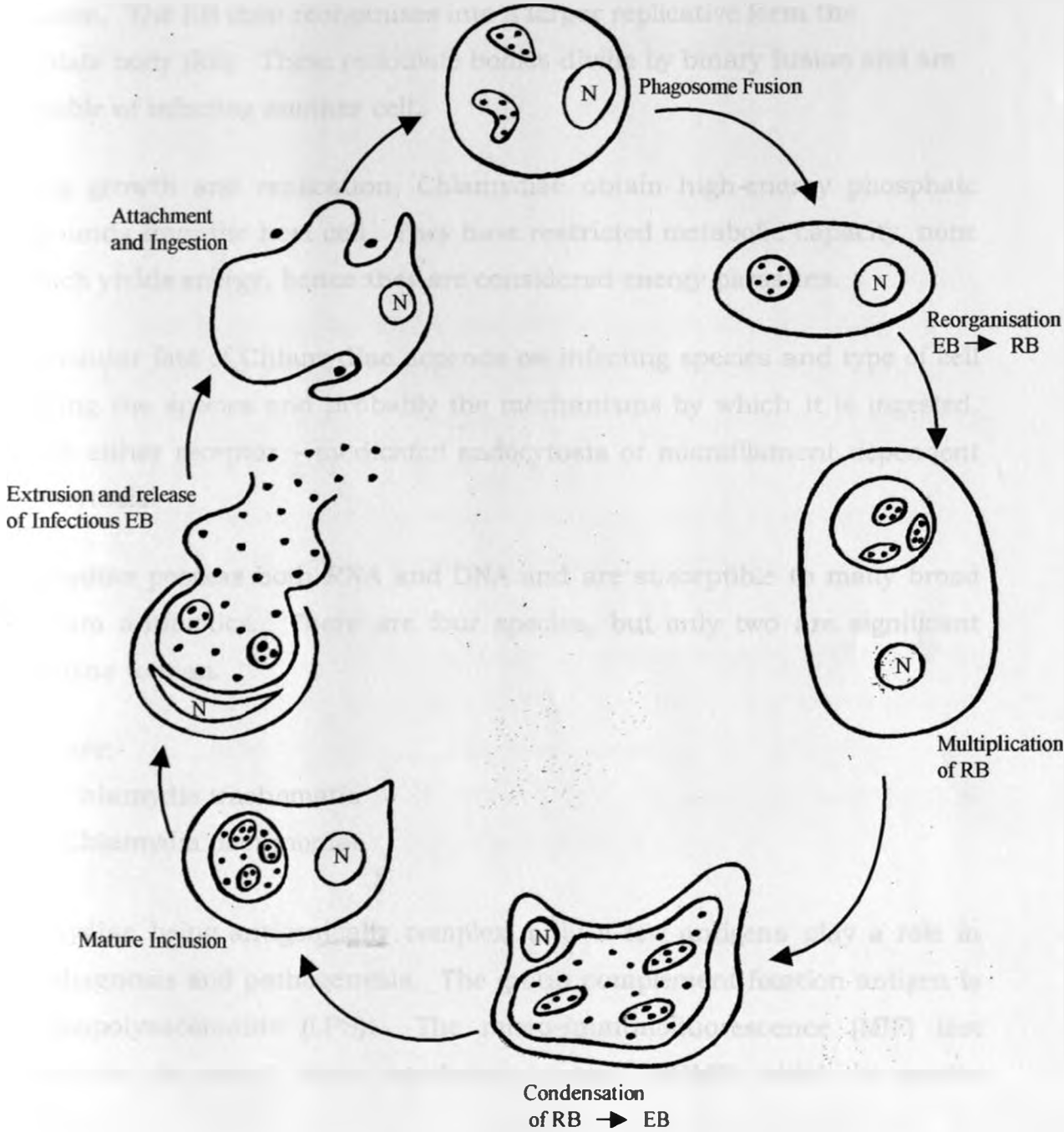
## **The Microbiology of Chlamydiae**

Chlamydiae are common pathogens in the animal kingdom.<sup>5</sup> They are non motile, gram negative obligate intracellular bacteria. They replicate within the cytoplasm of the host cells, forming characteristic intracellular inclusions that can be seen by the light microscope.

Chlamydiae are prokaryotes, exhibiting morphological and structural similarities to gram negative bacteria including a trilaminar outer membrane, which contains lipopolysaccharide and several membrane proteins. However, they lack peptidoglycan, a macromolecule that provides most prokaryotes with structural rigidity and osmotic stability. The infective extracellular form, the elementary body (EB), exhibits extensive disulfide cross-linking between cysteine residues within and without the membrane proteins resulting in a spore like structure that is metabolically inert.

(See life cycle)

# Life Cycle of Chlamydiae in Tissue Culture





The life cycle begins when an EB attaches to a susceptible epithelial cell using a heparine sulphate bridge on receptors on its surface and that of the target cell. The EB enters the epithelial cell by a receptor mediated endocytosis. Lysosomal fusion is inhibited by undefined mechanisms allowing the EB to reside in a protected membrane – bound vesicle called an inclusion. The EB then reorganises into a larger replicative form the reticulate body (RB). These reticulate bodies divide by binary fusion and are incapable of infecting another cell.

During growth and replication, Chlamydiae obtain high-energy phosphate compounds from the host cell. They have restricted metabolic capacity, none of which yields energy, hence they are considered energy parasites.

Intracellular fate of Chlamydiae depends on infecting species and type of cell ingesting the species and probably the mechanisms by which it is ingested. That is either receptor – mediated endocytosis or microfilament dependent phagocytosis.

Chlamydiae possess both RNA and DNA and are susceptible to many broad spectrum antibiotics. There are four species, but only two are significant pathogens to man.

These are:-

- a. Chlamydia trachomatis
- b. Chlamydia pneumoniae

Chlamydiae being antigenically complex, only a few antigens play a role in their diagnosis and pathogenesis. The group complement fixation antigen is the lipopolysaccharide (LPS). The micro-immunofluorescence (MIF) test determines the major outer membrane protein (MOMP) which is species specific.

# Diagnostic Tests for Chlamydia Trachomatis and Chlamydia Pneumoniae

## I. CHLAMYDIA TRACHOMATIS

Tests to identify this organisms are based on the following:-

### 1. Cell culture

Chlamydia trachomatis grows well in various cell lines but the most used ones are McCoy or Hela cells. Chlamydia trachomatis are incubated in tissue culture for 40 – 72 hours depending on cell type or biovar. Intracytoplasmic inclusions are seen with Giemsa, Macchiaveli Stains or Immunofluorescence or using iodine which stains glycogen on McCoy cells. Immunofluorescence stain with monoclonal antibodies is the most sensitive and specific in detecting inclusions.

Cell culture has a specificity of approximately 100% and in optimal conditions a sensitivity of 70 – 90%. This test is difficult and time consuming and also requires an expensive tissue culture system but it still remains the gold standard test for identification of Chlamydia trachomatis.

### 2. Antigen detection

These are based on Antigen detection using the:-

- i. Direct detection by fluorescent antibody (DFA) staining
- ii. Enzyme – linked immunosorbent assay (ELISA)

### 3. Molecular techniques

- i. Ribosomal RNA detection with hybridization with a DNA probe.
- ii. Nucleic acid amplification by
  - a. Polymerase chain reaction (PCR)
  - b. Ligase chain reaction (LCR)

PCR & LCR are more specific and sensitive than cell culture and are comparable to cell culture in nasopharyngeal specimens in infants.

Antigen detection tests have sensitivities of more than 70% and specificities of 97 – 99% in a prevalence of more than 5%. In low prevalence, false positive makes up a significant proportion. Positive tests are verified by cell culture, different second antigen non culture test or blocking antibody or competitive probe. These tests are less satisfactory for respiratory specimens.

### 4. Serological techniques

- i. Complement fixation using antibodies against lipopolysaccharides, where positive titres are 1:16. Its disadvantages are that more than 50% of the children do not mount detectable antibodies and less specificity as more than 50% of other Chlamydial manifestations have titres higher than 1:16. Its advantages are that it is rapid, uses single specimen and is sensitive.

- ii. Microimmunofluorescence test (MIF) using elementary bodies as antigens and antibodies against cell wall components are detected. It detects antichlamydial IgG in 100% infants with pneumonia or inclusion conjunctivitis. These antibodies may reflect passively transferred maternal antibody. Anti IgM is present in 30% infants with neonatal inclusion and 100% with Chlamydial pneumonia. Therefore IgM > 1:32 with immunofluorescence can diagnose infant Chlamydia pneumonia. MIF is now widely used and has become a “gold standard” due to its sensitivity and specificity.

#### 5. Cytologic diagnosis

Visualization of intracytoplasmic inclusions in specimens. This has low sensitivity and specificity.

## II. **DIAGNOSIS OF CHLAMYDIA PNEUMONIAE**

Isolation of Chlamydia pneumoniae is difficult as this organism does not readily grow on Hela 229 and McCoy cell lines. It grows on HL cell line or HEP - 2.

- i. Cell culture is the most specific test though labour intensive and time consuming.
- ii. Polymerase chain reaction using TWAR – specific DNA is 25% more sensitive than culture and is rapid.

- iii. Microimmunofluorescence is more sensitive and diagnostic for TWAR strain infections. The major drawback with serological tests is presence of significant subjective component. It is used in prevalence studies.
- iv. Chlamydia complement fixation test is useful in the young patients with TWAR infection.

This study used direct antigen detection by immunofluorescence technique for *Chlamydia trachomatis* and *Chlamydia pneumoniae*.

## **Disease patterns of Chlamydiae**

Disease patterns caused by Chlamydiae are varied and depend on the infecting species. Chlamydiae are divided into genital and respiratory species. Genital Chlamydiae consists of *Chlamydia trachomatis* with genital serotypes causing neonatal inclusion conjunctivitis and pneumonia. Serovars L<sub>1</sub>, L<sub>2</sub>, L<sub>3</sub> cause lymphogranuloma venereum. Respiratory Chlamydiae consist of *Chlamydia pneumoniae* which cause pharyngitis, sinusitis, bronchitis, bronchiolitis, bronchial asthma, atypical pneumonia and interstitial pneumonitis in the newborn. It also causes coronary vascular disease, predisposing to myocardial infarction, atherosclerosis. Another species is the *Chlamydia psittaci*, which causes hypersensitivity and atypical pneumonia.

Neonatal Chlamydial pneumonia clinically presents at 3-16 weeks of age and often the patient has been sick prior to this. Infants may present with nasal discharge, fast breathing, with or without a cough. These symptoms are insidious in onset. On examination, the infant appears well, afebrile but develops increasing tachypnoea. Conjunctivitis is present in approximately 50% patients and middle ear abnormalities in more than 50% patients.

Paroxysms of “staccato” cough which interferes with sleeping and eating may be present. Auscultation reveals scattered rales, occasionally wheezing, with good breath sounds. Chest radiographs show hyperinflation and diffuse interstitial or patchy infiltrates. Moderate eosinophilia is common. Partial pressure of oxygen is decreased in arterial blood samples but the partial pressure of carbon dioxide is normal. Serum immunoglobulins (1gG, 1gM) are characteristically elevated by two to four fold.<sup>5</sup> In very young infancy, the initial respiratory manifestations of *Chlamydia trachomatis* may be more severe left untreated and include prolonged apnea or respiratory failure. Diagnosis of this organism in this age group is important due to its long term complications and residual sequelae. Eight months follow-up of infants who had had *Chlamydia trachomatis* infection at an age less than six months showed that these neonates had an increased tendency to chronic obstructive airway disease by lung function tests and it was also noted that they had an added increase in physician diagnosed asthma.<sup>5</sup>

Prevention of neonatal *Chlamydiae* infections may be effected by screening for and treatment of *Chlamydiae* infections in pregnant mothers and their partners. These require oral macrolide antibiotics, topical treatment being ineffective. The effective drugs in treatment of paediatric *Chlamydiae* infections are macrolide antibiotics.

## **Literature review.**

Previous studies have indicated that neonatal pneumonia is frequently caused by *Chlamydiae*. In Brazil, Vaz F A et al<sup>6</sup> studied clinical and laboratory aspects of *Chlamydia trachomatis* infection in the neonatal period and found that most of these infections were acquired by the newborn infant during delivery with 25 – 50% of them developing conjunctivitis and 10 – 20% ending up with pneumonia.

In this study both term and preterm newborns admitted in the neonatal unit with conjunctivitis and/or pneumonia were evaluated using direct examination of conjunctival material, chest x-ray and antibody immunofluorescence tests for identification of Chlamydia. Pneumonia was detected in up to 75% of the patients. The group demonstrated no association between Chlamydia infection and mode of delivery, maternal age, multiple sexual partners and genital tract infection during pregnancy. They concluded that Chlamydia trachomatis is an important agent in the newborn infants requiring constant surveillance.

Jain S et al in Atlanta, USA<sup>7</sup> evaluated the spectrum of morbidity associated with perinatally acquired Chlamydia trachomatis infections in infants less than 12 weeks of age. They retrospectively reviewed maternal and infant records during a 2 year period. Symptomatic infants were tested for Chlamydia trachomatis colonization of their upper respiratory tracts. Thirteen point two percent tested positive for Chlamydia trachomatis from the conjunctiva and/or nasopharynx. Thirty-five percent of their mothers had documented Chlamydia trachomatis infection at delivery or during pregnancy and had not been treated. The presence of maternal infection with chlamydia at birth was associated with disease in the infant.

Zar H J et al in South Africa<sup>8</sup> described Chlamydia trachomatis as a significant cause of lower respiratory tract infection in infants born to mothers amongst whom there is a high prevalence of sexually transmitted diseases. The study looked at 100 cases with signs of lower respiratory tract infection and found 6% of them with Chlamydia trachomatis infections. The majority of these infants were less than 3-8 weeks old and most had a mild disease requiring only outpatient antibiotic therapy.

Ratelle et al in Massachusetts<sup>9</sup> reviewed medical records of both infants and mothers to evaluate the clinical presentation, maternal epidemiological profile, risks of transmission and screening practices of healthcare providers for cases reported to have Chlamydia trachomatis infection. They found an overall prevalence of 22% of Chlamydia pneumoniae in their group. They also found that primary prevention of neonatal infections through prenatal screening in the third trimester, treatment of infected mothers and their partners and active follow-up programs could reduce the risk of neonatal infection.

Colarizi et al in Rome<sup>11</sup> looked at preterm neonates admitted consecutively to neonatal care unit, for management of respiratory distress and reported that 8% had Chlamydia trachomatis within 24 hours of life. One percent of these neonates had evidence on chest radiographs of hyaline membrane disease and about 1% had pneumonia.

Dereli D. et al in Turkey<sup>12</sup> studied conjunctival and nasopharyngeal samples in infants with symptoms of lower respiratory tract infections. These were patients who had fallen ill from the 4<sup>th</sup> – 12<sup>th</sup> week of life and had shown resistance to therapy. Chlamydia trachomatis antigen tested by direct immunofluorescence and serum anti-chlamydial antibodies was detected in 30% of the infants.

There have been no local studies reporting the prevalence of neonatal respiratory infections with Chlamydia. Bukusi<sup>13</sup> and Cohen<sup>14</sup> reported about 95 Chlamydia carriage rates among women consulting for gynaecological illnesses.



## **STUDY JUSTIFICATION**

- At Kenyatta National Hospital newborn unit infants with pneumonia not responding to conventional antibiotics are regularly given macrolide antibiotics on presupposition of atypical organisms as the possible aetiology. No local study has been done to verify this.

## **STUDY OBJECTIVES**

1. To determine the prevalence of Chlamydiae colonization of the upper respiratory tract in neonates at Kenyatta National Hospital.
2. To estimate the proportion of late onset neonatal pneumonia that may be associated with Chlamydiae in the neonate at this centre.

## **MATERIALS AND METHODS**

### **a. Study Design and duration**

A descriptive cross – sectional study was conducted from the months of September to November in the year 2000.

### **b. Study Area**

This study was carried out in the Newborn Unit, Kenyatta National Hospital, the teaching hospital affiliated to the College of Health Sciences, University of Nairobi. It is also the national referral hospital for the country.

The newborn unit of this hospital is a level two neonatal care facility. The patient population consists of babies whose birth weights are less than 2000 grams and sick bigger infants. Infants whose mothers are acutely ill are also admitted for observation. Out born infants are only admitted if they are younger than 24 hours.

### c. **Study Population**

- All neonates diagnosed to have pneumonia after the first week of life and fulfilled the World Health Organization definition (annex III) admitted to the unit.
- Definitions. *“the case definition was any neonate who presented with fast breathing (respiratory rate  $\geq$  60 breaths per minute, at 2 observations), difficulty in breathing (sub-costal retractions or chest in-drawing) and had stayed more than one week in the newborn unit. They also had to have radiological evidence of interstitial pneumonitis. “Atypical pneumonia in this study was presence of interstitial pneumonitis on chest radiograph plus evidence of colonization by Chlamydiae”.*

### **Inclusion Criteria**

1. Cough
2. Tachypnoea
3. Difficulty in breathing

### **Exclusion Criteria**

1. Evidence of aspiration
2. Cardiovascular disease

#### d. Sample Size and Selection of Subjects

A sample size of 52 cases was studied. This was determined using the following formula – Fisher’s formula for prevalence studies.

$$N = \frac{Z^2_{1-\alpha/2} P(1-P)}{d^2}$$

where	N	=	sample size
	$\alpha$	=	significance level set at 5%
	$Z_{1-\alpha/2}$	=	normal distribution table value for $\alpha = 5\%$
		=	1.96
	P	=	estimated prevalence of atypical pneumonia among neonates with pneumonia = 16%
	d	=	degree of precision set at $\pm 10\%$
$\therefore$	N	=	$\frac{1.96^2 \times 0.16 (1 - 0.16)}{0.1^2}$
		=	52 cases

#### e. Data Collection

Procedure:

- *Recruitment.*

Cases were recruited sequentially until the required sample size was obtained. On identification of a case, maternal consent was obtained (see example of consent form in annex I). History and physical examination was done as shown in annex II. History of antibiotic use in the mother and baby was recorded.

- *Chest x rays*

Portable chest x-rays were taken and reported by two independent paediatric radiologists.

- *Laboratory specimens.*
  - Blood for white cell counts and qualitative analysis (see annexe V).
  - Nasopharyngeal and throat swabs for Chlamydia isolation (annexe V).

## **STUDY LIMITATION**

The study limitation was the inability to make a firm pathological diagnosis of atypical pneumonia. This would require lung biopsies. Interstitial pneumonitis can also be caused by viruses like Rubella, Herpes simplex, Human Immunodeficiency Virus and Cytomegalovirus.

## **DATA PROCESSING AND ANALYSIS**

Descriptive statistics were summarized using frequency distributions tables. Appropriate tests of significance were applied to determine the significance of differences between groups or associations between factors. These included the Standard normal deviate test and Fisher's exact probability test. The chosen level of significance was 5%.

## **ETHICAL CONSIDERATION**

Approval to carry out the study was sought and granted by the Ethical and Scientific committee of Kenyatta National Hospital, parental consent was also obtained for each infant.

## **RESULTS**

A total of 52 neonates were studied, of these 25 (48.1%) were males and 27 (51.9%) were females.

Postnatal ages of the subjects ranged between 7-30 days with a mean age of 12.6 days. Almost 70% of the subjects were less than two weeks old.

The prevalence of interstitial pneumonitis based on chest radiographs was 30/47 (63.8%). Five radiographs were not traced hence not available for analysis.

The colonization rate of Chlamydiae in the nasopharyngeal aspirates (NPAs) by direct antigen detection using immunofluorescence technique was 33/52 (63.5%).

Atypical pneumonia defined by the presence of interstitial pneumonitis on chest radiographs and evidence of colonization by Chlamydiae in nasopharyngeal aspirates, was detected in 24/47 (51%) of the cases with late onset neonatal pneumonia.

Some population parameters were studied in order to determine any association between these and colonization by Chlamydiae. These were parameters previously found to predispose to such colonization and presumably resulted in subsequent development of atypical pneumonia. They are summarized in table I.

**TABLE I - POPULATION PARAMETERS**

<b>PARAMETER</b>	<b>NO</b>	<b>%</b>
<b>A. MODE OF DELIVERY</b>		
Spontaneous Vertex delivery (SVD)	39	75.0
Caesarian Section (C/S)	13	25.0
<b>TOTAL</b>	<b>52</b>	<b>100.0</b>
<b>B. ESTIMATED MATURITY IN WEEKS AT ADMISSION<sup>19</sup></b>		
Preterm	46	88.5
Term	6	11.5
<b>TOTAL</b>	<b>52</b>	<b>100.0</b>
<b>C. BIRTH WEIGHT IN GRAMS AT ADMISSION</b>		
< 2500	48	92.3
≥ 2500	4	7.7
<b>TOTAL</b>	<b>52</b>	<b>100.0</b>

Table I, shows that majority (75%) cases were delivered by vaginal route while only 25% were delivered by caesarian section. Majority (88.5%) of the cases were preterms and only 11.5% were term babies. Low birth weight contributed a significant (92.3%) proportion of the cases while normal birth weight contributed only 7.7%.

## INFLUENCE OF MODE OF DELIVERY ON PRESENCE OF COLONIZATION

Mode of delivery, signifying maternal Chlamydial carriage, was compared against Chlamydial colonization in nasopharyngeal aspirates of the cases. Various modes of delivery, which may have an influence on colonization were compared. Table II shows the findings.

**TABLE II: - INFLUENCE OF MODE OF DELIVERY ON  
PRESENCE OF COLONIZATION**

		<u>Mode of Delivery</u>		TOTAL
		Vaginal	C/Section	
Colonization with Chlamydiae in NPA's	Positive	24 61 (46)	9 69 (17)	33
	Negative	15 39 (29)	4 13 (8)	19
TOTAL		39 100	13 100	52

$$X^2 = 0.03 \quad p\text{-value} = 0.87$$

Table II shows that the mode of delivery had no influence on the presence of colonization with Chlamydiae. Those cases born by vaginal route and had evidence of colonization were 61% while those born by caesarian section with evidence of colonization were 69%. Statistically analysed,  $X^2 = 0.03$  with a P-value = 0.87. There was no statistical significance.

## INFLUENCE OF BIRTH WEIGHT ON DEVELOPMENT OF ATYPICAL PNEUMONIA

Influence of birth weight on presence of colonization with Chlamydiae and subsequent development of atypical pneumonia was sort. The relationship between birth weight and development of atypical pneumonia is depicted in Table III.

**TABLE III: - INFLUENCE OF BIRTH WEIGHT ON DEVELOPMENT OF ATYPICAL PNEUMONIA**

		<u>Atypical pneumonia</u>		TOTAL
		Positive	Negative	
Birth weight	< 2500	21 (40)	27 (52)	48
	≥ 2500	3 (6)	1 (2)	4
TOTAL		24	28	52

Fishers exact probability = 0.25

Table III showing the relationship between birth weight and development of atypical pneumonia in the cases. Forty percent of the low birth weight cases developed atypical pneumonia compared to 6% of the cases that fell in the normal birth weight category. Statistical analysis using Fisher's exact probability gave a figure of 0.25, this was not statistically significant.



## PRESENCE OF ATYPICAL PNEUMONIA IN RELATION TO MATURITY AT BIRTH

Influence of gestational age on development of atypical pneumonia in the cases was also looked at. This relationship between gestational age and development of atypical pneumonia is shown in Table IV below.

**TABLE IV: - PRESENCE OF ATYPICAL PNEUMONIA IN RELATION  
TO MATURITY AT BIRTH**

		<u>Atypical pneumonia</u>		TOTAL
		Positive	Negative	
Maturity	Preterm	22 (42)	24 (46)	46
	Term	2 (4)	4 (8)	6
TOTAL		24	28	52

Fishers exact probability = 0.33

Table IV shows that 42% of the preterm cases had atypical pneumonia compared to only 4% in term neonates. On statistical analysis, the Fisher's exact probability was 0.33, this was not statistically significant.

## COLONIZATION RATE AND INTERSTITIAL PNEUMONITIS

Evidence of Chlamydiae colonization in NPA's using antigen detection by immunofluorescence was compared against evidence of interstitial pneumonitis on chest x-rays to see whether there was any correlation between these two features. Correlation between neonatal Chlamydiae colonization in nasopharyngeal aspirates and presence of interstitial pneumonitis on chest x-rays in the cases with late onset neonatal pneumonia is shown in Table V.

**TABLE V: - COLONIZATION RATE AND INTERSTITIAL PNEUMONITIS**

		<u>Interstitial pneumonitis on CXR</u>		TOTAL
		Positive	Negative	
Chlamydiae positivity in NPA's	Positive	24 (51.0)	9 (19.0)	33
	Negative	6 (13.0)	8 (17.0)	14
TOTAL		30	17	47

Positive P-value = 0.73, Negative P-value = 0.60, Sensitivity = 80%, Specificity = 50%

Table V showing that 51% of the colonized subjects also had radiological evidence of interstitial pneumonitis compared to only 13% cases who were not colonized yet had radiological evidence of interstitial pneumonitis. The positive predictive value was 0.734 and the negative predictive value was 0.60, giving an overall accuracy efficiency of 70%. The sensitivity of chest x-ray was 80% with a specificity of only 50%.

## **DISCUSSION**

Chlamydia is one of the intermediate microbes regularly associated with pneumonia in the newborn period. The usual scenario is the combination of the presence of the organisms in the respiratory tree and probably some contributory risk factors in the host (newborn). The diagnosis is usually based on a combination of radiological features and demonstration of the presence of the organism in the respiratory tree.

Isolation of Chlamydia is expensive. It is necessary to identify cheaper ways of diagnosing this condition for optimum therapy in less advanced centers like those in Kenya.

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UNIVERSITY OF NAIROBI**

The hypothesis in this study was that there is a high presence of Chlamydia in the respiratory tree of newborns with late neonatal pneumonia at this centre. This was indeed confirmed as 63.5% of all infants with late onset neonatal pneumonia were colonized. This rate was within the range that has been reported before. Some previous reports have actually given higher rates of 75%<sup>6</sup>. The actual range is between 6% and 75%<sup>8, 6</sup>. All these studies also involved infants with late neonatal pneumonia. The lower rates were more prevalent in the temperate regions like the United States<sup>7</sup> while the rate of 75% was observed in more tropical Brazil <sup>6</sup>. There may be some association between climate and chlamydia colonization of human beings.

It has been established that the presence of Chlamydiae or other atypical organisms in the respiratory tree predispose to development of the form of pneumonia described as atypical pneumonia or interstitial pneumonitis. Since this form of pneumonia contributes significantly to morbidity and mortality and cannot be treated by conventional antibiotics used for late neonatal sepsis, it is necessary to identify these cases and accord them appropriate therapy.

This study also recognized the handicap of using colonization to imply infection. The case definition was thus refined further for a more accurate diagnosis of atypical pneumonia. This required the presence of both nasopharyngeal colonization and a radiological picture of interstitial pneumonitis in the presence of clinical pneumonia.

Though interstitial pneumonitis has several other causes, on its own the strength of this definition is the requirement of the concurrence of clinical disease and microbial isolation. Lung biopsy or aspirates were not possible for technical reasons.

Using this case definition the study attempted to determine the source of Chlamydia in this group. It was hypothesized that this was either from the maternal birth canal or the hospital environment (nosocomial). It was assumed that if the Chlamydia invaded the infant from the mother, it would most likely do so during delivery in which case infants born vaginally should be more prone to colonization than those born by abdominal route. We found no difference in colonization rates between the caesarian section and vaginally delivered infants. The source of infection in this group was probably nosocomial. The infection control measures in this hospital have traditionally focused on the usual pyogenic bacteria. It is therefore important to modify these strategies to include atypical organisms since they also appear to be inhabitants of this unit.

Previous workers have demonstrated higher prevalence of atypical pneumonia in smaller and less mature infants<sup>6, 7, 8, 11</sup>. This was not reproduced in this study. This was probably because this population was very skewed towards smaller and premature infants (48 were less than 2500grams while only 4 were  $\geq$  2500 grams. 46 infants were premature while only 6 were term).

The prevalence of atypical pneumonia among infants with late onset pneumonia was 51%. This means that every infant diagnosed as having late onset neonatal pneumonia at this facility will have nearly 50% chance of having Chlamydiae as a contributor. It is therefore reasonable based on these results to include antimicrobial cover for Chlamydia for all empirical prescriptions in these circumstances.

Radiological evidence of atypical pneumonia has been the sole tool of diagnosis in this centre. Indeed it is the most easily accessible and probably cheaper than isolation of the microbe itself. Using the case definition above as the gold standard, chest x-rays had a positive predictive value of 0.73 with a sensitivity of 80% and specificity of 50%. This means that treatment of all patients on the basis of radiological features alone will over treat only about 50% of the individuals. This is acceptable for a disease with such significant morbidity and mortality and whose treatment is relatively cheap and safe.

The current practice of using radiology alone for diagnosis and treatment of atypical pneumonia is supported.

With an overall prevalence of 51% of all cases with late neonatal pneumonia there probably is justification for including a macrolide antibiotic cover for all patients with late neonatal pneumonia in this center whenever treatment is made in the absence of chest x rays.

White cell counts and qualitative analysis were done for all the patients, the findings were generally similar to those seen in acute bacterial sepsis with no distinctive features, special to atypical organisms. These can therefore not be used in the diagnosis of atypical pneumonia.

## **CONCLUSION**

- Fifty-one percent of infants with late neonatal pneumonia in this centre have Chlamydia infection as the probable aetiological agent.
- Chest x-rays used as a single tool, can suggest that pneumonia may be due to Chlamydiae.
- In this unit, Chlamydia colonization in newborns over one week old is nosocomial in origin.

## **RECOMMENDATION**

1. The inclusion of appropriate macrolide antibiotics in the empiric treatment of all cases of late onset neonatal pneumonia.
2. Chlamydia should be included in the infection control activities in this facility in particular, to identify the source of Chlamydiae in this unit.

**ANNEXE I**

**PARENT CONSENT FORM**

I, ..... P. O. Box. ....  
 mother of baby ..... IP. NO.  
 ..... admitted at the Newborn Unit, Kenyatta National Hospital,  
 have no objection for my baby to be recruited into the study – *“The prevalence  
 of Chlamydiae in late onset neonatal pneumonia at the Newborn unit –  
 Kenyatta National Hospital”*.

The nature of the study which involves specimen collection of blood,  
 nasopharyngeal aspirates, radiological examination and appropriate  
 interventions have been explained to me by the researcher.

Signed Parent \_\_\_\_\_ Date \_\_\_\_\_

Signed Researcher \_\_\_\_\_ Date \_\_\_\_\_

**ANNEXE II****STUDY DATA****A. Patients identification Data**

- i. Date
- ii. Name
- i. Hospital number
- ii. Study No. \_\_\_\_\_ Code
- iii. Sex M \_\_\_\_\_
- iv. D. O. Birth
- v. Age in days

**B. Presenting complaints for Nursery admission**

Large for gestational age (LGA)

Prematurity

Respiratory distress syndrome (RDS)

Congenital abnormalities

Infant of diabetic mother

Born before arrival (BBA)

Others

Diagnosis at admission

Treatment received preceding and current period

**C. Antenatal History**

Mothers age

Last menstrual period

Expected date of delivery

Gestation at delivery



Antenatal clinic

Problems - Cough  
 - Pervaginal discharge

Treatment received

Immunization – Tetanus Toxoid etc

**D. Natal history**

- Mode of delivery
- Duration of labour
- Problems during labour

**E. Postnatal history**

Problems experienced

**F. Physical examination**

- i. General examination – good, fair, sick looking
- i. Incubator care or cot care
- ii. Oxygen supplementation Yes \_\_\_\_\_ No \_\_\_\_\_
- iii. Weight in grams on admission \_\_\_\_\_ at present \_\_\_\_\_
- iv. Head circumference in centimetres
- v. Temperature at admission \_\_\_\_\_ at present \_\_\_\_\_
- vi. Mode of feeding  
 Expressed breast milk \_\_\_\_\_ Cow milk \_\_\_\_\_  
 Formula feeding \_\_\_\_\_
- viii. Signs Present – 1      Absent – 2 code  
 Appearance  
 Pallor  
 Cyanosis  
 Jaundice  
 Temperature  
 Sclerema

**G. Respiratory system**

- i. Respiratory rate – actual rate (tachypnoea = RR > 60 Bpm)
- ii. Cough
- iii. Basal rales on auscultation
- iv. Breaths sound

**H. Cardiovascular system**

- i. Heart rate actual rate (Tachycardia – HR > 140 Bpm)
- ii. Gallop rhythm
- iii. Cardiomegally (Down +/- or outward displacement of the apex)
- iv. Murmur

**I. Central nervous system**

- i. Lethargy
- ii. Irritability
- iii. Altered level of consciousness
- iv. Motor defects

**J. Skin - any lesions****Laboratory**

1. Chest X-ray
  - Cardiac shadow
  - Lung fields – pneumonia- interstitial pneumonitis
    - alveolar pneumonitis
  - Increased vascular markings
  - Pulmonary oedema
  - Radiologists findings and comments

## 2. Full Haemogram

Haemoglobin level

Red blood cell count  $\times 10^{12}/l$

Mean corpuscular haemoglobin in pg/cell

Mean corpuscular volume in femtolitres

Mean corpuscular haemoglobin concentration in g/dl

White cell count  $\times 10^9 / \text{litre}$

Differential counts	count	percentage
---------------------	-------	------------

Lymphocytes

Neutrophils

Monocytes

Eosinophils

Basophils

Band forms

Immature: Total lymphocyte ratio

Platelet count  $\times 10^{12}/\text{litre}$

## 3. Blood culture results

## 4. Microimmunofluorescence results

## ANNEXE III

### WHO CRITERIA FOR PNEUMONIA IN THE NEONATAL PERIOD

From a public health point of view, the WHO has laid down criteria which help to identify pneumonia at various age groups, classify pneumonia in order to aid in effective management of this major cause of death. In the neonate, these criteria are:-

#### i. Fast Breathing

Pneumonia is defined when the respiratory rate is  $\geq 60$  breaths per minute after a 2<sup>nd</sup> reading. Due to the variability in respiratory rates in  $< 2/12$  ages, WHO recommends that two readings of fast breathing be taken 10 minutes apart. This is a sensitive and specific indicator for pneumonia in late infancy onwards.

#### ii. Difficulty in Breathing

Normally neonates have mild subcostal indrawing but when the indrawings are marked, they signify severe infection and aid in classification of pneumonia.

#### iii. Cough

This symptom may or maynot be present in the neonate.

#### NB

Fever is not an efficient criteria as many other diseases have fever for example malaria, upper respiratory illnesses and diarrhoea. Fever may be absent in very severe disease and in cases of protein-energy malnutrition.

iv. Other signs

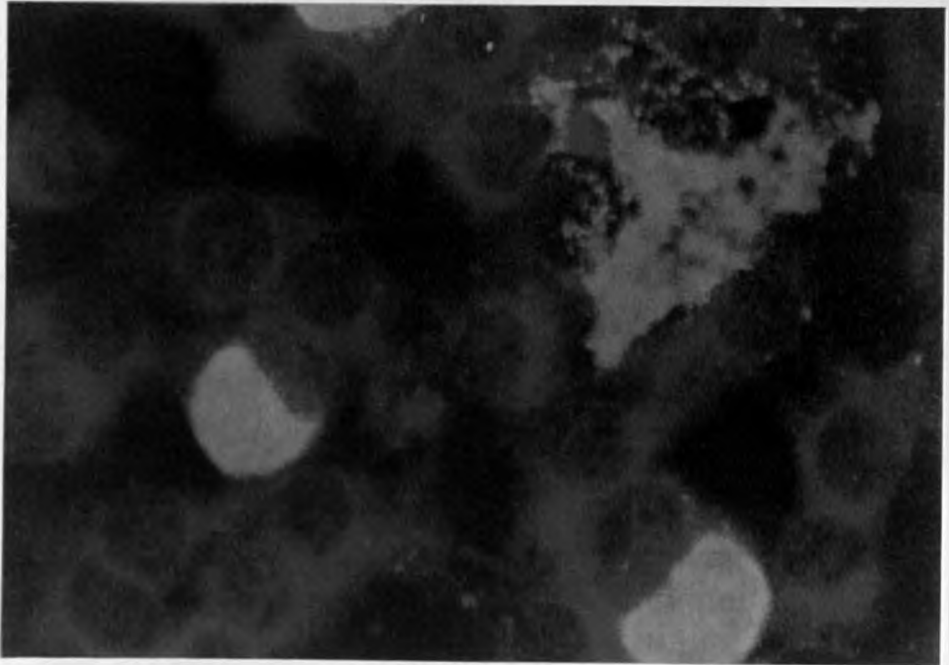
Needed in the neonatal period are:

- a. Stopping to feed
- b. Abnormally sleepy or difficult to wake
- c. Fever or hypothermia (body temperature  $< 35.5^{\circ}\text{C}$ )
- d. Convulsions

Thus with the above features, all neonates are managed as cases of very severe disease.

**ANNEXE IV****ILLUSTRATIONS****a. Direct antigen detection by immunofluorescence studies**

Photograph showing bright apple green fluorescence with inclusions against reddish background of Evan's Blue stain.

**Study Case No. 2**

8 days old, male, neonate delivered by vaginal route at 30 weeks

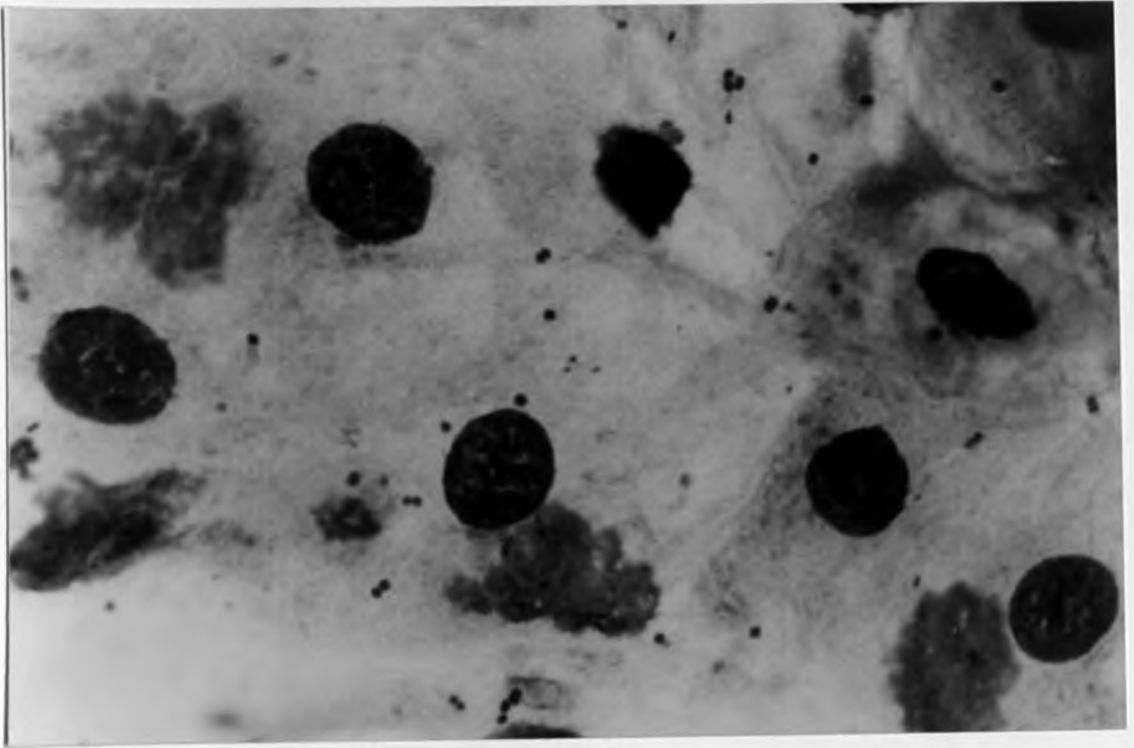
Bwt – 1200g

**CXR -** Bilateral perihilar streaky inflammatory infiltrate with associated bilateral air-trapping. Suggestive of interstitial (perihilar) pneumonitis.

**NPA's -** Positive for both *Chl. trachomatis* and *Chl. pneumoniae*.

## b. Giemsa stain

Photograph showing mauve reticulate bodies, nuclei of host cells are dark purple with a pale blue cytoplasm.



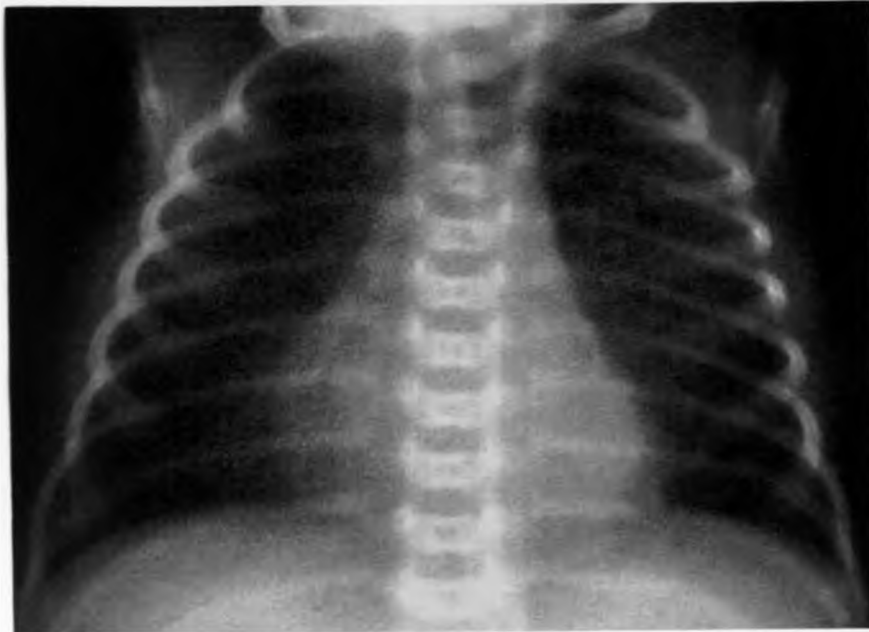
## Study Case No. 30

11 days old, female, neonate delivered by vaginal route at  
35 weeks

Bwt - 1750g

CXR - Bilateral perihilar inflammatory infiltrate with associated  
bilateral air-trapping. Suggestive of interstitial  
pneumonitis.

NPA's - Positive for both *Chl. trachomatis* and *Chl. pneumoniae*.



**Study case no. 2**

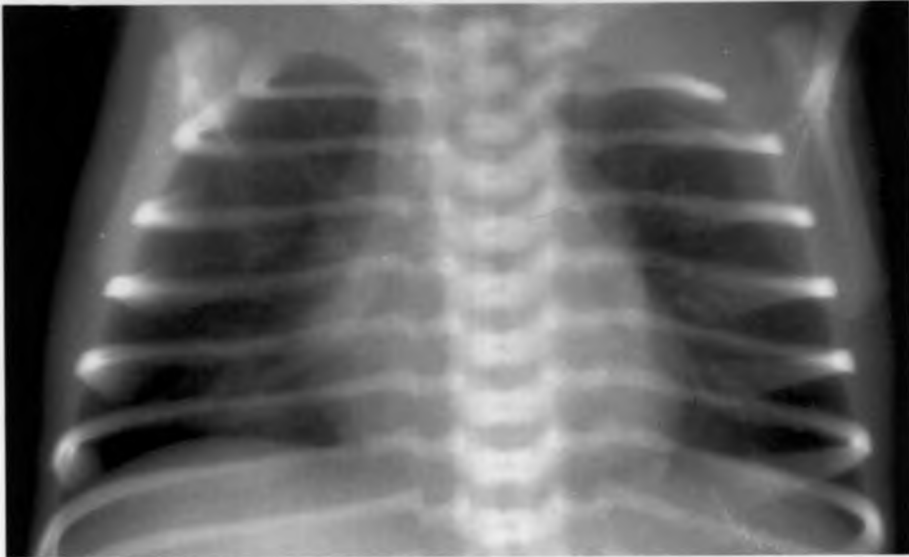
8 days old (M) neonate delivered vaginally at 30 weeks.

**Bwt** – 1200g

**NPA's** – Positive for both *Chl.trachomatis* and *Chl.pneumoniae*

**CXR** – Bilateral perihilar streaky inflammatory infiltrate with associated bilateral air trapping. Suggestive of Interstitial (perihilar) pneumonitis.





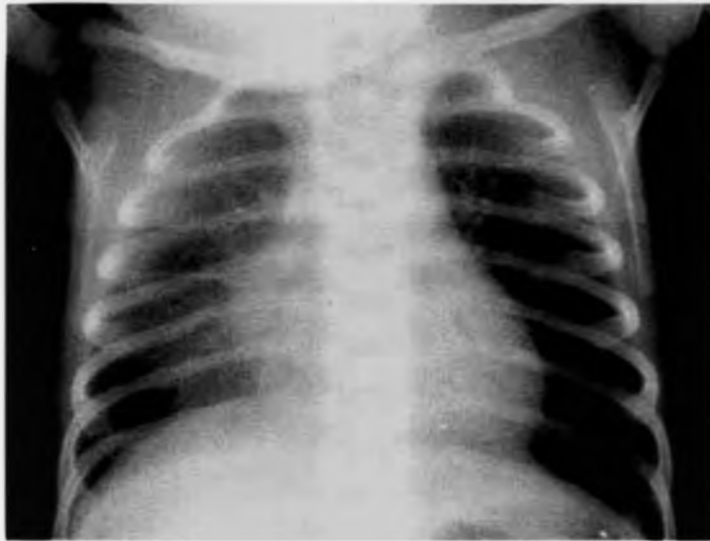
**Study case no. 10**

16 days old (F) neonate delivered by C/section due to prolonged rupture of membranes with chorioamnionitis at 33 weeks

**Bwt** - 1300g

**NPA's** - Negative for both *Chl.trachomatis* and *pneumoniae*

**CXR** - Right upper and lower lobe patchy opacity with bilateral air trapping suggestive of aspiration pneumonia



**Study case no. 18**

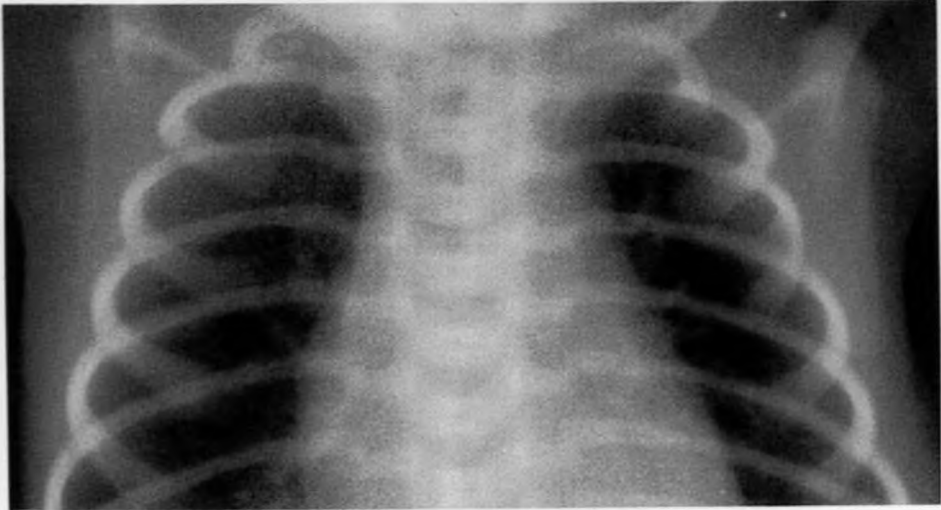
9 days old (F) neonate delivered by C/section due to severe  
PET with reduced fetal movements at 34 weeks

**Bwt** - 1550g

**NPA's** - Positive for Chl.trachomatis

- Positive for Chl.pneumoniae

**CXR** - Bilateral perihilar infiltrates and a right basal  
pneumonic process with bilateral air trapping  
suggestive of pneumonia



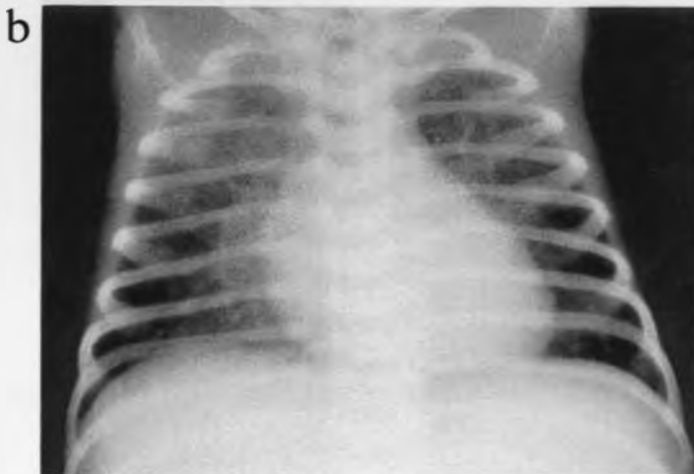
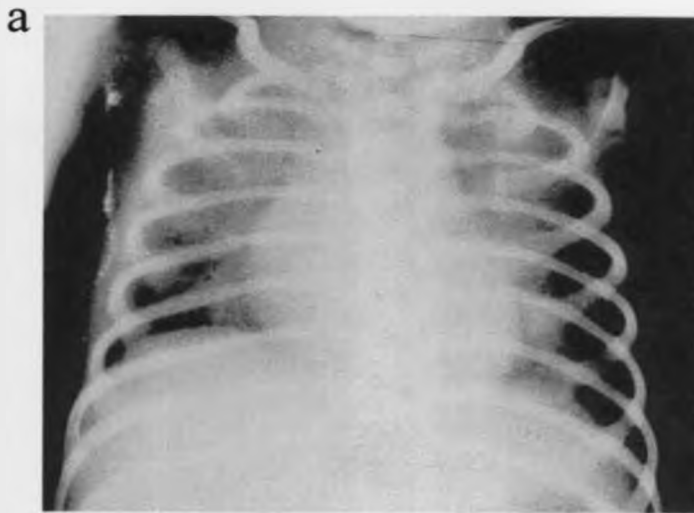
**Study case no. 20**

8 days old (F) neonate, delivered by C/section due to severe PET at 36 weeks.

**Bwt** - 2200g

**NPA's** - Negative for Chl.trachomatis and Chl.pneumoniae

**CXR** - Showed hyperinflated lungs with bilateral perihilar and basal granular opacities suggestive of HMD grade I



**Study case no. 37**

10 day old (F) neonate delivered vaginally at 35 weeks

**Bwt** - 1600g

**NPA's** - Negative for both *Chl.trachomatis* and *Chl.pneumoniae*

**CXR** - a.-Bilateral perihilar and basal pneumonic opacities with bilateral air trapping

b.-Clearing of the pneumonic process but rather slowly with bilateral air trapping.



**Study case no. 42**

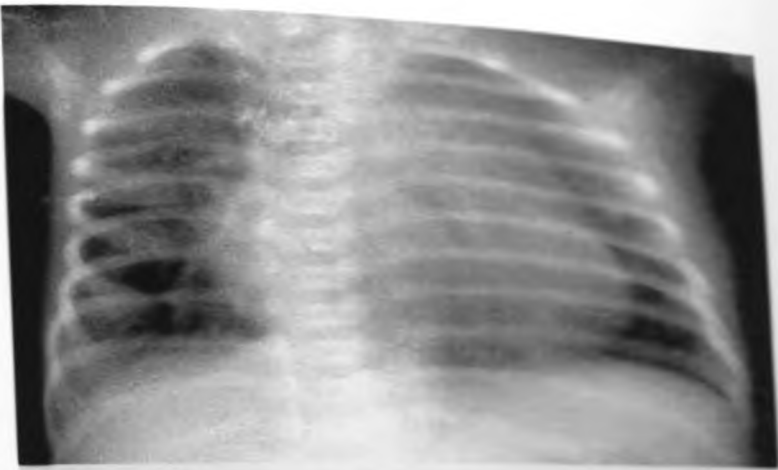
17 days old (M) neonate delivered Vaginally at 28 weeks.

**Bwt** - 1100g

**NPA's** - Positive for Chl.pneumoniae  
- Negative for Chl.trachomatis

**CXR** - Hyperinflated lungs with perihilar and basal granular opacities suggestive of HMD grade I

a



b



Study case no. 43

21 days old (F) neonate delivered vaginally at 34 weeks to a mother who had attempted to kill the baby.

Bwt - 1600g

NPA's - Positive for Chl.pneumoniae

- a. Right upper and bilateral lobes pneumonic opacities with air bronchogram suggestive of complicated HMD with pneumonia

- b. Generalized bilateral ground glass appearance suggestive of wet lung.

## ANNEXE V

### SPECIMEN HANDLING

i. Procedure for collection of NPA's specimen

Just prior to the next feed specimens were taken as follows:-

- The nasal area was cleaned with a sterile swab moistened with sterile normal saline.
- Two throat swabs moistened with normal saline were used to take throat swabs – one was used to make impressions on microscopic slides for routine Giemsa and Gram's stain and another throat swab was sent for routine microbiology.
- A sterile nasogastric catheter size 4 French was inserted upto the nasopharynx, care being taken to avoid trauma and catheter tip contamination.
- Instillation of 0.5mls of sterile normal saline and immediate aspiratioin of the fluid.
- This aspirate specimen was stored in a sterile screw capped plastic container and transported to the lab.

iii. These slide smears were then subjected to:-

- a. Gram's stain
- b. Giemsa stain

For procedure see Annex VI, VII.

iv. 0.2ml of nasopharyngeal aspirate was centrifuged and slides were made from the sediment, air dried then fixed in methanol then subjected to immunofluorescence studies for Chlamydial antigen detection.

Procedure were as per the manufacturer's instructions Annex VIII.

v. The remaining 0.3ml was frozen at  $-70^{\circ}\text{C}$ .

Specimen for full haemogram and blood culture.

i. Procedure of collection

- Venepuncture site was cleaned with spirit.
- Using a scalp vein gauge 22 or 24, 1ml blood was drawn for these routine tests.
- 0.5ml was transferred into a sterile container with anticoagulant for full haemogram.
- 0.5ml blood was injected using a fresh sterile needle into another sterile container with aerobic media and sent to the laboratory for routine culture.



## ANNEXE VI

### 1. GIEMSA STAINING TECHNIQUE

This is a Romanowsky stain widely used in malaria staining and other parasites. In microbiology, the Giemsa technique is used mainly to stain *Chlamydia trachomatis* inclusion bodies, *Borrelia* species and in *Yersinia pestis*.

For staining *Chlamydiae*, a weaker solution of Giemsa and a larger staining time are used.

**Reagents:-** Giemsa stain

Buffered water, pH 7.0 – 7.2

**Method:-**

- a. Fix the air-dried nasopharyngeal aspirate smear by covering it with methanol for 2 – 3 minutes. Allow the smear to air-dry.
- b. Dilute the freshly prepared Giemsa stain in buffered water immediately before use. For *Chlamydia trachomatis*, the stain is diluted 1 into 40 as follows:- A smaller cylinder is filled up to the 19.5ml mark with buffered water. Add 0.5ml of Giemsa stain i.e. to the 20ml mark
- c. Place the slide, smear down-wards, in a petri dish or other small container, supported on each side by a thin piece of stick.
- d. Pour the diluted stain into the dish and cover with a lid.
- e. Stain for 1 hour (1-5 hours for heavy or thick smears).

**Note:** This inverted method of staining avoids stain being deposited on the smear.

- f. Rinse with 95% ethyl alcohol.
- g. Wipe the back of the slide clean, and place in a draining rack for the smear to air-dry.
- h. Examine the smear microscopically, first with the 40X objective to see distribution of material and to select a suitable part of the smear to examine with the oil immersion lens.

i. **Results**

Chlamydia trachomatis depending on the stage of development – Blue – mauve to dark purple, elementary bodies.

Nuclei of host cells – dark purple

Cytoplasm of host cells – pale blue

Eosinophil granules – red

Melanine granules – black green

Bacteria – pale or dark blue

## **ANNEXE VII**

### **2. GRAM STAIN TECHNIQUE**

Most bacteria can be differentiated by their Gram reaction due to differences in their cell wall structure. Those that stain dark purple with crystal violet and are not decolourised by acetone or ethanol are called Gram positive.

Those that after being stained with crystal violet lose their colour when treated with acetone or ethanol and stain red with neutral red, saffranin are called Gram negative. Iodine solution used in the technique acts as a mordant for the crystal violet.

#### **Reagents**

- Crystal violet stain
- Gram's iodine
- Acetone – alcohol decolorizer
- Neutral red, 1g/1(0.1% w/v)

Alcohol and acetone mixture is preferred because it decolorizes more rapidly than ethanol 95% v/v and is less likely to over decolorize smears than acetone alone.

#### **Method**

1. Fix the dried nasopharyngeal aspirate smear
2. Cover the fixed smear with crystal violet stain for 30 – 60 seconds
3. Rapidly wash off the stain with clean water
4. Tip off all the water and cover the smear with Gram's iodine for 30 – 60 seconds
5. Wash off the iodine with clean water

6. Decolorize rapidly with acetone / 95% ethanol alcohol wash immediately with clean water
7. Cover the smear with neutral red stain for 2 minutes
8. Wash off the stain with clean water
9. Wipe the back of the slide clean and place in a draining rack for the smear to air-dry
10. Examine the smear microscopically with the 40X objective to check staining and see material distribution and then under oil immersion.

### Results

Gram positive bacteria	-	Dark purple
Nuclei of pus cells	-	Red
Epithelial cells	-	Pale red
Gram negative bacteria	-	Pale or dark red

## **ANNEXE VIII**

### **IMMUNOFLUORESCENCE STUDIES**

#### **DIRECT ANTIGEN DETECTION IN NASOPHARYNGEAL ASPIRATES BY IMMUNOFLUORESCENCE**

##### **A. CHLAMYDIA TRACHOMATIS**

- The CHLAMYDIA-CEL IF TEST (CELLABS – AUSTRALIA).

This is a rapid invitro direct immunofluorescence test for detection of Chlamydia trachomatis in clinical specimens.

- Principle of the test:

This test enables direct staining of clinical specimens and can therefore be used for visualisation of extracellular organisms. All Chlamydia trachomatis serotypes share a common protein antigen located in the outer membrane of cell wall. Fluorescein – labelled monoclonal antibodies directed against this outer membrane protein can therefore be utilized for antigen detection. The labelled antibody binds specifically to Chl. trachomatis present in methanol fixed specimens which have been directly applied to a slide. A washing stage removes unbound antibody. When viewed under a fluorescence microscope, Chl. trachomatis are seen as bright apple-green intracellular or extracellular EB's or RB's contrasting with the reddish brown colour of counterstained material. This test is specific and sensitive.

• Contents of the kit:-

- Reagents – Fluorescein labelled purified monoclonal antibody reagent diluted in a protein stabilised buffer solution (pH 7.4) with Evan's Blue as counter stain and 0.1% (w/v) sodium azide.
- Mounting fluid – a photobleaching inhibitor in glycerol.
- Positive control slide – prepared on unstained slide of mammalian cells and Chlamydia free EB's and RB's.
  - ◆ Methanol for specimen fixation on slides
  - ◆ Phosphate buffered saline (PBS) pH 7.4 for washing stained specimens.
  - ◆ Non fluorescing emersion oil
  - ◆ Precision pipette to deliver 25 ul
  - ◆ Wash basin, cover slips, microscope slides with wells
  - ◆ Fluorescence microscope with filter system for FITC (maximum excitation wavelength 490nm, mean emission wavelength 530nm) and x600 – x1000 magnification.

• Instructions:-

- Add 25 ul of reagent to the fixed specimen smear and positive control slide, cover entire well area.

- Incubate slides at 37°C in a moist chamber for 30 minutes in the dark, avoid drying of the slides as this will cause non-specific binding.
- Rinse gently in a bath of PBS for about one minute.
- Drain slide and remove excess moisture around well with tissue.
- Add a drop of mounting medium to the slide well. Place a cover slip on top of the drop and remove all air bubbles.
- Scan the entire specimen using a fluorescence microscope under oil immersion at X600 - X1000 magnification. Read immediately.

**NB** - Slides were processed in batches

• **Result interpretation:-**

- Chlamydial forms seen in specimens are EB's or RB's either intracellular or extracellular and appear as bright apple-green fluorescent pinpoint smooth edged disc shaped bodies (EB's) or larger bodies which either fluoresce evenly or possess dark centres with a halo of fluorescence (RB's); against a background of counter stained cells.
- Comparison is made with the positive control slide as regards appearance and size of EB's / RB's.

• **Diagnosis:-**

- Slides are positive when at least 10 Chlamydial bodies are seen. These excludes false positive results.
- Slides are negative when no Chlamydial organisms are seen but cells are present either intact or ruptured.

### Limitation:-

- Successful diagnosis depends on collection of clinical specimens with sufficient cellular material which is visible microscopically.
- Proper slide preparation.
- Improper reagent storage leads to altered sensitivity.
- Adequate reagent coverage

### Specificity:-

The kit detects all known Chl. Trachomatis serovars, A, B, Ba, C, D, E, F, G, H, I, J, K. It does not detect Chl. psittaci stains, Chl. pneumoniae. There is no detected cross reactivity with any other organisms.

## B. CHLAMYDIA PNEUMONIAE

### Principle of the test:

Chlamydia pneumoniae are difficult to culture and are sensitive to temperature. Diagnosis relies on microimmunofluorescence (MIF) test using Chl. pneumoniae elementary body antigen to detect antibodies. This test utilises monoclonal antibody to Chl. pneumoniae which binds specifically to the organisms in fixed specimens. Unbound antibody is removed by a washing step. The bound antibody is visualised by a second antibody, FITC – conjugated goat anti-mouse Ig. After washing to remove excess conjugate the specimen is observed under a fluorescence microscope. Chl. pneumoniae are seen as bright green EB's and RB's or inclusions.



**Contents of kit:-**

- Monoclonal reagent – reagent diluted in a protein stabilised buffer solution and 0.1% (m/v) sodium azide.
- FITC reagent – goat anti-mouse Ig – FITC with Evans Blue as counterstain and 0.1% (m/v) sodium azide.
- Mounting fluid – a photobleaching inhibitor in glycerol.
- Positive control slide – prepared unstained slide of mammalian cells showing Chl. pneumoniae – EB's and RB's and inclusions.
- Methanol for specimen fixation.
- Phosphate buffered saline (PBS) pH 7.4 for washing stained specimens.
- Non-fluorescing immersion oil
- Precision pipette for delivery 25ul
- Coverslips
- Microscope slides with 6-8mm diameter wells
- Fluorescence microscope with filter system for FITC (maximum excitation wavelength 490nm, mean emission wavelength 530nm) with X600 and X1000 magnification.

## Specimen collection and slide preparation

Specimens from symptomatic patients should contain as many epithelial cells as possible as Chlamydiae are intracellular organisms that infect epithelial surfaces. Throat swabs and NPA's are recommended.

### Nasopharyngeal Aspirates:-

- Centrifuge aspirates specimen for 5 minutes to ensure that any extracellular organisms present are pelleted with the cells and not discarded in the supernatant.
- Pellet is resuspended in PBS and 20 ul is placed on the slide.
- Dry and fix in methanol.

### Instructions for use:-

- Add 25 ul of the monoclonal antibody reagent to the fixed specimen smear and positive control slide, covering the entire well areas.
- Incubate the slides at 37°C in a moist chamber for 30 minutes. Do not allow the slides to dry as this will cause nonspecific binding.
- Gently rinse for 1 minute in a bath of PBS.
- Drain slide and remove excess moisture until just dry. Excess moisture dilutes antibody.

- Add 25 ul of anti-mouse Ig – FITC reagent to the specimen.
- Incubate the slide in the dark as in step 2.
- Repeat steps 3 and 4.
- Add a drop of mounting medium to the slide well. Place a coverslip on top of the drop and remove air bubbles.
- Scan the entire specimen well using a fluorescence microscope under oil immersion at X600 – X1000 magnification. Read immediately.

NB - Slides were processed in batches.

#### Specificity:-

This test detects only *Chl. Pneumoniae* only. It does not detect any of the serovars of *Chl. Trachomatis* and *Chl. Psittaci*.

There has been any known cross-reactivity with other organisms.

**BIBLIOGRAPHY**

1. Berman R, vaughan V, Nelson N  
Infections of the Newborn  
Nelson textbook of Paediatrics#. WB Saunders Company  
14<sup>th</sup> Edition  
Pg. 495
2. Pneumonia  
Nelson textbook of Paediatrics  
14<sup>th</sup> Edition  
Pg. 508
3. Respiratory tract colonization and a distinctive pneumonia syndrome in infants infected with Chlamydia trachomatis  
New England Journal of Medicine  
306, 1977 Jan - Jun
4. Sergio stagno et al  
Infant pneumonitis associated with CMV, Chlamydia, PCP, Ureaplasma. Prospective study. Pediatrics.  
68 : 322, 1981 (3) Sept.
5. Mandell Bennett, Dohn  
Principals and practice of Infectious diseases  
4<sup>th</sup> Edition
6. Vaz FA. Ceccon ME. Diniz EM.  
Chlamydia trachomatis infection in the neonatal period: Clinical and laboratory aspects. Experience of a decade : 1987 - 1998  
Revista da Associacao Media Brasileira  
45 (4) : 303 - 11, 1999 Oct - Dec.

7. Jain S. et al

Perinatally acquired Chlamydia trachomatis associated morbidity in young infants

Journal of Maternal Fetal medicine

8 (3) : 130 - 3, 1999 May - Jun.

8. Zar HJ, Van Dyk A. Yeats JK. Harislo D.

Chlamydia trachomatis lower respiratory tract infections in infants

Annals of Tropical Pediatrics

19 (1) : 9 - 13, 1999 Mar.

9. Ratelle S. et al

Neonatal Chlamydial infections in Massachusetts;

1992 - 1993

American Journal of Preventive Medicine

13 (3) : 221, 1997 May - Jun.

10. Colarizi P et al

Chlamydia trachomatis - associated respiratory disease in the very early neonatal period.

Acta Paediatrica

85 (8) : 991 - 4, 1996 Aug.

11. Dereli D. et al

Chlamydial infections in infants

Journal of Tropical Pediatrics

42 (4) : 233 - 6, 1996 Aug.

12. Ejzenberg B. et al

Aerobic bacteria, Chlamydia trachomatis

Pneumocystis carinii and cytomegalovirus as agents of severe pneumonia in small infants.

Revista do Instituto de Medicina Tropical de São Paulo

38 (1) : 9 - 14, 1996 Jan - Feb

3. Bukusi E. A et al  
HIV-1 Infection and acute pelvic inflammatory disease  
1999 Dec.
4. Cohen C.R et al  
HIV-1 Infection and acute salpingitis  
1998 Nov.
15. Afebrile pneumonia in infants  
Primary care  
23 (4) p 849 – 60, 1996 Dec.
16. Atypical pneumonia in children  
Adv. Paediatric disease  
10, 1 – 39, 1995
17. Pneumonia in children  
Lancet April (2) pf 741 – 3, 1998
18. Sample size determination  
A user's manual  
Epidemiology and statistical methodology unit  
WHO, Geneva, 1986
19. Dubowitz LMS, Dubowitz V Golberg C  
Clinical assessment of gestational age in the newborn infant  
J. Pediatr 77 : 1, 1970

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12 April 2001

Dr. Fridah A. Govedi  
Dept. of Paediatrics  
Faculty of Medicine  
University of Nairobi

Dear Dr. Govedi,

RE: RESEARCH PROPOSAL "A STUDY TO DETERMINE THE PREVALENCE OF CHLAMYDIAE ORGANISMS IN LATE ONSET NEONATAL PNEUMONIA AT NEWBORN UNIT - KENYATTA NATIONAL HOSPITAL" (P889/7/2000)

This is to inform you that the Kenyatta National Hospital Ethical and Research Committee has reviewed and approved the revised version of your above cited research proposal.

On behalf of the Committee I wish you fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Thank you.

Yours faithfully,

PROF. A.N. GUANTAI  
SECRETARY, KNH-ERC

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