INCIDENCE OF *Mycoplasma, pneumoniae* IN CHILDREN WITH PNEUMONIA AT AHMADU BELLO UNIVERSITY TEACHING HOSPITAL, ZARIA.

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THESIS APPROVAL

This thesis by Farrah Mirza meets the regulations governing the degree of Master of Science of Ahmadu Bello University and is approved for its contribution to scientific knowledge and literary presentation.

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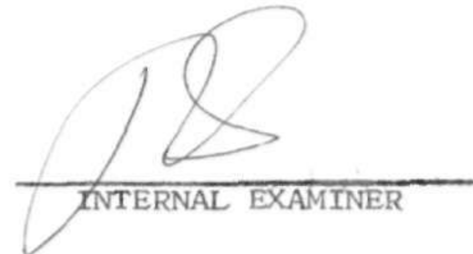

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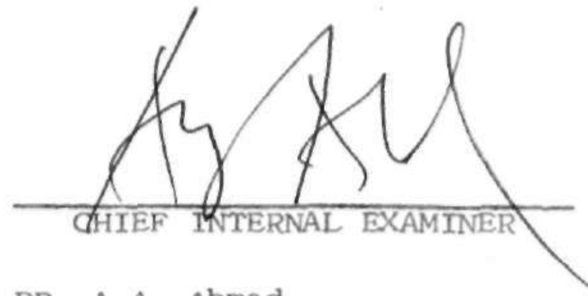

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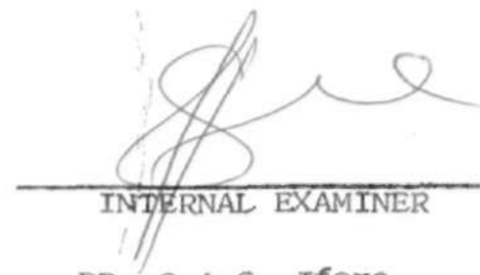

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DECLARATION

I hereby declare that the work in this thesis was carried out by me in the Mycoplasmology laboratory of the Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria and the FPLO Unit of the Bacteriology Division, National Veterinary Research Institute, Vom, under the Supervision of Dr. A. A. Ahmad, Dr. A. M. Yakubu and Dr. O. A. S. Ifere.

No part of this thesis has been presented in any previous application for a higher degree. All sources of information are specifically acknowledged by means of references.

Farrah Mirza

DEDICATION

To my parents

and my sister.

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A B S T R A C T

Throat swabs and sera specimens were taken from 104 children suffering from clinically diagnosed pneumonia in the Pediatric Out Patients Unit of the Ahmadu Bello University Teaching Hospital, Zaria.

The swabs were cultured in standard mycoplasma medium supplemented with glucose. Any mycoplasma-like organism encountered was characterized biologically and serologically by standard techniques. The Complement Fixation Test was done on the sera samples to determine the *Mycoplasma -pneumoniae* antibody titre.

The isolation and characterization techniques yeilded 2 isolates of M. *pneumoniae* (2%) and the complement fixation test gave 32 positive titres (>-_1/64) of M. *pneumoniae* antibody (31%).

Thus, from isolation and serology an incidence of 33% of M. *pneumoniae* was found in the pneumonia patients of pediatric age,

52 control patients were also taken for the study (that is those without clinical symptoms of pneumonia) from whom throat swabs and sera specimens were taken. No M. *pneumoniae* was isolated from the throat swabs and no serum sample gave a positive titre (> 1/64) in the control patients.

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CHAPTER ONE

INTRODUCTION

THE MYCOPLASMAS

Mycoplasmas are the smallest and simplest self replicating procaryotes (Anon, 1974). The mycoplasma cell contains only the minimum set of organelles essential for cell growth and replication: a triple-layered plasma membrane to seperate the cytoplasm from the external environment, ribosomes to assemble the cell proteins, and a double stranded deoxyribonucleic acid (DNA) molecule to provide the information for protein synthesis. Unlike all other prokaryotes, mycoplasmas have no cell wall (Razin, 1978).

The mycoplasmas were first recognised by Louis Pasteur from a case of bovine pleuropneumonia, although he did not observe it microscopically nor isolate it. Nocard and Roux (1898) succeeded in isolating the organism in a cell-free medium and called it the "microbe de la peripneumonie". Microorganisms from other sources, having properties similar to the pleuropneumonia organisms of cattle, later came to be known as "Pleuropneumonia like organisms" or "PPLO" (Hayflick and Chanock, 1965).

The taxonomic status of the pleuropneumonia like organism was not defined for a long time. There was no generally accepted nomenclature or classification used to identify the strains. The term Mycoplasma introduced by Nowak in 1929 became the first valid generic name. This name was finally adopted by Edward and Freundt in 1956 (Edward and Freundt, 1956; Freundt, 1957).

Mycoplasmas are widely dispersed in nature, they have been isolated from man, chicken (and their eggs), turkeys, pigeons, parakeets, ducks, mice, rats, cattle, sheep, goats, dogs, cats, horses, swine and guinea pigs (Hayflick and Chanock, 1965). Mycoplasmas have also been isolated from sewage and decomposing organic material (Laidlaw and Elford, 1936). Although, this mycoplasma was later found to belong to the genus Acholeplasma and was renamed Acholeplasma laidlawii (Edward and Freundt, 1969; 1970).

1.2 CLASSIFICATION

Disagreements regarding the relationship of the mycoplasmas to the L-forms of bacteria have for many years been one of the major obstacles to a meaningful taxonomic classification of the former organisms. It was decided in 1967 (Edward and

Freundt, 1967) to separate the wall-less prokaryotes from the eubacteria into a new class, the Mollicutes (Mollis: soft; cutis: skin). The common term mycoplasmas has been used rather loosely to denote any species in the Class Mollicutes, whereas the terms acholeplasmas, ureaplasmas, spiroplasmas and anaeroplasmas are used when reference is being made to members of the corresponding genus rather than to a defined species within the genus (Razin, 1938a).

The mycoplasmas were placed in the order Mycoplasmatales due to their peculiar properties, including their typical morphology and plasticity; their sensitivity to lysis by osmotic shock, alcohols, organic solvents, detergents, antibody and complement; their filterability through 450nm pore diameter filters; their fried-egg colony shape and their inability to revert to a bacterium in a medium without penicillin and thallium acetate (Razin, 1983b). Subdivisions into the three main families:- Mycoplasmataceae (sterol requiring), Acholeplasmataceae

(non-sterol requiring) and Spiroplasmataceae (spiral shaped, motile); has been based on nutritional and morphological criteria and on differences in the genome size (Freundt, 1983).

The Ureaplasmas differ from the Mycoplasmas and Acholeplasmas in their ability to hydrolyze urea (Shepard *et al.*, 1974). Other biochemical reactions such as breakdown of carbohydrate substrate (Erno and Stipkovits, 1973), serum digestion and phosphate production (Alluotto *et al.*, 1970), film and spots formation (Rottem and Razin, 1964) aids in speciation.

The currently accepted taxonomic strata of the class Mollicutes adapted by Freundt (1983) is as follows:-

CLASS: Mollicutes

ORDER: Mycoplasmatales

FAMILY I: Mycoplasmataceae

(1) Sterol required for growth.
(2) Genome size approximately 5.0 x 10^8 daltons.
(3) NADH oxidase localized in cytoplasm.

GENUS 1: Mycoplasma (approximately 70 species current): Do not hydrolyze urea.

2: Ureaplasma (one specie with serotypes): Hydrolyze urea.

FAMILY II: Acholeplasmataceа

(1) Sterol not requiredfor growth.
(2) Genome size approximately 1.0 x 10^9 daltons.

(3) NADH oxidase localized in membrane.

GENUS 1: Acholeplasma (8 species current).

FAMILY III: Spiroplasmataceae

(1) Helical organisms during some phase of growth.

(2) Sterol required for growth.

(3) Genome size approximately 1.0 x 10^9 daltons.

(4) NADH oxidase localized in cytoplasm.

GENUS 1: Spiroplasma (3 species current).

Genera of uncertain taxonomic position:

:Thermoplasma (1 specie)

:Anaeroplasma (2 speices).

1.3 MORPHOLOGY, STRUCTURE AND FUNCTION

1.3.1 Colonial Morphology:

The morphology of mycoplasma colonies on agar ranges from the fried-egg shape (characterised by a dense centre where the colony has grown into agar and the less dense peripheral zone where the colony spreads out along the surface of the agar) to homogenous round shapes or colonies with irregular borders (Hayflick, 1969; Smith, 1971).

The morphology depends largely on the species of mycoplasma, the number of passages in artificial medium, and the conditions of crowding on the agar plate. The more crowded an agar plate, the smaller and more irregular the colonies (Hayflick, 1969). Colonies on initial isolation, are usually round with smooth borders. Mycoplasma colonies, on agar medium, measure from 50 to 500μm in diameter (Razin, 1983b). On nutritionally poor media or with inadequate pH or atomospheric conditions or when the medium surface is too dry, the initial central "down growth" or denser region may form without the accompanying peripheral surface growth (Razin and Oliver, 1961; Smith, 1971).

Some mycoplasma colonies lack the typical denser centre in the initial passages into artificial medium e.g. Mycoplasma pneumoniae (Razin, 1978), M. hypopneumoniae and M. gallisepticum (Rosenfield, 1971).

1.3.2 Cellular Structure:

The mycoplasmas are highly pleomorphic because they lack a rigid cell wall. Viewed under a light microscope they appear as filaments, balôons, granules, rings, clubs and discs. Minute forms are capable of passing through bacterial filters and can revert to their larger forms. This phenomenon was one reason for the early classification of mycoplasmas as viruses (Adegboye, 1981).

1.3.3 Ultra Structure and Related Functions

The mycoplasma cell is bounded by a plasma membrane, and the enclosed cytoplasm contains ribosomes, chromosomes, macromolecular RNA (ribonucleic acid), plasmids and cytoplasmic granules (Razin, 1983a).

The cell membrane has a triple-layered "unit-membrane" structure composed of lipids, proteins and carbohydrate residue. The inner and outer layers of the membrane are electron dense and the middle layer is less dense (Hayflick and Chanock, 1965). Besides providing the enclosure for the intracellular structures, the cell membrane is the major immunogenic determinant of the mycoplasmas. The membrane glycolipids are the major antigenic determinants responsible for the production of metabolism and growth inhibiting, complement fixing, and agglutinating antibodies, whereas the membrane proteins appear to be primarily involved in the attachment of the microorganisms to the host cells (Razin *et al.*, 1970; 1972; Powell *et al.*, 1976). Thus, the use of plasma membrane as vaccine has been suggested by Razin *et al* (1970) who showed it to be feasible. The resistance to antibiotics like chloramphenicol, tetracycline and streptomycin is due to reduced permeability rather than ribosomal alteration (Fraterrigo, 1971).

The cytoplasm occupies most of the internal space of the cell and the denser region at the periphery is made up of ribosomes. Some colonies, which exhibit a characteristic dark center and transparent peripheral zone, have in the peripheral zone numerous lace like or highly vacuolated structures which on higher magnification show tiny granules in Brownian motion (Hayflick and Chanock, 1965).

Some mycoplasmas have unique specialized polar organelles shaped as tapered tips or "blebs", built around a central striated rod, on one or both ends of the organism (Green and Hanson, 1973; Levishon and Razin, 1973; Tajima et al.,1979). Green and Hanson (1973) showed the bleb to be the site of increased protein synthesis. These blebs or tips appear to play a role in the attachment of the mycoplasmas to host cells and to inert surfaces, and they are gradually formed when the organism is in close contact with the host cells (AbuZahr and Butler, 1978; Tajima et al., 1979; Razin, 1983a).

Electron microscopy reveals a fuzzy layer or a nap covering all or part of the cell surface of a number of mycoplasmas. The chemical nature of the extracellular material is largely unknown, though it usually stains by ruthenium red, which is a general stain for polyanions (such as polysaccharide glycocalyxes of eukaryotic cells and capsules of prokaryotic organisms). The possibility that this extracellular

or capsular material plays a role in pathogenesis - by inhibiting phagocytosis of the mycoplasmas or by facilitating their adherance to host cell surfaces - remains to be investigated (Razin, 1978; 1983a).

The mycoplasma genome is typically prokaryotic in consisting of a circular double-stranded DNA molecule, which contains the genetic information, but it differs from the genomes of other prokaryotes in its small size and low guanine plus cytosine (G+C) content. The genome size of mycoplasma and ureaplasma species is about 5 x 10^8 daltons which is the smallest genome size recorded for any self-replicating prokaryote (Askaa et al., 1973), being approximately one-half the size of the genome of Rickettsia and one-sixth the size of the genome of Escherichia coli (Myers et al., 1980). The DNA makes up 4-7% and the RNA makes up 8-17% of dry weight of the cell.

The RNA is composed of 22S, 16S, 5S (rRNA), 4S (tRNA) and an unstable mRNA species. The ribosomes are seen as cytoplasmic granules in electron micrographs and they function in protein synthesis. The plasmids are extra nuclear genetic materials indicated by the existence of Mycoplasma virus (Razin, 1978).

The replication of the mycoplasma genome resembles that of other prokaryotes in being semi-conservative (Razin, 1983a).

1.3.4 Replication.

The smallest mycoplasma cell capable of reproduction is about 0.3μm in diameter. Mycoplasmas are reported to be composed of minimal reproductive units varying in size from 125 to 250nm (Hayflick, 1965).

Several mechanisms of reproduction have been suggested. Freundt (1969) described a mode of production in which filaments formed from coccoid cells are transformed by beading and disintegration into single coccoid cells. The transformation of the filament to chains of cocci starts either on one end of the filament, spreading rapidly over it's entire length, or simultaneously at several points on the filament. The transformation is rapid and may be completed within 2.5 minutes. In most, and under certain conditions perhaps in all, mycoplasma cultures elongated or filamentous forms can be observed (Boatman et al., 1977).

Binary fission has been observed in mycoplasmas and it is essentially not different from that of other prokaryotes dividing by binary fission. For typical binary fission to occur, cytoplasmic division must be fully synchronized with genome replication, but in mycoplasmas cytoplasmic division may lag behind genome replication resulting

in the formation of multinucleate filaments (Brodt, 1968a; Razin, 1978).

1.3.5 Pathogenicity.

All members of the class mollicutes, apart from the thermoplasmas are parasites, and many are pathogens in animals, insects and plants. The pathogenicity and virulence of some mycoplasmas has been traced to many factors:

Toxic and Products of cell metabolism:

Hydrogen peroxide (H_2O_2), the end product of respiration in mycoplasmas, has been incriminated as a major pathogenic factor produced by mycoplasmas as it lyses erythrocytes. However, the production of H_2O_2 does not determine pathogenicity by itself as nonpathogenic *Acholeplasma laidlawii* produces it too. For the H_2O_2 to exert its toxic effect, the mycoplasmas must adhere close enough to the host cell surface to maintain a toxic, steady-state concentration of H_2O_2 sufficient to cause damage to cell membrane as in *M. pneumoniae* (Elliot and Birch, 1972; Brodt, 1976).

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Ammonia is another end product of mycoplasma metabolism which may play a role in pathogenicity when produced in large quantities, as during urea hydrolysis by ureaplasmas. Hydrolysis of arginine by mycoplasmas also yields ammonia as an end product. In this case, it is the depletion of the essential amino acids rather than the toxicity of ammonia which is to blame for the symptoms (Barile, 1973) in cell cultures contaminated by arginine - splitting mycoplasmas.

Toxins:

A true exotoxin with neurotoxic properties has been found only in cultures of Mycoplasma neurolyticum, the agent of "rolling disease" in mice. The toxin when injected intravenously into mice or rats causes swelling of the capillary endothelium and partial or total occlusion of the capillary lumen (Thomas and Bitensky, 1966).

Killed mycoplasma cells or fractions thereof can also be toxic to animals. Injection of large quantities of killed M. fermentans cells killed mice with symptoms resembling those of gram-negative endotoxemia. The nature of the toxic factors in M. fermentans is not clear (Razin, 1978).

Adherence to cell Surfaces:

Mycoplasmas rarely invade the blood stream and tissues. They adhere to and colonize the epithelial linings of the respiratory and urogenital tracts of infected animals and can thus be regarded as surface parasites. Attachment of mycoplasmas is firm enough to prevent their elimination by the action of the ciliated epithelium and by the urine. Mycoplasmas have been shown to adhere to erythrocytes, spermatozoa, macrophages, tracheal epithelial cells, Hela cells, fibroblasts in monolayer cultures, tracheal organ cultures and inert surfaces such as glass and plastic. (Collier, 1972; Engelhardt and Gabridge, 1977; Clyde, 1983).

Close attachment between M. pneumoniae and the neuraminic acid receptor sites on erythrocytes and tracheal epithelium leads to destruction of cell membrane and subsequent atypical pneumonia. This is achieved without them being attacked by the catalase present in the extracellular fluid. M. gallisepticum attaches tightly to RBC leading to their distortion in shape. It's presence in the respiratory epithelium may lead to ciliostatis, or loss of cilia, as well as damage to the cell membrane (Jordan, 1975; Power and Jordan, 1976; Razin et al., 1980).

1.3.6 HOST INFECTIONS WITH MYCOPLASMAS

As with other microorganism groups mycoplasmas may exist on mucous membranes as components of the autochthonous microflora, or colonization with virulent species may lead to induction of disease through some form of tissue injury. Human infants may be colonized transiently with ureaplasmas after birth, but they loose it and do not regain it until adolescence or adulthood, suggesting a role for anatomical and physiological modulation of susceptibility as well as the opportunity for acquisition by venereal transmission (Razin, 1978; Clyde, 1983).

Pathological consequences of mycoplasma infections result in most instances from extracellular surface parasitism, although some highly virulent species exhibit invasive properties. Those species that penetrate the mucosal barriers tend to exhibit a high degree of specificity for the tissues and organs that become infected for example M. mycoides for the lungs and pleura, M. arthritidis for the arterial walls (Razin, 1981; Clyde, 1983).

The effects of mycoplasmas on host tissues vary according to the type of organism and the organ system that is involved. Injury may be reflected by disturbances of specialized cellular function and histopathological changes, as

described for *M. pneumoniae* infection of respiratory epithelial cells (Collier, 1972). The neurotoxin elaborated by *M. neurolyticum* alters the permeability of cerebral capillary endothelial cells, producing the "rolling disease" in mice (Razin, 1978). Septicaemia with *M. gallisepticum* in turkey poults may be followed by selective infection of cerebral arterial wall, resulting in fibrinoid necrosis and an encephalopathy syndrome. An inflammatory toxin of *M. bovis* increases vascular permeability and activates complement (Clyde, 1983). Ureaplasma have been shown to compromise oviductal ciliary activity, probably through the production of ammonia from urea and reduce the motility of spermatozoa when attached to the cell membrane (Shepard *et al.*, 1974).

1.4 OBJECTIVES OF THIS WORK

(1) To isolate *M. pneumoniae* from pediatric patients with pneumonia.

(2) To confirm the isolates by growth inhibition test.

(3) To perform a complement fixation test on the serum samples of the pneumonia patients and to determine the antibody titre of *M. pneumoniae* in the patients.

CHAPTER TWO

2.1 HUMAN MYCOPLASMA

The human mycoplasmas consist of the following eight large colony forms and one serologically heterogenous group of "Tiny (T)-strain" mycoplasma (Purcell and Chanock, 1969):

M. pneumoniae	M. salivarium
M. hominis	M. fermentans
M. orale	M. lipophilum
M. buccale (M. orale 2)	Ureaplasma urealyticum
M. faucium (M. orale 3)	(T-Mycoplasma)

M. pneumoniae is isolated from the respiratory tract (Chanock et al., 1962; Loda, 1968; Hayflick, 1969; Westerberg, 1973). M. orale, M. buccale, M. faucium and M. salivarium are generally flora of the throat or buccal cavity (Organick and Worman, 1967). M. hominis has been isolated from the throat by Eldridge (1970) and serologically related to respiratory disease, but it's prevalence in upper respiratory illness is yet to be defined (Purcell and Chanock, 1969). U. urealyticum has also been isolated occasionally from the throat, but has caused no apparent ill effects. Sputum specimens, throat swabs, and tracheal aspirates are acceptable specimens for the isolation of the

respiratory mycoplasmas (Allen, 1967; Praznik, 1969; Purcell and Chanock, 1969).

The mycoplasmas isolated from the genitourinary tract include _M. fermentans_, _M. hominis_ and _U. urealyticum_. _M. fermentans_ is a rare isolate and probably part of the normal flora (Hayflick, 1969). _M. hominis_ and _U. urealyticum_ have been shown to be pathogenic in genitourinary disease (Mardh and Westrom, 1970; Caspi, 1971, 1972; Friberg and Gnarpe, 1973; Adegboye _et al._, 1979). Upper female reproductive tract specimens, blood, vaginal swabs, cervical swabs and urine are suitable for isolating genital mycoplasmas (Praznik, 1969).

2.2 NUTRITION OF MYCOPLASMAS

Mycoplasmas are fastidious in their cultural requirements, they are nutritionally exacting and need to be supplied with a large array of precursors for the synthesis of macromolecules. The media described usually contain peptone, beef heart infusion, DNA to supply nucleic acid precursors, yeast autolysate together with aqueous yeast extract to supply labile and other growth factors, animal serum as a nontoxic source of lipid and sodium chloride or a nonionic solute to adjust the tonicity (Edward, 1947). It is known that many mycoplasmas exist in nature which

cannot be grown in media presently available; growth of those that can be cultivated is often poor or may fail completely in subculture after primary isolation. A rapid decline in viability may be due to the accumulation of toxic metabolic products, to an excessive change in pH of the culture, or to the depletion of a lipid precursor required for membrane synthesis (Rodwell, 1983).

2.2.1 Ionic Composition of Media

Mycoplasmas grow best with an osmotic pressure range of 10 - 14 atmospheres. Sodium chloride is included in most media to provide required tonicity (Leach, 1962).

2.2.2 Energy Source

Mycoplasmas have an inefficient energy-yielding metabolism and so consume large amounts of substrate to maintain an adequate supply of energy for macromolecule synthesis. Addition of 0.5%(w/v) glucose to media is usually sufficient to allow maximum growth of fermentative species. Strongly growing fermentative species produce large amounts of acid from glucose fermentation and the medium must be well buffered. Growth ceases and the cells die rapidly once the pH falls below 6.5. Sodium

phosphate is a suitable inexpensive buffer (Hayflick, 1965; Rodwell, 1983).

2.2.3 Aeration of Cultures

Growth of some mycoplasmas (e.g. M. mycoides sub species mycoides small colony type, M. pneumoniae) is improved by gentle aeration of cultures, whereas aeration has no effect on growth of other fermentative species (e.g. M. mycoides subspecies mycoides large colony type, A. laidlawii). For growth on solid media, an atmosphere of 5% CO_2 in nitrogen may be best, whereas other mycoplasmas may require strict anaerobiosis (Rodwell, 1983).

2.2.4 Serum Requirement

All species of Mycoplasmas, Spiroplasmas and Uroaplasma require sterol for growth as all are incapable of synthesizing long-chain fatty acids. Mycoplasmas incorporate complex lipids (cholesterol, esters, phospholipids) into their plasma membrane when available (Rodwell and Abbot, 1961). It is possible that some species have a growth requirement for lipids which they are unable to synthesize, thus the lipid composition of the membrane can be altered within wide limits by the lipids supplied for growth. In most media lipids are usually supplied

by animal sera. Serum is usually added at a concentration of 10-20%. Horse serum has been most frequently used in mycoplasma media, although bovine, swine and turkey sera have been used under special circumstances (Razin, 1978; Rodwell, 1983).

2.3 Mycoplasma pneumoniae

M. pneumoniae is regarded as the etiological agent of the so called "cold-agglutinin-positive primary atypical pneumonia", a disease entity of an acute infection of the respiratory tract with atypical pneumonia. The name "Primary Atypical Pneumonia" was originally given by Reimann in 1938. Eaton et al (1944; 1945) isolated the organism and reported it's transmission to chick embryos, cotton rats and hamsters. Liu (1957) visualised the antigen of the infectious particle at the periphery of the epithelial cells lining the mesobronchi. Chanock et al (1962) grew the organism on a cell free artificial agar medium and identified the colonies obtained as PPLO or Mycoplasma. A year later the organism was named Mycoplasma pneumoniae, suggesting it's relationship to pneumonia (Chanock et al., 1963a).

The colonies of M. pneumoniae are homogenously granular and are partially embedded in the agar. They rarely have a "fried-egg" appearance of the mycoplasmas but this peripheral halo can develop

on plates with sparsely populated colonies or on swine serum agar (Chanock *et al.*, 1962). When stained with Dienes stain the colonies appear to contain many blue densely stained small granules which decolorize after a few hours because of their capacity to reduce the methylene blue component of Dienes stain (Hayflick, 1969).

Except for *M. fermentans*, *M. pneumoniae* is the only member of the human mycoplasmas that ferments glucose. Other sugars fermented are xylose, mannose, maltose, dextran and starch (Purcell and Chanock, 1969).

Razin *et al* (1970) have shown that membrane glycolipids are the major antigenic determinants responsible for the production of metabolism and growth inhibiting, complement-fixing and agglutinating antibodies, whereas membrane proteins appear to be primarily involved in the attachment of *M. pneumoniae* to host cells (Powell *et al.*, 1976).

M. pneumoniae grows as filaments, some of which show a specialized terminal "tip" (Biberfeld and Biberfeld, 1970). The tip consist of a dense rod, surrounded by an electron-lucent system, ending with a plate-like thickening. Bredt (1968b) showed that *M. pneumoniae* displays a gliding motility on inert surfaces with the tip as the leading part.

Chanock et al (1963b) described M. pneumoniae as an organism which grows on cell free agar medium containing 10% yeast extract and 20% horse serum. It produces soluble hemolysin for human, guinea-pig and horse erythrocytes and can grow on bronchial epithelium of chick embryo and in tissue cultures of chick embryo, guinea pig, monkey and human origin. Cytopathic effect is not evident in chick embryo in vivo but can occur in infected monkey or human tissue cultures. The organism can also produce pneumonitis in cotton rats and hamsters. The size of the organism has been estimated to be 180–250 mμ and it's growth can be inhibited by streptomycin, aureomycin, declomysin and some tetracyclines.

2.4 INFECTIONS OF Mycoplasma pneumoniae

Reports by Foy et al (1970) and Foy (1976) have shown that M. pneumoniae infections are endemic in densely populated areas; and cyclic increases occur at long intervals resulting in prolonged epidemics every 3 to 4 years. Recent report by White et al (1981) confirms that any age may be affected, however Foy et al (1970) showed that pneumonia caused by M. pneumoniae is rare in persons ≥40 years of age. Epidemics also occur in military recruits and college populations (Evans et al., 1967; Mogabgab, 1968; Steinberg et al., 1969). Immunity after infection is not

always long lasting and infections can occur in those with pre-existing antibody (Foy *et al.*, 1977).

2.4.1 Respiratory Tract Infections:

The sites of involvement in the respiratory tract during *M. pneumoniae* infections are the nasopharynx, throat, trachea, bronchus, bronchiole and alveolus (Denny *et al.*, 1971). The severity of infection varies from an acute mild respiratory infection to severe pneumonia, formerly called "cold-agglutinin-positive primary atypical pneumonia". This is because the X-ray findings differed from those caused by bacteria and in addition, Penicillin did not have any therapeutic effect (Janson, 1978). *M. pneumoniae* is recognised as a significant cause of lower respiratory tract infections accounting for 10% (Chanock, 1965) to 15 - 35% (Foy *et al.*, 1970; 1979) of such infections in the American civilian populations. *M. pneumoniae* has a wide geographical distribution (Chanock *et al.*, 1967) causing 28% of atypical pneumonias in Tokyo (Kitamoto *et al.*, 1966), 17% of lobar pneumonias in Nigeria (MacFarlane *et al.*, 1979) and 15% of atypical pneumonias in India (Gupta *et al.*, 1975). It is also common among the Chinese (Tai and

Wei, 1976) and has been reported from South Africa (Joosting et al., 1975) and Egypt (Hassan et al., 1972).

Clinical Symptoms

Approximately 1 in 30 of those infected with M. pneumoniae develop a clinically apparent pneumonia (Chanock et al., 1961; Clyde, 1979). Although, second attacks of M. pneumoniae pneumonia are rare, they have still been seen and their incidence is 1.9 cases/1000 previously infected persons (Burman and Lofgren, 1979). The incubation period of the disease as reported by Foy et al (1966) is probably between 2 to 3 weeks. The onset of clinical illness is gradual. No symptoms or signs are diagnostic of the infection, but headache and malaise, fever and cough are the predominant findings; sore throat frequently occurs (Hers, 1968). Usually, the severity of symptoms is greater than the physical signs that occur later in the disease. Rales are the most prominent signs, but dullness to percussion and production of sputum occur frequently, these usually clear before the patient feels well. As with the other aspects of clinical infection, there is nothing diagnostic about the X-ray findings, but involvement is usually in one of the lower lobes; the pneumonia is described as interstitial or broncho pneumonic (Denny et al., 1971).

M. pneumoniae has been associated with non-pneumonic respiratory infections like bronchitis, laryngitis and upper-respiratory tract infections; which, like pneumonia, show no diagnostic clinical characteristics to allow their easy identification (Denny *et al.*, 1971).

2.4.2 Auditory Infections:

In the auditory system, serous otitis media, otitis externa and myringitis bullosa may occur (Rifkind *et al.*, 1962; Foy *et al.*, 1970). Reports on sensori-neural hearing impairment are scarce. The clinical picture is that of sudden deafness and tinnitus but no decay. The hearing loss may be temporary or permanent (Van Dishoeck, 1963; Rowson *et al.*, 1975; Shanon *et al.*, 1982).

2.4.3 Skin Infections:

The skin lesions include maculopapular rashes, erythema nodosum and Steven-Johnson syndrone (SJ-syndrome) (Katz *et al.*, 1967; Lyell *et al.*, 1967; Sieber *et al.*, 1967). The association of *M. pneumoniae* with the SJ syndrome has been made in many instances and the organism has been isolated directly from the bullous lesions (Lyell *et al.*, 1967).

The SJ syndrome was associated with *M. pneumoniae* when in 1964 Ludlam *et al*

reported finding substantial positive complement fixation titres for *M. pneumoniae* in the sera of patients with SJ syndrome. The SJ syndrome or erythema multiforme major is a multisystem inflammatory disorder associated with a widespread erythematous eruption that can result in death. Although, usually considered to be a pediatric disease, it frequently affects adults (Southeimer *et al.*, 1978). The prevalence of SJ syndrome has been estimated to be from 1.2 to 4.2% (Toma *et al.*, 1976; Lind, 1978; Ali *et al.*, 1986).

2.4.4 Central Nervous System Infections:

Central nervous system (CNS) involvement is the most common and significant complication of *M. pneumoniae* infection and includes manifestations such as meningitis, meningo encephalitis, polyradiculo neuropathy, psychosis and cerebellal syndrome (Hers, 1968). *M. pneumoniae* infection with neurological symptoms may occur with or without clinically diagnosed respiratory tract infection (Dorff and Lind, 1976).

The risk of CNS complication is estimated to be 1 to 7% and the maximum incidence occurs among the younger age group (Sterner and Biberfeld, 1969; Ali *et al.*, 1986). Despite such alarming initial symptoms as unconsciousness,

convulsions and paresis, complete recovery is the usual outcome (Urquhart, 1979). Whereas Burman and Lofgren (1979) showed that a relatively mild mycoplasma pneumonia may become complicated by alarming CNS disorder that could cause death unless subjected to intensive care. The mechanism of CNS involvement in patients with respiratory tract infection due to M. pneumoniae is not known as no neurotoxin has been found to be produced by the organism and neither has it been isolated from the cerebro-spinal fluid.

Mycoplasma encephalitis may be focal and very difficult to distinguish from cerebral haemorrhage or abscess by radiological investigation thus necessitating neurosurgical intervention (Murray et al., 1975). Recurrent mycoplasma encephalitis has been reported by Burman and Lofgren (1979). Fatal cases of meningo encephalitis associated with M. pneumoniae have also been reported by Murray et al (1975).

2.4.5 Blood Infections:

Haemolytic anaemia is the most common extra-pulmonary manifestation observed in M. pneumoniae infections and it usually conincides with recovery from the respiratory

tract infection (Ali et al., 1986). Increase in cold-agglutinin titre that is IgM antibodies directed against antigens present on the red blood cells surface is often present in approximately half of the patients with mycoplasma pneumonia (Murrary et al., 1975). Hemolysis in patients with mycoplasma pneumonia occurs 2-3 weeks after the onset of illness, in several instances it has been reported to follow a cold feeling felt by the patient (Lindstrom and StahlFurenhed, 1981). M. pneumoniae is uniquely capable of inducing a change in the erythrocyte membrane since haemadsorption of human erythrocytes to colonies of M. pneumoniae takes place. This haemadsorption facilitates the action on the erythrocyte membrane of a soluble hemolysin, apparently a peroxide produced by M. pneumoniae. Peroxide toxicity has been amply documented (Chanock et al., 1963b; Razin, 1978; Deas et al., 1979) and peroxide produced by M. pneumoniae could affect red blood cells by altering their biological activities, osmotic fragility and antigenicity. As a rule the action of peroxides is rapidly inhibited by catalase but the haemadsorption could prevent the action of these enzymes to make possible the action of the peroxide on the cell membrane (Lindstrom and Stahl-Furenhed, 1981).

2.4.6 Heart Infections:

Cardiac disease occurs in 4.5% of patients with M. pneumoniae infections (Ponka, 1979a). Myocarditis, pericarditis and transmission abnormalities have all been described (Levine and Lerner, 1978; Pickens and Catteral, 1978; Ponka, 1979b). Friedli et al (1977) described a complete heart block in a child, presumably due to M. pneumoniae. Carditis is often associated with severe haemolytic anaemia (Ali et al., 1986).

2.4.7 Joint Infections:

Three patterns of joint disease have been described in M. pnuemoniae infections. The first is transient and coincides with the acute illness, it is polyarticular, migratory and affects large joints (Urquhart, 1979). The second is a polyarthropathy of medium sized joints associated with morning stiffness, redness and swelling; it follows the acute illness and may persist upto a year (Hernanadez et al., 1977). Thirdly, M. pneumoniae is the recognised cause of septic arthritis in hypogammaglobulinaemic patients (Taylor-Robinson et al., 1978).

Generalized lymphadenopathy in M. pneumoniae infection has been reported but is rare (Leach

and Lewis, 1969; Ali et al., 1986); cervical lymphadenopathy occurs more frequently and has been reported in upto 40% of affected children (Clyde and Denny, 1967). Murray et al (1975) first reported hepatitis caused by M. pneumoniae, it's incidence is not known although, it appears to be rare. Disseminated intravascular coagulopathy (Pickens and Catteral, 1978) and acute glomerulonephritis (Cassell and Cole, 1981) have also been rarely associated with M. pneumoniae.

2.5 Suggested Mechanisms Associating M. pneumoniae Infection and Extra Pulmonary Disease:

2.5.1 Autoantibodies:

Autoantibodies are produced during mycoplasma pneumonia and most evidence suggests that they are stimulated by antigens cross-reacting with host tissues, such as the I-antigen on erythrocytes and glycolipids in the brain (Biberfeld, 1971). Their common occurence with uncomplicated M. pneumoniae pneumonia would suggest that they are not pathogenic for the host in most cases. The mechanism by which antibodies to host antigens are formed is not clear, but infectious agents may modify host tissues and induce autoantibodies (Fernald, 1983). The normal T-lymphocyte suppression of self immunity can be

bypassed by cross reacting antigens, alteration of host cell antigen by the infecting organism and non-specific activation of lymphocytes by microbialmitogens (Niklasson and Williams, 1974). While the presence of autoantibodies is obvious, direct evidence of antibody - mediated injury like immune complex disease has not been described.

2.5.2 Immuno Suppression:

Advances in defining the immunologic interactions between mycoplasmas and the immune system suggest organism-induced immunosupression as a mechanism linking extra pulmonary complications with the primary respiratory disease (Sabato et al., 1981). Biberfeld's and Fiala's observations of anergy to tuberculin skin testing and depressed lymphocyte responses to M. pneumoniae antigen during convalescence from acute pneumonia suggest that it is a regular feature of M. pneumoniae infections (Biberfeld and Sterner, 1971; Fiala et al., 1974).

Such altered immune responsiveness during mycoplasma infections could be the permissive factor for several associated phenomena like the escape of organisms from lung tissues to systemic sites, the generation of autoantibodies and the reported prolonged carriage of the

organism in affected tissues which could all be facilitated by a state of anergy (Fernald, 1983).

2.6 Communicability of M. pneumoniae:

Contrary to common belief, M. pneumoniae is rather sturdy and can withstand a variety of conditions, it can survive in aerosols at most relative humidity levels (Denny et al., 1971). It would appear that M. pneumoniae is not highly contagious, as it takes several days (14-26) for it to spread among members of a family as demonstrated by Foy et al (1966), or among military recruits as shown by Steinberg and his co-workers (1969). Although the organism does not spread rapidly, a very high percentage of susceptible contact, 84-95% in children and 41-65% in adults (Foy et al, 1966; Balassanian and Robins, 1967) may become infected eventually, Steinberg et al (1969) demonstrated that the platoon was the basic epidemiologic unit for M. pneumoniae infection, and that the risk of infection with this organism is greater in more congested areas within the barracks. It has therefore been hypothesized that the infections are contracted principally by droplet spread through close contact (Foy et al., 1966; Balassanian and Robins, 1967). The prolonged carriage of M. pneumoniae in the infected host

may be important in the epidemics caused by the organism (Denny *et al.*, 1971).

2.7 Recovery of Mycoplasmas:

The successful recovery of mycoplasmas from clinical specimens is dependent on a number of factors, including those host-dependent components that may condition recovery and a variety of cultural or technical factors that may either enhance or limit primary isolation of these organisms. Some of these factors, as listed by Tully (1983a) are as follows: host factors which include, antibiotics or other drugs in tissue or body fluids; presence of enzymes or other inhibitors in ground tissues; low levels of organisms in the tissues selected; and antibody in host tissues or fluids. The cultural factors include, poor quality of growth medium due to batch variability; poor choice of culture medium supplements, pH, atmospheric conditions, and temperature, inhibitory activities of thallium acetate or antibiotics in medium; mycoplasma strain sensitivity to growth medium components like yeast extract or serum; compe ting microbial flora, including other mycoplasmas; latent mycoplasmas that may occur in culture procedures employed (animal host, cell cultures, serum, chicken embryo, etc.) for primary isolation. These factors must be considered in the

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development of proper cultural techniques for mycoplasma recovery.

2.8 Control of Infections due to *M. pneumoniae*

2.8.1 Effect of Antibiotics:

The use of antibiotics is often desirable. Penicillin and it's derivatives are not effective because *M. pneumoniae* has no cell wall the site of action of this antibiotic. *M. pneumoniae* is sensitive to tetracyclines and erythromycin. When given in daily doses of 2g they may shorten the length of the febrile illness and reduce the symptoms. Their effect is, however, not dramatic and radiological clearing of the lungs is influenced to a lesser extent, although erythromycin is approximately 50 times more active than tetracycline *in-vitro* (Denny *et al.*, 1971). The effectiveness is often, therefore, very difficult to establish and is influenced by age and pre-existing disease. Previously healthy young people treated early in the disease react more promptly. A well-developed pneumonia requires longer treatment, e.g. ten to fourteen days, than a simple minor upper respiratory syndrome or bronchitis (Hers, 1968).

2.8.2 Vaccines:

Mycoplasma pneumoniae accounts for upto 35% of all pneumonias at times when influenza epidemics are not prevalent (Foy et al., 1979). Considerable efforts have been directed towards the development of a vaccine against mycoplasmal respiratory tract disease of man. The protective efficacy of inactivated Mycoplasma pneumoniae vaccine, prepared so far, does not exceed 67% (Barile et al., 1981; Brunner, 1981a). Denny et al (1971) reported that the inactivated vaccines are generally poor antigens which afford little protection, and also there is a possibility that they might sensitize the vaccinees.

Experimentally, a live attenuated vaccine has been prepared which gives a distinct but incomplete protection against M. pneumoniae infection in hamsters (Fernald and Clyde, 1970). However, it has been shown by Brunner (1981a) that the attenuation is not sufficient for man.

It has been hypothesized that the lung histopathology after Mycoplasma pneumoniae infection in man may be, in part, immunlogically mediated. Therefore, it must be made certain that the M. pneumoniae vaccine to be administered to humans produces a protective and not a pathological immune reaction (Brunner, 1981a).

2.9 Specimen Collection and Transport:

Throat or nasopharyngeal swabs, tissues from lung or brain, and spinal, pleural or pericardial fluids can be placed immediately in liquid and solid mycoplasma media, or in a suitable transport medium. Conventional mycoplasma broth, containing 10% fresh yeast extract, 20% horse serum, and 500 to 1,000 units/ml of penicillin G is generally an effective transport medium. If the specimen cannot be cultured within 24 hours of collection, it should be frozen (-70°C) until appropriate culture materials and equipment are available (Tully, 1981).

2.10 Cultural Techniques:

Broth Cultures:

Primary isolations are usually made by breaking swabs into broth in sterile test tubes. A serial tenfold dilution of the specimen is made upto 10^{-4} or 10^{-5}, because at low dilutions bacterial contaminants can overgrow the cultures (Adegboye, 1981; Jordan, 1983; Tully, 1983a). The airtight sealed tubes are incubated at 37°C. Usually, all three types of broth; glucose, arginine and urea; are inoculated, unless a specific mycoplasma is being sought.

Glucose broth is observed daily for colour change of phenol red indicator from pink (pH 7.6) to yellowish (pH 7.0) which indicates growth (Robert et al., 1967). Incubation period for the pH change can vary from a few hours to several days, depending on the mycoplasma specie (Adegboye, 1981). Cultures are then used for inoculating solid media.

Solid Media Cultures:

Routinely appropriate agar plates are inoculated and incubated at 37°C in glass anaerobic culture jars in a CO_2 enriched atmosphere provided by 1NHCl added to marble chips in a beaker; or aerobically in a bell jar. Humidity is provided in the atmosphere by inclusion of cotton wool soaked in water (Adegboye, 1981; Jordan, 1983).

Maximum recovery of mycoplasmas is by direct plating onto agar (Amin and Jordan, 1978) but initial passage into broth is necessary to reduce bacterial contamination (Tully, 1983a). It is necessary to incubate and keep cultures on solid medium for 20 to 30 days before discarding as negative, as field strains are occasionally not seen until after 15-20 days of inoculation (Jordan, 1983).

2.11 Identification of Isolates:

2.11.1 Purification:

Crawford and Kraybill (1967) reported the isolation of more than one species from a particular site in various hosts including man. The problem of mixed cultures and over-growth of less fastidious organisms may be tackled by cloning (Anon, 1979) filteration through membrane filters (220-450nm) (Tully, 1983b), immunofluorescence staining of mixed growth on agar (Barile and DelGiudice, 1972; Jordan and Amin, 1980) and by a combination of growth inhibition and immunofluorescence techniques (Bradbury and MacClenaghan, 1982).

Cloning as a purification procedure involves picking of well isolated colonies and subculturing three times from terminal dilution plates. The cultures can be hard to purify by cloning when more than one species is present within a colony (Fabricant and Freundt, 1967).

In filtration, as recommended by Tully (1983b) with a sterile syringe and needle, a few mls of the test broth culture is used. The needle is replaced with a membrane filter (220-450nm) on the syringe, gentle pressure is applied and the broth passes through the filter into a sterile tube. Serial dilution

of the filtrate is made and the capped tubes are incubated and observed for colour change.

For fluorescent antibody technique specific mycoplasma antisera are prepared in rabbits (Morton and Roberts, 1967) and the gamma globulin fraction of the serum is usually conjugated with fluorescein isothiocyanate (FITC) (Niarn, 1969). The FITC conjugated antiserum is then used to stain the mycoplasma colony impressions directly or agar blocks of cultures (Clarke et al., 1961).

2.11.2 Biochemical Characterization:

The mycoplasmas can be broken into three groups on the basis of the substrates they use. The groups are:

(1) Glucose metabolizers (*M. pneumoniae* and *M. fermentans*).

(2) Arginine metabolizers (*M. orale*, *M. salivarium* and *M. hominis*).

(3) Urea hydrolyzers (*U. urealyticum*). (Purcell and Chanock, 1969; Shepard and Lunceford, 1970).

Other optional tests are phosphatase activity, tetrazolium reduction, serum digestion (Alluotto et al., 1970, Williams and Wittler, 1971) and esculin hydrolysis (Williams and Wittler, 1971; Rose and Tully, 1983).

The metabolic end product of glucose is lactic acid, which changes the medium from the original optimal pH of 7.6. A phenol red indicator is included in the medium to demonstrate pH change and a drop in pH indicates mycoplasma growth (Alluotto *et al.*, 1970). The growth medium containing horse serum and yeast extract may also show a slight fall in pH even in uninoculated media after several days of incubation. Also, non-fermentative organisms may produce small amounts of acidic metabolic products from constituents other than sugars, leading to a subsequent drop in pH (Razin and Grillo, 1983).

The arginine metabolizers metabolize arginine with the subsequent release of ammonia into the broth; this results in an alkaline shift of the medium which is demonstrated by a colour change in the phenol red indicator from salmon to red. Colour changes do not always occur in the diphasic medium and so it has to be subcultured onto agar at intervals (Purcell and Chanock, 1969).

The urea hydrolyzers use the urea and release ammonia into the broth (Shepard and Lunceford, 1970).

2.11.3 Biologic Identification:

M. pneumoniae can be distinguished from other human mycoplasmas by hemolysis (Clyde, 1964; Cole, 1968) and hemadsorption (Manchee and Taylor-Robinson, 1968; Purcell and Chanock, 1969) reactions. Both tests require that the isolate be grown on complete mycoplasma agar and that the resulting young colonies be used for testing.

The beta-hemolysis test is performed by overlaying a plate containing colonies with a 4% suspension of guinea pig or sheep erythrocytes in agar. This agar overlay is allowed to solidify and the plates are incubated aerobically at 37°C. The plates are observed for hemolytic plaques at 24-48 hours. M. pneumoniae induces a zone of clear (beta) hemolysis around its colonies. The organism produces a peroxide which diffuses into the zone surrounding the colony causing the erythrocytes to lyse. The hemolysin is produced only in an atmosphere containing sufficient oxygen. Other human mycoplasmas can also cause hemolysis, but the plaques are smaller and take longer to develop and usually demonstrate incomplete or alpha hemolysis.

The haemadsorption test is performed by flooding the positive plate with a 0.5% suspension of guinea pig erythrocytes. The cells are incubated in contact with the colonies for 30 minutes and then the plate is washed with phosphate buffered saline (PBS). The colonies are then observed under a microscope (X 100). M. pneumoniae is the only large-colony human mycoplasma that adsorbs the guinea pig erythrocytes to its colony surface.

2.11.4 Serological Characterization:

Mycoplasma isolates can be identified into species by a number of different serologic test. The tests recommended are:

(1) Growth inhibition (Clyde, 1964; Purcell and Chanock, 1969).

(2) Metabolic Inhibition (Purcell, 1966, Taylor-Robinson, 1966; Purcell and Chanock, 1969).

(3) Immunofluorescence (Purcell and Chanock, 1969; Baas and Jasper, 1972; Lehmkuhl and Frey, 1974).

(4) Complement fixation (Biberfeld, 1968; Purcell and Chanock, 1969).

1. Growth-Inhibition Test:

Edward and Fitzgerald (1954) first described the use of the colony-inhibition test. Henderchee et al (1963) demonstrated that filter paper disc saturated with specific antiserum and placed on agar previously inoculated with a homologous species, lead to zones of growth inhibition around the disc. The depth of the agar medium influences the number and size of mycoplasma colonies which in turn influences the growth inhibition test (Clyde, 1964). It has also been observed that new mycoplasma isolates are less susceptible to growth inhibition till after several subcultures (Frey and Hanson, 1969).

2. Metabolic Inhibition Test:

The test takes advantage of the fact that when mycoplasmas utilize their substrate medium they give off a by-product (Taylor-Robinson et al., 1966) that changes the pH of the medium. A specific antiserum against a mycoplasma species will inhibit it's metabolic activity and the subsequent release of lactic acid, in glucose fermentors, or ammonia, in arginine utilizers. The specific antigen-antibody reaction therefore prevents mycoplasma from growing, and the lack of metabolic activity is demonstrated by the failure of the pH of the medium to change.

Metabolic inhibition test has been used successfully under controlled conditions for the serotypic characterization of the mycoplasmas (Purcell and Chanock, 1969).

3. Immunofluorescence Test:

This is a rapid method for the species identification of the mycoplasma colonies on agar. The test is both sensitive and specific and has an advantage over the other tests that it allows identification of mixed cultures without the isolates having to be cloned (Del Giuidice et al., 1967).

The test is based on the principle that when the gamma globulin fraction of a specific antiserum is labelled with fluorescein dye, the properties of the labelled antibody are essentially unchanged. When this antibody is placed in contact with it's homologous antigen, an antigen-antibody reaction occurs which can be visualized with proper illumination under a fluorescence microscope. A yellow-green fluorescence of the colony is indicative of a homologous reaction.

4. Complement Fixation Test:

This is not used for serologic characterization as it requires mycoplasma broth cultures

to be concentrated. It is mostly used for the serologic testing for the detection of *M. pneumoniae* antibodies in the patient's serum. Serologic tests are more sensitive indicators of mycoplasma infection than recovery of the organism, as in many instances the antibody response can be detected when the organism cannot be recovered. Isolation of mycoplasmas in the absence of a serologic response test does not necessarily mean that the organism is associated with the disease. Serologic testing, then is imperative to good laboratory diagnosis of *M. pneumoniae*. A fourfold rise in antibody titre to a mycoplasma species is suggestive of recent infection with the organism.

CHAPTER THREE

MATERIALS AND METHODS

Laboratory diagnosis of *Mycoplasma pneumoniae* infections rests primarily on an adequate serological analysis of the patient's serum and on the isolation and identification of the organism from secretions of the respiratory tract or other appropriate sites (Tully, 1981).

3.1 Culture Media

The culture technique and medium was according to Adegboye (1977, 1981) which were adapted from Chanock *et al* (1962) with minor modifications.

Constituents

(1) Basal medium

Bacto PPLO broth without crystal violet (Difco Laboratories, Detroit, Michigan) was used for broth medium. Bacto PPLO agar (Difco Laboratories, Detroit, Michigan) was used for agar plates.

(2) Horse Serum

Two horses; at the Veterinary Medicine Department, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria;

were bled aseptically from time to time. Serum from the horses had earlier been shown to be able to support the growth of different mycoplasmas. The blood was allowed to clot and kept at +4°C overnight. Serum was aseptically seperated and the pooled sera was seitzfilter sterilized and frozen at -20°C.

(3) Glucose

A 50% (w/v) stock solution was prepared using Glucose (Difco Laboratories, Detroit, Michigan). This was dispensed in 10ml amounts into universal bottles, autoclaved at 121°C, 15lb per sq. in. pressure for 15 minutes and then stored at +4°C.

(4) Yeast Extract

Active baker's yeast measuring 250gm (Engedura Bakers Yeast, Holland) was sprinkled on the surface of 750mls of distilled deionized water in a 2 litre beaker. The mixture was heated gently to boiling for 15 minutes and frozen at -20°C when cool. The mixture was thawed and then clarified by centrifugating at 1,000 x g for 30 minutes, twice. The supernatant drawn was sterilized by seitzfilter and this was used as yeast extract.

(5) Phenol Rod:

Phonol red measuring tgm (Dif ce Laboratories, Detroit, Miohlgcn) was disWve&.-a in Wtol of distilled deionizod water to make a 1$ stock solution* The 1$ stock solution was dispensed in 10ml aliquots and stored at +4°C.

(6) Ponicillin:

Benzyl penicillin sodium {crystapon 600m<2^ Glaxo Laboratories Limited, Groonford, England) was rocons-fcitutod with 1.0ml -sterile wator to • givo 1 x 10 units per ml. This was stored at +4°C until use.

(7) Thallium .Acetates

A 1C$ stock solution was prepared by disso3^ving I.0gm thallium acotato (Fishor Scientific Co-, Now Jorsey) in distilled deionizod water to make a final volume of 10mls. This was. storilizocL by autoclavin^: and disponsod in 2ml aliquots and stored at +4°C.

Both the penicillin and thallium a.cetato were used as bacterial inhibitors.

3.2.1 Preparation of Media:

A 250ml broth and a 250ml of agar modia woro prepared at a, time, as follows:

Broth Medium:

To 175ml of distilled deionized water in a sterile conical flask, 5.25gm of the PPLO broth powder was added. The mixture was stirred and heated to boiling using a hot plate (Gallenkamp and Co. Ltd., London). To the broth solution 0.5ml of the 1% phenol red solution was added and the pH adjusted to 7.6 with 1N Hydrochloric acid or 1N sodium hydroxide. The broth was then autoclaved at 121°C, 15 lb per sq. in. pressure for 15 minutes. It was then transfered to a water bath set at 40°C where the other components were added aseptically in appropriate amounts as follows:

(1)	Horse serum	50.0ml
(2)	Glucose (50% solution)	2.5ml
(3)	Yeast Extract	25.0ml
(4)	Benzyl Penicillin 10^6u/ml	0.25ml
(5)	Thallium acetate (10% solution)	0.5ml

Agar Medium:

A 170ml of distilled deionized water was used to dissolve 8.5gm of PPLO agar base (Difco Laboratories, Detroit, Michigan). After the pH adjustment and autoclaving, other materials (items 1 to 5 above) were added aseptically. The molten agar at approximately 40°C was dispensed into sterile 90mm plastic petridishes (Sterilin Ltd., Teddington, Middlesex, England) and allowed to solidify. The plates were stored at +4°C and can be used for 2 weeks.

3.1.2 Specimens:

Throat swabs and 5mls of blood were taken from 104 patients with clinically diagnosed pneumonia in the Paediatric Unit of the Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. A convalescent sample of the blood was taken 2 weeks after the first or acute sample.

The blood was drawn by a sterile syringe and needle (21G) and allowed to clot in a sterile universal bottle which was stored later at +4°C overnight. The serum was obtained by centrifuging at 1,000 rpm for 30 minutes and stored in a sterile bijoux bottle at -20°C for further use.

The throat swabs were taken with a sterile cotton swab and this was snapped into a sterile screw capped test tube containing 2ml of PPLO broth medium. The tube was capped and stored at -70°C until further use.

Throat swabs and 5mls of blood were also taken from 52 children of the same age group with no clinical evidence of respiratory tract infection to serve as controls.

3.1.3 Cultural Procedure:

In Broth:

Each swab was snapped off into a screw capped test tube containing 2ml of broth.

The tubes were frozen at $-70^{\circ}C$ usually for not more than two weeks. The frozen specimens were thawed at room temperature, mixed thoroughly and 0.2ml of each specimen was transfered into a fresh 1.8ml of broth. This was diluted serially in tenfolds, down to 10^{-3}. The tubes were screwed and incubated at $37^{\circ}C$. Cultures were examined every 24 hours for colour change from pinkish (pH 7.6) to pinkish yellow (pH 7.2 to 7.0). Tubes with good homogenous colour change (that is without precipitate indicative of bacterial contamination) were checked for colonies. After 7 days, cultures that failed to show colour change were passaged into fresh broth and incubated again for 7 days. Broth cultures with appropriate colour change but with heavy precipitate indicative of bacterial growth were filtered through 450nm millipore membrane using 25mm filter holders and then subcultured. Alternatively, the contaminated broth cultures were re-diluted serially in tenfolds, down to 10^{-3} and reincubated.

<u>On Agar:</u>

To check for colonies, 0.1ml of each broth culture with appropriate colour change was diluted serially to 10^{-3}. A drop of each dilution and the undiluted culture was inoculated onto a quadrant of an agar plate divided into four quadrants using a marker.

Broth cultures with no apparent colour change after approximately 14 days of incubation were also blind passaged by inoculating a drop of the undiluted culture onto agar plates. The plates were incubated in a wet chamber at 37°C and were examined every 48 hours for mycoplasma-like colonies using a stereomicroscope (Olympus optical Co., Japan) at a magnification of X40 to X80. Plates were incubated for 25 to 30 days before being considered negative for mycoplasma.

3.1.4 Characterization:

The various steps used in characterization were based on standard methods earlier described (Tully, 1983b), with minor modifications. They were as follows:

Purification of Isolates:

Mycoplasma-like colonies on any plate were purified by cloning. A single representative colony was picked using the bevel of a sterile hypodermic needle mounted on a syringe. This was inoculated into 0.2ml of broth, which was then diluted down to 10^{-3}. The cloned culture was incubated and subcultured onto agar medium after approximately 3 to 4 days. If colonies were recovered the cloning process was repeated

twice more in those cases where the orignal plates had some bacterial contaminants. However, if the original plate yielded pure mycoplasma-like colonies and care was taken to remove only one well isolated representative colony from the plate, cloning was done only once.

Reversion Studies:

Each cloned mycoplasma-like isolates was plated on mycoplasma agar devoid of penicillin and thallium acetate, incubated at 37°C, and examined daily for 7 days. The mycoplasma colonies were examined for changes in typical morphology, if any. Blood agar plates were also plated with broth culture of each cloned organism and examined for bacterial colonies, if any.

Biological Test: Beta haemolysis

An aliquot of saline agar was melted by heating to 100°C and cooling to 45°C and an amount of guinea pig blood in Alsever's solution was added to make a final cell concentration of 5%. A sufficient amount of the melted blood agar was poured over the positive mycoplasma culture to form a thin overlay. The overlay was allowed to solidify and incubated at 37°C aerobically for 24 to 48 hours or till the haemolytic plaques were visible. Small areas of clear haemolysis were seen around colonies of *M. pneumoniae* (Clyde, 1963).

complement is bound in the antigen-antibody complex; if absent free complement remains in the solution. An indicator system is then used to detect the presence or absence of complement, consisting of sheep erythrocytes and antisheep erythrocyte antibody (hemolysin). In the presence of complement, the sheep cells are lysed by the hemolysin. Thus, in the completed test, antigen - serum mixtures with lysis of the sheep cells indicate absence of antibody (complement not bound), whereas mixture with intact erythrocytes indicate the presence of antibody (complement bound) (Clyde and Senterfit, 1983).

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Equipment and Materials:

Microtitration equipment were used as these provide maximum conservation of reagents and make possible the titration of many serum samples in a single test. Microdiluters (0.025ml), microdroppers (0.025 and 0.05ml) and multiwell microdilution trays (8 rows of 12 round bottom wells) were used (Sterilin Limited, Teddington, Middlesex, England).

Other necessary equipment items used were a 37°C incubator, a 56°C water bath, a 4°C refrigerator and a -20°C freezer. A contrifuge was used for the preparation of erythrocyte suspension.

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<u>Serological Test:</u> Growth Inhibition Test

The test organism was grown in complete mycoplasma broth, and at the point where the colour of the medium just began to change 0.1ml of the culture was inoculated evenly onto an agar plate. Serial dilutions of the mycoplasma culture were made up to 10^{-3} and these were also inoculated separately onto agar plates. The inoculum was dried on the agar surface at 37°C for 1 hour by slanting the top of the petridish to facilitate drying. Presaturated discs of <u>M. pneumoniae</u> and <u>M. salivarium</u> antisera (Mycoplasma Reference Laboratory, Central Public Health Laboratory Service, Colindale, London NW9 5HT, England) were placed opposite each other on the surface of the agar plates. The plates were incubated at 37°C in 5% CO_2, 95% N_2. The plates were observed twice a week for colony growth and the periphery of each disc examined for colony inhibition (Clyde, 1964).

3.2 <u>Serodiagnosis:</u>

<u>Complement Fixation Test</u>

<u>Principle:</u>

The test antigen and unknown sera are allowed to interact in the presence of a measured amount of complement. If antibody is present,

1. Sheep Erythrocytes:

Blood was drawn from sheep (merino), using sterile materials and aseptic technique, in 75ml amounts into two bottles containing 125ml of sterile Alsever's solution. The standardization of the sheep red blood cells was done according to Garvery et al (1977).

2. HEMOLYSIN:

A commercial preparation of hemolysin (Burroughs Wellcome, England) was used for the test. The vials indicated that the titre was between 1,000 to 2,000; a titration of the hemolysin was done to assess the exact titre of the serum and to determine a satisfactory working dilution. The titration was done according to Garvey et al (1977) and the final dilution of hemolysin was found to be 1/80.

3. Complement:

Thirty guinea pigs, supplied by National Veterinary Research Institute, Vom, Jos, Nigeria, were slaughtered and their sera pooled. To determine the titre of the complement, it was titrated against hemolysin using the method of Clyde and Sentorfit (1983).

The working dilution of the complement was found to be 1/16.

4. Diluent:

Commercial preparation of veronal buffer (Burroughs Wellcome, England) was used in the whole test.

5. Antigen:

The M. pneumoniae antigen was supplied by The Mycoplasma Reference Laboratory, Central Public Health Laboratory, Colindale, London NW9 5HT England, at a titre of 1/20.

6. Positive Antiserum:

The M. pneumoniae positive control serum for the test was supplied by the Mycoplasma Reference Laboratory Central Public Health Laboratory, Colindale, London NW9 5HT, at a titre of 1/40.

7. Negative Antiserum:

The M. mycoides positive antiserum was used as a negative control antiserum this was supplied by The National Veterinary Research Institute, Vom, Jos, Nigeria, at a titre of 1/50.

Complement Fixation Test for *M. pneumoniae*

(1) The standardized SRBC were washed and standardized so that a 3% suspension was sensitized with $6HU_{50}$ of hemolysin for use in the test.

(2) The titrated C^1 was made into it's working dilution and kept ice-cool for use.

(3) The 1/4 dilution of the test sera, the appropriate dilutions of the positive serum and the negative serum were inactivated in a water bath at 56°C for 30 minutes.

In a microtitration tray 0.025ml of each dilution was added in each well and to this 0.025ml of *M. pneumoniae* antigen and 0.025ml of complement were added. Antigen was not added to the last well and this served as the serum control. Antigen and complement controls were also set by not adding serum to the antigen control and serum and antigen to the complement control. One set of these controls was set regardless of the number of microtitration trays used in a day. The trays were covered and incubated at 37°C for 30 minutes with shaking at 10 minutes intervals. To all the wells 0.025ml of sensitized SRBC were added and shaken, the plates were covered and incubated at 37°C for 30 minutes again with shaking at 10 minutes intervals.

The test can be read immediately or after storage at +4°C for 24 hours, when the fixation is more distinct. Titration end points are represented by wells showing 75% complete cell buttons.

RESULTS

Mycoplasma-like organisms were isolated from 3 out of 104 patients with pneumonia. Two types of colonies were isolated from each patient, viz, (i) a large-yellowish colony (Plate 4.1A) without peripheral zone and (ii) a small colony with a large peripheral zone. The larger colonies were visible after 6-7 days of incubation, while the smaller colonies were visible after 3-4 days of incubation on agar plates. The colonies were cloned to get pure mycoplasma isolates, but the large-yellowish colony of isolate number 79 was lost during cloning.

Beta - hemolysis test was done on all the isolates and the large colonies from numbers 10 and 56 showed hemolytic plaques (Table I). The plaques around the colonies of number 56 were few, that is only few colonies showed plaques; but the plaques around the colonies of number 10 were large and most of the colonies had plaques around them.

Growth - Inhibition test using *M. pneumoniae* and *M. salivarium* antisera discs was done for all the isolates. The periphery of the discs of *M. pneumoniae* antiserum showed inhibition of large colonies in numbers 10 and 56 (Table II; Plate 4.1B). No other colonies were inhibited by either *M. pneumoniae* or *M. salivarium* antisera (Plate 4.2).

TABLE 3.1: COMPLEMENT FIXATION TEST TO DETERMINE THE ANTIBODY LEVEL OF *M. pneumoniae*

	RECIPROCAL OF SERUM DILUTIONS (0.025ml)								
	4	8	16	32	64	128	256	514	4
SERUM	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
ANTIGEN (1/20)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0
BUFFER	0	0	0	0	0	0	0	0	0.025
COMPLEMENT (1/16)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
	INCUBATION AT 37°C FOR 30 MINUTES								
SENSITIZED SRBC	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
	INCUBATION AT 37°C FOR 30 MINUTES								

No mycosplasma-like colonies were isolated from the 52 control patients (that is, those without clinical evidence of pneumonia).

Thus, after purification, from isolation alone 2 out of 104 (2%) were positive.

A four-fold rise in the complement fixing antibody titre of acute and convalescent sera or a single complement fixation titre of ⩾ 1/64 shows recent infection of M. pneumoniae in an individual (Murray et al., 1975; Southeimer et al., 1978; MacFarlane et al., 1979; Broome et al., 1980; Roach et al., 1980).

Out of 104 sera specimens analysed 8(7.7%) had both acute as well as convalescent specimens (Appendix) and the rest had a single specimen. Out of these 8 specimens, 3 showed a four fold or a greater rise in the complement fixing antibody titre (1/16 to 1/64, 1/16 to 1/64 and 1/16 to 1/512; 2 showed a decrease in the titre (1/128 to 1/64 and 1/64 to 1/8); 1 showed no change in the titre (1/64 to 1/64) and the other 2 showed titres from 1/8 to 1/16 and negative to 1/16.

Of all the sera analysed, using the complement fixation test, 32 were considered to be positive for M. pneumoniae (31%) and the highest percentage of positives (41%) was found in the school age (5-10 years) group (Table III). Out of the 104 serum samples analysed by CFT 55 were from males and 49 from females

and the number of positives were 18 (33%) and 14 (29%) respectively (Table V).

All the 52 control sera analysed by CFT showed no positive titre (< 1/64) (Table IV).

Therefore, M. *pneumoniae* pneumonia was found in 34 patients (33%), 2 (2%) by isolation and 32 (31%) by serology.

TABLE 4.1: BIOLOGICAL IDENTIFICATION OF
M. pneumoniae USING BETA-HEMOLYSIS TEST

NUMBER OF ISOLATE	TYPE OF COLONIES	
	LARGE COLONIES WITHOUT PERIPHERAL ZONE	SMALL COLONIES WITH PERIPHERAL ZONE
10	++	-
56	+	-
79	ND	-

- Negative

\+ Positive but few colonies show clear hemolysis

++ Positive; all colonies show large hemolytic plaques.

ND Not Done.

TABLE 4.2 SEROLOGICAL IDENTIFICATION OF
M. pneumoniae USING GROWTH-INHIBITION TEST

NUMBER OF ISOLATE	TYPE OF COLONIES			
	LARGE COLONIES WITHOUT PERIPHERAL ZONE		SMALL COLONIES WITH PERIPHERAL ZONE	
	M. pneumoniae	*M. salivarium*	*M. pneumoniae*	*M. salivarium*
10	+	-	-	-
56	+	-	-	-
79 ND	ND	-	-	-

\+ clear zone of inhibition

- No inhibition

ND Not Done.

TABLE 4.3: INCIDENCE OF M. pneumoniae PNEUMONIA IN VARIOUS AGE GROUPS BY THE COMPLEMENT FIXATION TEST.

AGE GROUP (YEARS)	TOTAL NUMBER	TOTAL NUMBER POSITIVE FOR M. pneumoniae (≥ 1/64)	PERCENTAGE
INFANTS (0–2)	54	15	28
PRESCHOOL (2–5)	33	10	30
SCHOOL (5–10)	17	7	41
	104	32	31

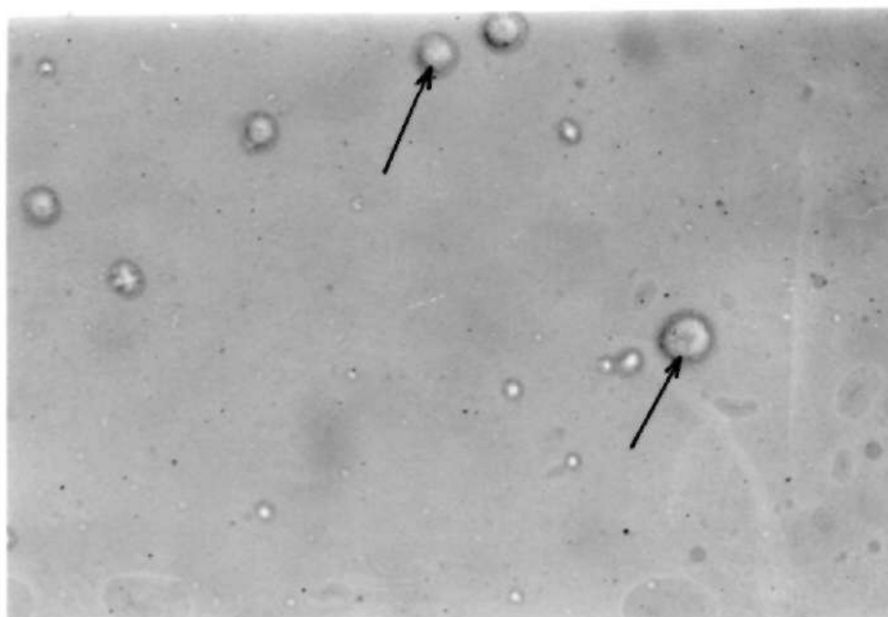
TABLE 4.4: INCIDENCE OF M. pneumoniae IN CONTROL PATIENTS IN VARIOUS AGE GROUPS BY THE COMPLEMENT FIXATION TEST

AGE GROUP (YEARS)	TOTAL NUMBER	TOTAL NUMBER POSITIVE FOR M. pneumoniae (≥ 1/64)	PERCENTAGE
INFANTS (0–2)	29	0	0
PRESCHOOL (2–5)	15	0	0
SCHOOL (5–10)	8	0	0
	52	0	0

TABLE 4.5 SEX DISTRIBUTION OF *M. pneumoniae* PNEUMONIA CASES BY THE COMPLEMENT FIXATION TEST.

SEX	TOTAL NUMBER	TOTAL NUMBER POSITIVE FOR *M. pneumoniae* ($\geqslant 1/64$)	PERCENTAGE
MALE	55	18	33
FEMALE	49	14	29

(a)



(b)

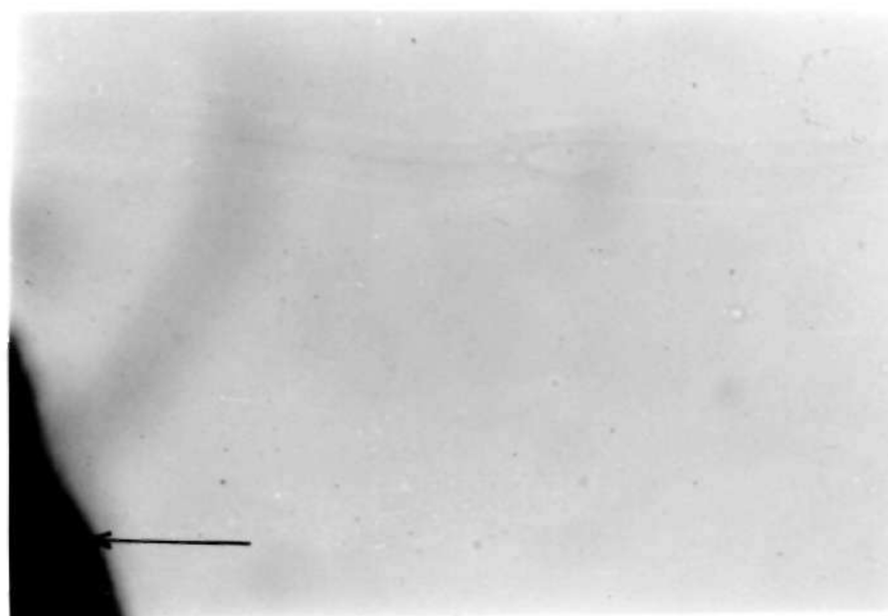


Plate 4.1A: Colonies of mycoplasma isolate identified serologically as M. pneumoniae × 100. Arrows point at the colonies.

4.1B: Growth inhibition of colonies identified as M. pneumoniae. Arrow points at M. pneumoniae antiserum paper disc.

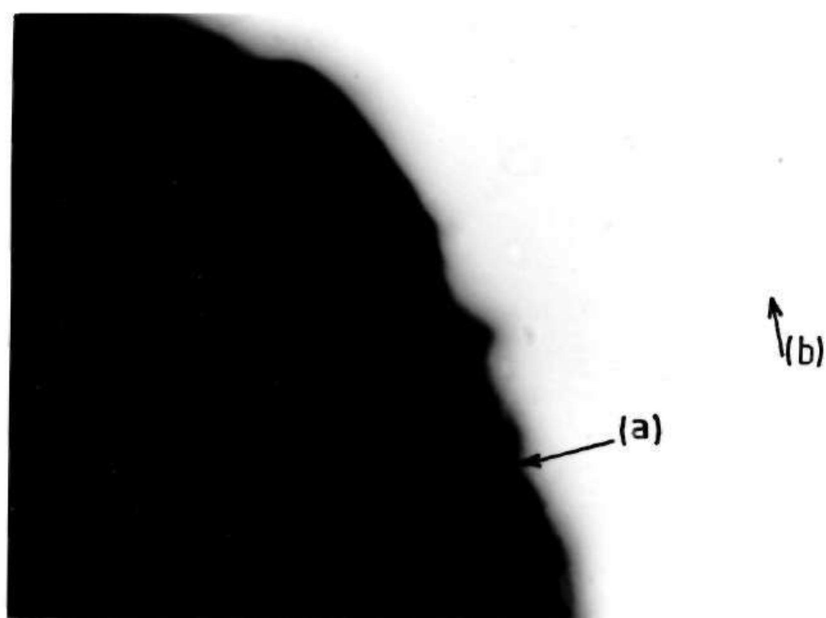


Plate 4.2: Growth of colonies identified as M. pneumoniae. Arrow points at (a) M. salivarium antiserum paper disc. (b) M. pneumoniae colonies × 100.

DISCUSSION

Mycoplasma pneumoniae is a known cause of pneumonia in many parts of the world (Chanock, 1965; Kitamoto *et al.*, 1966; Chanock *et al.*, 1967; Foy *et al.*, 1970; Gupta *et al.*, 1975; Joosting *et al.*, 1975; Tai and Wei, 1976; MacFarlane *et al.*, 1979; Ali *et al.*, 1986). A prevalence of 33% in this study shows that *M. pneumoniae* is involved in pneumonias in children 0 - 10 years of age in Zaria, Nigeria. This finding is comparable to those obtained in other areas, 28% in Tokyo, Japan (Kitamoto *et al.*, 1966) and Connecticut, U.S.A (Cordero *et al.*, 1967) 29% in Chapel, Hill, U.S.A. (Loda *et al.*, 1968), 33% in Linkoping, Sweden, (Lindstrom and StahlFurenhed, 1981), 35% in Seattle, U.S.A. (Foy *et al.*, 1979) and 38% in Wisconsin, US.A. (Broome *et al.*, 1980).

In contrast, a higher prevalence of *M. pneumoniae* pneumonia has been reported, 49% in Omaha, U.S.A. (Saliba *et al.*, 1967), 52% in New South Wales (Reid and Murphy, 1970) and 74% in Atlanta, U.S.A. (Dowdle *et al.*, 1967). Lower prevalence of 1 to 1.5% in Seattle, U.S.A. (Alexander *et al.*, 1966), 10% in Bethesda, U.S.A. (Chanock *et al.*, 1960), 17% in Zaria, Nigeria (MacFarlane *et al.*, 1979) and 23% in Chapel Hill, U.S.A. (Glezen *et al.*, 1971) have also been reported.

M. pneumoniae was isolated from the throats of 2 patients whose sera analysis showed a titre which was not diagnostic ($\frac{1}{8}$ and 1/32). The reason for a low or undiagnostic antibody titre could be that at the time the serum was obtained from the patients the *M. pneumoniae* infection was ongoing. The *M. pneumoniae* was isolated from patients 1 and 3 years of age. This is different from the reports by Alexander *et al* (1966), Denny *et al* (1971), Foy *et al* (1979) and Brunner (1981b) who showed that the isolation rate was lowest in children below 5 years of age and highest between 5 to 9 years of age. The finding in this study can be compared to studies in Chapel Hill, U.S.A. (Glezen *et al*., 1967) where it was shown that the isolation rate of *M. pneumoniae* from pneumonia patients 2 to 4 years of age was the same as from children 5 to 8 years of age. It is possible that this might be due to the higher incidence of pneumonia in the lower age group and so patients of older age group suffering from pneumonia are less in number. This could have resulted in the variance of the number of positives in the children.

In the Beta-hemolysis test, the isolate number 56 had a lot of bacterial contamination and after cloning and filteration the colonies obtained were used for the test. It is possible that there was

still some contamination in the pure culture as all the colonies did not show hemolytic plaques. Cultures or isolates from the oropharynx have a lot of contamination and also most human mycoplasmas like M. orale, M. buccale, M. salivarium and M. faucium are generally found in the throat (Organick and Worman, 1967).

The isolates showed inhibition by M. pneumoniae antiserum disc in the Growth Inhibition test. There was no inhibition of colonies around the M. salivarium antiserum discs (Plate 4.2).

Paired sera samples could not be obtained from most patients because the patients failed to come back two weeks after being given the treatment. This might be due to the fact that most of the children came from rural areas and their parents were mostly uneducated and come from a low socio-economic background. These parents cannot afford to come to the hospitals unless the children were seriously ill.

In cases where a convalescent sera could not be obtained single or acute sera samples were used in the complement fixation test. A complement fixing titre of $\geqslant 1/64$ was considered positive in this study as other investigators find this to be acceptable (Murray et al., 1975; Southeimer et al., 1978; MacFarlane et al., 1979; Broome et al., 1980;

Roach *et al.*, 1980). From the sera analysis done by the Complement Fixation Test, 32 patients were found to be positive for *M. pneumoniae* by serology alone. The highest percentage (41%) of positive individuals were found in the school age children (5-10 years). This can be compared to the works of Alexander *et al* (1966) and Foy *et al* (1979), in Seattle, U.S.A., who also showed the highest rate of *M. pneumoniae* pneumonia in the 5-9 years age group. It has also been reported that the frequency of infection with *M. pneumoniae* in preschool children is equal to or more than in the older siblings, thus implying that *M. pneumoniae* infections may occur more commonly than generally appreciated in the early years of life (Balassanian and Robins, 1967; Foy *et al.*, 1966, Biberfeld and Sterner, 1969); and that the antibody responses of the very young and older children are similar (Fernald *et al.*, 1975).

M. pneumoniae infection was found in all the groups, with the second highest percentage (30%) in the preschool children (2-5 years). This is because the children in the rural areas are left to wander out of the house at a very young age and as *M. pneumoniae* usually spreads by droplet infection between households with playmates the infection is easily picked up.

The sera specimens of the 52 control patients (those without clinical evidence of pneumonia) were also analysed by the complement fixation test (Appendix). None of the sera gave a CF antibody titre of ≥1/64 which denotes recent infection when only a single serum sample is available; thus, no control patient was positive for Mycoplasma pneumoniae infection. Out of the 52 sera samples 11 (21%) gave a titre of 1/32, 1(2%) a titre of 1/16 and 40 (77%) a negative CF titre. A CF titre of 1/32 is fairly high and could be as a result of previous infections with M. pneumoniae as CF titres can remain elevated for several months (Roach et al., 1980).

Although, this study showed a slightly higher frequency of males (33%) suffering from M. pneumoniae pneumonia than the females (29%), the difference was statistically insignificant. However, Foy et al (1979) reported a higher frequency of M. pneumoniae pneumonia in females than the males.

The only study done in Nigeria on M. pneumoniae was by MacFarlane et al (1979) in the Ahmadu Bello University Teaching Hospital, Zaria. This study was done on adults suffering from lobar pneumonia and an incidence of 17% of Mycoplasma pneumoniae was found., thereby establishing it's presence in Nigeria.

M. pneumoniae is a very fastidious organism which takes a long time to grow (7-14 days), thus

isolation cannot possibly be used for diagnosis of M. pneumoniae pneumonia; serology of M. pneumoniae is usually done when M. pneumoniae infection is suspected. Routine laboratory diagnosis of M. pneumoniae in Pediatric Out Patient's Clinics in Nigeria is not feasible because of lack of facilities both in manpower and equipment and the logistics involved. The need for diagnosis of M. pneumoniae only arises if the common serious bacterial pneumonias have been ruled out and if the patients fail to respond to the antibiotics administered against them. M. pneumoniae infection must be thought of in children whose pneumonias respond slowly for no obvious reasons and a change of antibiotic to include Erythromycin or tetracycline must be considered.

Vaccination with an effective inactivated or attenuated M. pneumoniae preparation has been used and the efficacy shows 67% (Barile et al., 1981) in those populations which are high at risk, that is, military recruits, college students and prisoners. However, until a safe immunoprophylactic preparation is available the M. pneumoniae diseases in children have to be treated.

Although, M. pneumoniae was found in 33% of the patients with pneumonia but this does not necessarily implicate M. pneumoniae as the primary

pathogen of current pneumonia. Studies to investigate whether *M. pneumoniae* is the primary or secondary cause of pneumonias have to be done in Nigeria.

This study has established the presence of Mycoplasma pneumonia in 33% of children. While it is desirable to study a larger population for a longer duration to determine the seasonal variations, the epidemic pattern and the rate and level of antibody rise in the infected children, financial as well as time constraints make this impossible. More surveys of this kind on a larger scale need to be carried out on populations at risk to estimate the full extent and significance of *Mycoplasma pneumoniae* infection in Nigeria.

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APPENDIX

COMPLEMENT FIXATION TEST

NUMBER of SAMPLE	*M. pneumoniae* COMPLEMENT FIXATION TITRE	*M. pneumoniae* Infection (≥1/64)
1	1/64	+
2	1/64	+
3	1/64	+
4	1/64	+
5a 5b	1/16) 1/64)	+
6	1/32	–
7	1/32	–
8	–	–
9	1/64	+
10	1/32	–
11	–	–
12	1/32	–
13	1/128	+
14	1/64	+
15	1/128	+
16a 16b	1/128) 1/64)	+
17	1/64	+
18	1/32	–
19a 19b	–) 1/16)	–
20	1/16	–
21	1/32	–

22	–	–
23	–	–
24	–	–
25	–	–
26	1/64	+
27	–	–
28	–	–
29	1/32	–
30	–	–
31	1/32	–
32	1/64	+
33	–	–
34	–	–
35	–	–
36	1/32	–
37	1/32	–
38	–	–
39	1/32	–
40	1/32	–
41	1/32	–
42	1/16	–
43	1/32	–
44	1/32	–
45	1/32	–
46	1/32	–
47	1/64	+
48	1/64	+

49	1/32	–
50	1/32	–
51	1/64	+
52	1/32	–
53	1/32	–
54	–	–
55	–	–
56a 56b	1/8 1/16	–
57a 57b	1/16 1/64	+
58	1/32	–
59	–	–
60	1/16	–
61	–	–
62	1/16	–
63a 63b	1/64 1/8	+
64	–	–
65	–	–
66	1/32	–
67	1/16	–
68	–	–
69	–	–
70	–	–
71	1/32	–
72	1/8	–
73a 73b	1/64 1/64	+

74	1/8	–
75	1/8	–
76	–	–
77	1/64	+
78	–	–
79	–	–
80a 80b	1/16) 1/512)	+
81	–	–
82	–	–
83	1/16	–
84	–	–
85	–	–
86	1/16	–
87	–	–
88	–	–
89	1/64	+
90	1/64	+
91	1/64	+
92	1/64	+
93	1/128	+
94	–	–
95	1/64	+
96	1/128	+
97	1/32	–
98	1/128	+
99	–	–
100	1/128	+

101	-	-
102	1/64	+
103	-	-
104	1/64	+

CFT IN CONTROL PATIENTS

NUMBER of SAMPLE	C.F. TITRE	M. pneumoniae INFECTION (≥ 1/64)
1	-	-
2	1/32	-
3	1/32	-
4	-	-
5	-	-
6	1/32	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	-
12	-	-
13	-	-
14	1/32	-
15	-	-
16	-	-
17	-	-
18	-	-
19	-	-
20	-	-
22	1/32	-
23	-	-
24	1/32	-

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25	-	-
26	-	-
27	1/32	-
28	-	-
29	-	-
30	-	-
31	-	-
32	1/16	-
33	-	-
34	1/32	-
35	1/32	-
36	1/32	-
37	1/32	-
38	-	-
39	-	-
40	-	-
41	-	-
42	-	-
43	-	-
44	-	-
45	-	-
46	-	-
47	-	-
48	-	-
49	-	-
50	-	-
51	-	-
52	-	-