CHAPTER II

REVIEW OF THE LITERATURES

HISTORY OF *MYCOPLASMA PNEUMONIAE*

In 1944, Eaton and associates isolated a filterable agent from the sputa of pateints with "primary atypical pneumonia" and this agent was first identified as a probable cause of the pneumonia (37). In 1957, Lui identified Eaton's agent in the bronchial epithelia of chicken embryos by indirect immunofluorescence (38). In 1961, Marmion and Goodburns found that Eaton-Lui agent was inhibited by Gold salts, suggesting of a pleuropneumonia-like organism (PPLO) (39). Later Chanock and associates were able to grow the Eaton-Lui agent on agar media, and called the new organism "*Mycoplasma pneumoniae*". They also showed that the organism produced primary atypical pneumonia (40,41).

Mycoplasma pneumoniae has traditionally been regarded as a respiratory tract pathogen of children and young adults. This organism is also an important cause of pneumonia and febrile upper respiratory tract infections in adults of all age groups. In addition, it now appears clear that *M. pneumoniae* infection can also be associated with a wide spectrum of both pulmonary and extrapulmonary syndromes that may be benign and self-limited, moderately troublesome, or sometimes life-threatening (42-44,12). Moreover, on occasion, extrapulmonary manifestations may actually overshadow or occur in the absence of symptomatic respiratory tract involvement (11).

THE ORGANISM

M. pneumoniae is a procaryote that more closely resembles a bacterium rather than a virus. However, it lacks a cell wall, and not surprisingly, is resistant to cell wall-active antibiotics. *M. pneumoniae* is bound by a single triple-layered membrane, poor to gram stain, grows on artificial media supplemented with yeast extract and serum, exhibits hemadsorption, and produces a peroxide hemolysin. The organism is 10x200 nm in size, appears filamentous, and on the end displays a neuraminic acid receptor site for attachment to host cell membranes (45).

EPIDEMIOLOGY

M. pneumoniae has been shown by serological tests to have a wide geographical prevalence (46). It has been considered as an important etiology of lower respiratory infections. *M. pneumoniae* infections are worldwide endemic throughout the year and cause epidemics in some areas (47-49,4). Infections are most frequently found in children, and young adults particulary those between 5 and 14 years old (50). Mycoplasma infections among patients with chronic bronchitis are common. Closed populations such as military personnels, prisoners, school children and college students living in dormitories (51-53) are prone to have mycoplasma infections.

CLINICAL FEATURES

The incubation period of *M. pneumoniae* infection is 1 to 3 weeks. Onset is insidious, starting with fever, headache, general malaise and followed by sore throat, dry cough or productive cough

of mucoid or mucopurulent sputum. Hemoptysis is rare. Chest pain occurs rarely expect with the presence of severe cough or pleural involvement but substernal soreness may be present in some cases. Earache may be present with or without bullous myringitis. Duration of fever is 4 to 10 days but patients with sickle cell anemia may have prolonged fever and a great extent of pneumonia (54,55). The cough may persist for several weeks even when pneumonia has disappeared especially in patients with hypogammaglobulinemia. In patients without underlying disease, the pneumonia is rarely severe or fatal (56,57,44).

PATHOGENESIS

Infection appears to be acquired via inhalation of infected material after exposure to an acutely ill coughing individual (12). Nasopharyngeal carriers of *M. pneumoniae* (whose clinical illness has resolved) do not appear to transmit the organism readily.

Since fatal cases of *M. pneumoniae* pneumonia are rare, there is little pathogenic data from which pathogenic inferences draw. Histologic findings from human cases have included tracheal and bronchial mucosa hyperemia. Alveolar exudates comprised principally of mononuclear inflammatory cells, plasma cell interstitial space infiltration, and accumulation of monocytes and macrophages in the bronchial epithelial submucosa. Studies using an experimental animal model have demonstrated that *M. pneumoniae* colonies bind via neuraminic acid receptors to respiratory tract

epithelial cells and initiate local tissue injury. The latter may be mediated by hydrogen peroxide elaborated by the organism itself. *M. pneumoniae* may also penetrate the bronchial mucosa. Polymorphonuclear leukocytes are attracted to deciliated cells, and leukocyte products probable contribute to or perpetuate the superficial inflammatory process. Simultaneously, monocytes, macrophages, and lymphocytes infiltrate submucosal areas as well(58).

Although an IgM followed by an IgG antibody response typically occurs in *M. pneumoniae* infection, serum antibody dose not necessarily confer lifelong immunity (59). It is pertinent to note, however, that locally secreted nasopharyngeal IgA appears to be effective in inhibiting *M. pneumoniae* binding to the respiratory tract epithelium.

There is a growing body of evidence to suggest that immune machanisms, rather than actual direct infection, play a role in the development of clinically apparent *M. pneumoniae* as well as some of the extrapulmonary complications. Thus, despite the potential to cause disease in practically any organ, body fluid, or mucosal surface, *M.pneumoniae* is seldom isolated from clinical material except from sputum or nasopharyngeal secretions. Moreover, *M. pneumoniae* or its antigen has seldom been demonstrated in the lungs of pateints with fatal pulmonary infection (11). Evidence has recently been reported, however, to support the notion that repeated subclinical *M. pneumoniae* infections with consequent sensitization of T lymphocytes (60) and probably other components of the immune system (e.g., autoantibodies) may be necessary before manifestations such as pneumonitis develop (12). For instance,

circulating *M. pneumoniae* immune complexes, which in one study were detected in 41% of infected patients (61), may contribute to target organ injury in the lung, synovium, or elsewhere. Moreover, antithymocyte globulin abrogates or diminishes the severity of experimental *M. pneumoniae* infection in animals (12), and corticosteriods have been used with some beneficial clinical effects in patients with severe *M. pneumoniae* pneumonia (62,44). Further, pneumonia failed to develop in a group of children with immunodeficiency syndromes who had acquired severe *M. pneumoniae* respiratory tract infections (56). These observations suggest, then, that previous sensitization and the vigor of the host immune response may explain in part why some patients with *M. pneumoniae* infection develop pneumonitis rather than bronchitis alone and why some experience extrapulmonary complications.

LABORATORY DIAGNOSIS

In general, the laboratory diagnosis of mycoplasma infections is based on essentially the same principles that are valid for the diagnosis of infections cause by other procaryotic pathogens. However, several of the characteristics of the mycoplasmas such as their small size, low affinity to bacterial stains, and their exacting nutritional requirements, do on the other hand pose a number of problems for the diagnostic laboratory(63).

Laboratory diagnosis of *M. pneumoniae* infection 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. Although a number of technical improvements have occurred recently in the serological evaluation of the disease, few modifications in cultivation techniques for the organism have been proposed since the initial discovery of the agent. However, recent evaluation of a new culture medium (SP-4), developed for growth of other mycoplasmas (spiroplasmas) (64), has greatly improved the isolation rate of *M. pneumoniae* from clinical specimens (65). When this culture medium is used in conjunction with direct and specific identification of *M. pneumoniae* colonies on agar plates by an immunofluorescent serological test, an increased isolation rate of 30 to 40% over conventional culture technique has been observed (65).

- SPECIMEN COLLECTION AND TRANSPORT(66)

Throat and nasopharyngeal swabs, tissue from lung or brain, and spinal or pericardial fluids should 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 1000 units/ml penicillin G, is generally an effective transport medium. If the transport specimen cannot be cultured within 24 h of collection, it should be frozen (-70°C) until appropriate culture materials and equipment are available.

Sera for antibody detection and antibody responses to *M. pneumoniae* in human respiratory infections are most frequently determined by the standard complement fixation (CF) proceduce. Others specialized serological techniques have been described for

measuring antibody to *M. pneumoniae*. Most of these are designed to detect specific immunoglobulin classes or to enable more rapid or sensitive measurement of mycoplasmal antibody.

CULTIVATION, ISOLATION AND IDENTIFICATION PROCEDURES

Cultivation Procedures (66) A 0.1 ml sample of the transport medium (or suitable body fluids) should be inoculated into a vial of the SP-4 diphasic medium. It is useful to refreeze the transport specimen at -70° C after inoculation of SP-4 medium in case repeat tests are required. Swabs obtained directly from the patient should be streaked immediately onto a mycoplasma agar plate and the swab swirled vigorously in the SP-4 diphasic broth. Tissues should be minced coarsely and then added to about 5 ml of SP-4 broth medium. At least two tenfold dilutions of the initial tissue suspension should be made in the same broth medium to reduce possible inhibitory substances from the tissues. All tubes should be incubated at 37° C for a minimum of eight weeks. Agar plates are incubated (37° C) anaerobically, either in an atmosphere of 95% N+5% CO_2 or in the Gaspak@. Diphasic SP-4 cultures or plain broth cultures should be observed every two to three days for indicator changes (either to the acid or alkaline pH range) and for turbidity. When this occurs, a sample (0.2 ml) of broth supernatant is transferred to a mycoplasma agar plate and the plate is incubated as noted above. Mycoplasma colonies usually appear after 5 to 14 days of anaerobic incubation.

Identification of Isolates

The most rapid and specific identification of *M.*

pneumoniae colonies growing on the agar plates is accomplished through a direct plate immunofluorescent antibody test (67). The plates are flooded with about 1 ml of phosphate-buffered saline and then are allowed to soak for 20 min at room temperature. This wash is then poured off and about 1 ml of an appropriate dilution of a fluorescein-conjugated antiserum specific for *M. pneumoniae* is added to the plate. The dilution of the conjugate selected will depend upon the potency of the antiserum, but in most instances a conjugate dilution of 1:20 to 1:40 will provide strong fluorescence of *M. pneumoniae* colonies. Following incubation at room temperature for 20 min, the conjugate is poured off the plates again washed with about 2 to 3 ml of saline, the wash poured off, and the plates inverted and dried for 20 min. The plates are examined with a fluorescence microscope equipped with incident illumination. Colonies are scanned under magnification of about x160, using a quartz halogen or mercury vapor light source for indicent illumination and incandescent lamp for transmitted illumination. In a few instances, all colonies on the plate may be identified as *M. pneumoniae*, but, more frequently, a few *M. pneumoniae* colonies on the plate may be mixed with a number of other colonies of *Mycoplasma* species that are part of the normal flora of the human throat. The latter colonies will not stain with the *M. pneumoniae* conjugate. Confirmation of *M. pneumoniae* colonies may be accomplished by preparing duplicate agar plates of the SP-4 diphasic broth and selecting colonies of the size and shape of those staining with the specific conjugate. These colonies are then transferred to fresh broth and the culture identified by

conventional growth inhibition test(68) with *M. pneumoniae* antiserum. Conversely, duplicate agar plates may be flooded with a suspension of 5% guinea pig red blood cells in saline and the colonies examined microscopically for adsorption of erythrocytes to specific colonies (69).

SEROLOGICAL DIAGNOSIS OF INFECTION

Diagnosis of mycoplasmal infections by isolation of the organism is difficult because mycoplasmas grow slowly and require complex media for isolation, whereas most bacterial pathogens may be readily cultivated in the clinical microbiological laboratory relatively quickly. As a consequence, few laborotories offer cultivating services. Therefore, serodiagnostic methods could be as important for diagnosis of mycoplasmal infections as they are for diagnosis of viral infections, the agents of which also may be difficult to cultivate. However, serodiagnostic methods for diagnosis of mycoplasmal infections are much less advanced because of the much greater antigenic complexity of mycoplasma than of viruses.

1. The Complement Fixation Test (CF)

CF for *M. pneumoniae* antibody is now usually performed with the standard microtitration equipment, requiring only 25 ul of serum. Commercial whole cell CF antigen is available but is frequently of low titer and anticomplementary. A lipid antigen prepared from whole organisms by chloroform-methanol extraction increases antigen sensitivity and decreases anticomplementary activity. Methods of preparing the lipid antigen have been described

in detail (70). The lipid antigen is complexed with bovine albumin and then frozen at -20°C. Following standardization of antigen against a known *M. pneumoniae* antiserum and assessment of the amount of complement to be used in the system (48), the CF titers of paired test serums are examined. Test results are available after 18 to 24 h. It is important for each serum titration to include controls of antigen and serum anticomplementary activity and a control human antiserum with an established *M. pneumoniae* CF titer. Sera obtained from patients at the onset of respiratory infection and one to three weeks later (during convalescence) will usually show a fourfold rise in CF antibody to *M. pneumoniae* antigen. Titers generally rise from < 1:8 or 1:16 to 1:256 or 1:512. This IgM antibody usually peaks at four to six weeks after infection and may decline slowly after two or three years to level of 1:8 or 1:16. False positive CF test with lipid antigen have been reported in some patients with pancreatitis but without other evidence of *M. pneumoniae* infection (71).

2. Cold Agglutination Test (CA)

Sera were used at 1:8 and then made twofold dilution until 1:1024 in phosphate-buffer saline (PBS), pH 7.0, in 0.5 ml volumes in tubes, to each of which was then added 0.5 ml of a washed 0.5% suspension of human Group O red blood cells in PBS. The tubes were placed 4° C overnight or for a minimum of 2 h. For reading the result, the tubes were tapped once to dislodge any sedimented red cells; any agglutination patern observed with the naked eye was records as positive and buttoned deposit that dispersed as a fine suspension as negative. Tubes were placed at 37° C for 30 min to

check that elution of the agglutinin had occured, resulting in disappearance of the agglutination pattern. A titer of > 64, although non-specific, was taken as suggestive of recent or current *M. pneumoniae* infection (23).

3. Indirect Immunofluorescence test (IFA)

The slide test with *M. pneumoniae* suspensions as antigen, as described by Wreghitt and Sillis (72), was used to assess *M. pneumoniae*-specific IgM, IgA and IgG in patients' sera tested which were twofold-diluted at 4 - 128. The presence of a specific IgM titer of > 4, or a specific IgA titer > 8, or fourfold rise or fall in the latter, indicates recent or current *M. pneumoniae* infection.

4. The Enzyme Immunoassay (EIA)

M. pneumoniae organisms lysed by adding an equal volume of 2% sodium dodecyl sulfate were used as the antigen in EIA. The protein concentration of the undiluted antigen was 1.6 mg/ml. [EIA was performed by modification of the method described by Voller et al.(73)]. The wells were coated overnight with antigen diluted 1: 1000 in phosphate-buffer saline (PBS; 10mM sodium phosphate with salt concentration 0.9% and pH 7.3) and blocked with 0.0313% gelatin in PBS for 30 min at room temperature. The wells were washed three time with PBS containing 0.05% Tween20.

Serum dilutions of 1:100, 1:1000, and 1:10000 in PBS containing 0.5% Tween20 and 10% fetal calf serum were pipetted in to the wells and incubated for 1 h at 37° C. After washing as above, alkaline phosphatase-conjugated rabbit anti-IgG or anti-IgM (Orion), both diluted 1:300 in the same buffer as the sera, was added and

incubated for 1 h at 37° C. After washing, the substrate (Sigma 104; Sigma Chemical.St Louis MO.) diluted in diethanolamine-$MgCl_2$ buffer (Orion) was added and incubated at 37° C for 30 min. The reaction was stopped with 1 N NaOH, and the color reaction was measured at 405 nm in a Multiscan multichannel photometer (Labsystems, Helsinki, Finland). The EIA results were calculated as end-point titers using an absorbance value of 300 optical density units as the cutoff value (74). A threefold or greater titer rise between the paired sera was regarded as significant.

5. Microparticle Agglutination Assay (MAG assay; Serodia Myco II; Fujirebio, Inc., Tokyo, Japan)

MAG test is maketed as a kit containing a reagent which are made of artificial gelatin particles carrier sensitized with cell membrane components of *M. pneumoniae* (Mac strain). This test is based on the principle that the sensitized particles cause indirect agglutination in the presence of anti-mycoplasma antibody in the specimen. For MAG testing, sera were heat inactivated by incubation in a 56°C water bath for 30 min. For each test serum sample, serial two-fold dilutions of 1:20, to 1: 10,240, which yielded final dilution of 1:40 to 1: 20,480, were prepared in U-bottom microwell plates. Twenty-five microliter of each serum dilution were added with one drop (25ul) of sensitized particles. One additional microwell containing a final serum dilution of 1:20 was prepared for each serum sample, and unsensitized particles were added to this well; this well served as a nonspecific agglutination control. The mixtures were carelully mixed and then incubated room temperature for 3 hr. Results were evaluated if the unsensitized particles had settled to form a

compact center point. If the particles had not settled completely after 3 hr, incubation was extended until setting was complete. The manufacturer indicates that the reaction mixtures may stand overnight without any change in results. Specimen which shows agglutinates with the sensitized particles at final serum dilution 1:40 or higher is interpreted as positive.

DNA PROBE FOR DIAGNOSIS OF INFECTION

The development of DNA probes specific to *M. pneumoniae* was expected to provide a solution to diagnostic problems by overcoming the need for cultivating the agents and the difficulties resulting from the serological cross-reactions. DNA probes should enable rapid detection of the pathogen directly from the clinical specimen in sufficient time to have an impact upon the care and treatment of the patient. Throat swab was obtained by rubbing with dry sterile polyester fiber-tipped Falcon swabs (Becton Dickinson Labware, Lincoln Park, N.J.) over the posterior portion of the pharynx. Swab was placed in 1 ml of transport medium consisting of Hanks balanced salt solution with 0.5% gelatin and sent immediately to the laboratory on wet ice for assay within 72 h. According to the instructions of the manufacturer, a 1 ml volume of each specimen was centrifuged at 12,000Xg for 10 min to concentrate the organisms. The *M. pneumoniae* ribosomal RNA, released on lysis of the organisms by a reagent, was allowed to hybridize with the [^{125}I] complementary DNA probe during 1 h of incubation at 72° C. The hybridized probe was separated from the nonhybridized probe by using a hydroxyapatite suspension and the radioactivity of the

hybridized probe was counted in gamma counter. A ratio of sample counts to nagative control counts greater than or equal to 3.0 was taken as positive for *M. pneumoniae* infection (50).

In routine laboratory practice, serology is used for the diagnosis of *M. pneumoniae* infections, since culture is relatively insensitive and 3 weeks or more may be required to obtain a result. In patients with primary infections, immunoglobulin M (IgM) can be detected from 7 days after the onset of symptoms and reaches a peak within 2 to 3 weeks. In patients with reinfections, IgM is mostly absent. Therefore, paired sera are used to confirm reinfection by *M. pneumoniae*, which is demonstrated by a fourfold rise in titer in IgG antibodies (22,75). This can be observed only when the first serum sample is taken within 10 days after the onset of disease (38). Sillis (22) showed that in the absence of IgM antibodies in patients with reinfections,IgA measurement in a single serum sample can be used for diagnosis. Thus, both in patients with primary infections and in patients with reinfections, serological diagnosis is not obtained before 1 week after the onset of symptoms.

POLYMERASE CHAIN REACTION FOR DIAGNOSIS OF INFECTION

Recently, PCR has been developed and seems to be the most promissing direct technique for diagnosis because of its high sensitivity and specificity(76). Different PCRs for the detection of *M. pneumoniae* have been described. The targets are the gene coding for the P1 adhesion protein, the 16s rRNA gene, and a DNA sequence specific for *M. pneumoniae* selected from a genomic

library (77-79). In different studies, the use of 16s rDNA sequences has been described for the detection of several microorganisms such as mycobacteria, *Helicobacter pylori*, and mycoplasmas(79-81). The advantage of using 16s rRNA sequences is the high degree of conservation of the target and the presence of a high copy number of rRNA molecules in the cell, which are available as templates for the PCR after reverse transcription (RT) to DNA (RT-PCR). However, a disadvantage of an RT-PCR is the need for purification of RNA, making it less suitable for routine procedures. To date,.many laboratories have successfully performed PCR directly on clinical material, after a sample pretreatment (direct PCR) for the detection of microorganisms (82,32).

In 1992, Buck, G.E. et al.(31) have used the PCR for detection of *M. pneumoniae*. The pair of synthetic oligonucleotide primers derived from segment of P1 virulence protein were used. This DNA segment was amplified in pure cultures of 5 different strains of *M. pneumoniae* but not in other tested species : *Mycoplasma, Acholeplasma,* or *Ureaplasma*. Simulated clinical specimens were used to compare PCR, culture, and the gene probe. The sensitivity of the tests were between 1 to 10, 10^3, and 10^4-10^5 organisms respectively. In 1993, Kai, M. et al.(33) have also reported the use of DNA amplification method to detect *M. pneumoniae* in clinical samples. *M. pneumoniae* 16S rRNA gene sequences were selected as the amplification target region. The PCR with purified DNA fragments as templates yielded an expected 88-bp fragment from *M. pneumoniae* but not from other *Mycoplasma* spp. nor from any of the other bacteria assayed. With this method, the sensitivity was

0.05 pg of *M. pneumoniae* DNA. Subsequently this PCR technique was used for detection of *M. pneumoniae* in throat samples. Twenty-two of 30 culture-positive and two of 32 culture-negative clinical samples gave positive in PCR test.

From the studies of several investigators groups showed that the PCR technique provided high sensitivity and specificity for detection of *M. pneumoniae*. This method is not time-consuming and comparatively easy to perform.