CHAPTER III

REVIEW OF THE LITERATURES

History of *Mycoplasma pneumoniae*

In 1943, Finland and associates found cold agglutinins in the sera of some patients with primary atypical pneumonia (49). In 1944, Eaton and colleagues isolated a filterable agent from the sputa and lungs of pateints from the disease using rats, hamsters, and chick embryos as laboratory hosts (50). This agent was first identified as a probable cause of the pneumonia (51). In 1957, Lui first observed the Eaton's agent in the bronchial epithelia of chicken embryo by indirect immunofluorescence (52). In 1961, Marmion and Goodburns found that Eaton-Lui agent was inhibited by Gold salts, suggesting of a pleuropneumonia-like organism (PPLO) (53). 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 (54).

M. pneumoniae has been regarded as an important cause of community-acquired pneumonia in children and young adults. This organism is also a cause of pneumonia and febrile upper respiratory tract

infections in adults of all age groups especially, in densely populated areas. The range of illness associated with *M. pneumoniae* extends from mild upper respiratory tract symptoms to pneumonia (2). That may be benign and self-limited, moderately troublesome, or sometimes life-threatening (16,56,57,58). Morever, on occasion, extrapulmonary manifestations may actually overshadow or occur in the absence of symptomatic respiratory tract involvement. The agent is usually found in the oropharynx, bronchus, and other parts of the upper respiratory tract during acute infection. The organism has also been isolated from lung and pleural fluid during acute respiratory disease and from the bronchoalverlar lavage fluid of immunocompromised patients with pneumonia, including those with AIDS (4, 59-63).

The organism

M. pneumoniae is the smallest and simplest free-living procaryote that more closely resembles a bacterium rather than a virus. However, it is distinguished phenotypically from other bacteria by a minute size and total lack of a cell wall, so that not surprisingly, it is resistant to cell wall-active antibiotics. *M. pneumoniae* contains DNA, RNA and processes a lipophilic single triple-layered limittingmembrane (64), poor to gram stain, grows on artificial media supplemented with a fresh yeast dialysate and horse serum, exhibits hemadsorption, ferments glucose, produces peroxide, and hemolyzes erythrocytes layered over colonies on agar media. The organism is 10x200 nm in size, the most commonly exists as filamentous form and on the rod at one end of the filament displays a neuraminic acid receptor site for attachment to host

cell membranes. The chief component of this rod is the adherence protein or adhesin, which has a molecular weight of 168 kD (65).

Epidemiology

In the general, initiation of *M. pneumoniae* infection has been occurred by intrafamily spread. It has been considered as an important etiology of lower respiratory infections. The organism is transmitted from person to person by infected respiratory droplets during close contact. The infections are worldwide endemic throughout the year but generally is most frequent during the fall and winter, and cause epidemics in some areas. A periodicity is present with peaks every 3 to 4 years (6,51,66,67,68). At intervals of several years, epidemics of *M. pneumoniae* pneumonia may occur with about double the usual incidence of disease (69). The incidence of *M. pneumoniae* pneumonia varied greatly by age. The rates of infection were highest among children, and young adults particularly those between 5 and 14 years old (2). Reinfection can occur in older patients with detectable *M. pneumoniae* antibody. Mycoplasma infections commonly occurs in patients with chronic bronchitis and asthma. Closed populations such as military personnel, prisoners, school children and college students living in dormitories (70-71) are prone to have mycoplasma infections. The disease appears to be endemic.

Clinical features

The incubation period of *M. pneumoniae* infection is 1 to 2 weeks. Illness usually begins with symptoms of upper respiratory infection, which in some patients progresses to bronchitis and pneumonia. Onset is insidious, starting with fever, headache, sore throat and cough. Cough is initially dry, may be paroxysmal, and frequently is worse at night. The posterior pharynx can be mildly erythematous but generally is without exudate. In patients with pneumonia, the cough may become productive of mucoid or mucopurulent sputum. Blood-streaked sputum may occur in the more severe cases, and hemoptysis is rare. The most of common finding include chills, malaise, rhinorrhea, otitis media, and generalized myalgia develop in at least half of the patients. If ear involvement is present, an erythematous immobile tympanic membrane or inflammation with formation of bullae can be seen (2,64,69). In patients without underlying disease, the pneumonia is rarely severe of fetal (1,4,72). Chest pain occurs rarely except with the present of severe cough or pleural involvement.

The disease is variable in severity, symptoms and signs of disease vary according to the stage of illness. The complications include meningoenceplitis, polyneuritis, monoarticular arthritis, Stevens-Johnson symdrome, pericarditis, myocarditis, hepatitis, diffuse intravascular coagulation and hemolytic anemia are rare (69,70). Approximately 20% of all *M. pneumoniae* infection may be asymptomatic (2,70).

Pathogenesis

Infection appears to be acquired via inhalation of infected material after exposure to an acutely ill coughing individual (16). Aerosol particles less than 5 nm in diameter reach the lower respiratory tract directly, but the larger particles deposit on the nasal and upper respiratory tract passages (64).

The pathogenesis of *M. pneumoniae* infections is perhaps the most thoroughly characterized of the host-parasite interactions involving mycoplasmas. Several experimental animal models have been described for the examination of *M. pneumoniae* virulence. Multiple factors seem to contribute to the effect of *M. pneumoniae* infection on the respiratory tract. The organism attaches to neuraminic acid residues on ciliated epithelial cells and stops ciliary activity with subsequent destruction of cilia and cell surfaces. The infection is superficial. Tissue injury caused by the release of hydrogen peroxide by the organism itself. Polymorphonuclear leukocytes are attracted to deciliated cells, and leukocyte products probable participate of the superficial inflammatory process. Simultaneously, monocytes, macrophages, and lymphocytes are provided to the areas (64,70).

Infections lead to the production of both circulating and secretory antibodies specific for mycoplasma antigens. The usual course of serum antibody appearance is immunoglobulin M (IgM), IgG, and IgA. IgA is the predominant antibody class in respiratory secretions. Although an IgM and IgG antibodies occur in *M. pneumoniae* infection,

serum antibody does not necessarily confer lifelong immunity the protection wanes and reinfections can occur five years after the first infection (64,71).

Multiple autoantibodies develop during *M. pneumoniae* infections including anti-red cell (cold agglutinins), antibrain, antilung, and antiliver antibodies. These nonspecific, antibodies may contribute to the pathogenicity of disease through autoimmune mechanisms. The pneumonia caused by *M. pneumoniae* may be influenced by antigen-antibody reactions in the lung (64,73). Some of the extrapulmomary manifestations of this infection may be contributed by circulating immune complexes (51,73,74). Immune mechanisms may be involved in the pathogenesis of central nervous system disease (74).

Laboratory diagnosis

Diagnosis of *M. pneumoniae* infection relies mainly on laboratory test, serological analysis of the patient's serum and identification of the organism from secretion of the respiratory tract or other appropriate site.

Cultivation techniques for *M. pneumoniae* have been proposed since the initial discovery of the agent on a medium devised by Hayfick, PPLO agar supplemented with fresh yeast extract and 20% horse serum (54). The organism also grows very poorly and slowly on the best mycoplasma media available because of lacking all the genes involved in amino acid synthesis (75). So, complex media are used for

the cultivation. The media are usually based on beef heart infusion, peptone, yeast extract, and serum with various supplement (76). The medium formulation have been evaluated for isolation of *M. pneumoniae*. Tully et al. modified culture medium (SP-4) for growth of other mycoplasmas (spiroplasmas), which has greatly improved the isolation rate of *M. pneumoniae* from clinical samples (18,77). In 1990, Soy peptone medium was employed for isolation by Kenny et al. (19). Recently, evaluation of a new culture kit (Pneumofast kit) has been developed for growth of *M. pneumoniae* (12,78). However, successful recovery of mycoplasmas from their natural habitats requires that appropriate samples which obtained in the field and transported to the processing laboratory under optimal conditions for maintaining viability.

Cultures are identified as *M. pneumoniae* if the following criteria are fulfilled (19): typical small colonies are observed on agar plates by using a dissection stereomicroscope or low power of an ordinary light microscope, acid is produced from glucose in diphasic medium, typical spherules (79) are observed in the fluid phase of the diphasic cultures, and hemolysis or hemadsorption of guinea pig erythrocytes (80,81,82). Immunofluorescent serological test is used in conjunction with culture medium for identification of *M. pneumoniae* colonies, an increased isolation rate 30 to 40% over conventional culture technique (18).

- Specimen collection and transport (76,83)

The specimen should be placed immediately in liquid and solid mycoplasma media, or in suitable transport medium. Specimen selection also should be influenced by the desirability of avoiding unwanted microorganisms and growth inhibitory substances (antibodies, antibiotics). Conventional mycoplasma broth, containing 10% fresh yeast extract, 20% horse serum, and 500 to 1,000 units/ml penicillin G, is generally an effective transport medium. If the transport specimen cannot be cultured within 24 hrs of collection, it should be frozen (-70°C) until culture is available.

- Cultivation Procedures (18)

A 0.1 ml sample of the transport medium (or suitable body fluids) should be inoculated into a vial of the modified Hayflick medium or other *mycoplasma* media. The remainder should be freeze at -70°C. Swabs obtained directly from the patient should be streaked immediately onto a mycoplasma agar plate and the swab swirled vigorously in the modified Hayflick broth. Tissues should be minced coarsely and then added to about 5 ml of 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) 5% CO_2. Modified Hayflick cultures of plain broth cultures should be observed every two to three days for indicator changes (the acid or alkaline pH range). 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 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 (84). The plates are flooded with about 1 ml of phosphate-buffered saline and 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. Following incubation at room temperature for 20 min, the conjugate is poured off the plates again then washed with about 2 to 3 ml of saline and poured off. After the plates are 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 indicant 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 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 with *M. pneumoniae* antiserum, as described by Wallace and Clyase (85), PPLO agar plates (10-cm) were place open in a 37°C incubator for 1 hr to dehydrated the medium surface, after which each plate was inoculated with 0.1 ml of logphase broth cultures of *M. pneumoniae*. Sterile 6-mm filter paper disks of the type used for antibiotic sensitivity testing were saturated with 0.025 ml of undiluted antisera, using calibrated loop (microtiter). Disks were then pressed onto the inoculated agar surface, and the plate were incubated until colonies became visible (1 to 4 day) The plat were examined with a low power stereomicroscope, and zone of inhibition around disks were measured in millimeters form the disk edge to the beginning of growth. Normal rabbit serum-impregnated disks served as controls on each plate. 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 (80).

- Serological diagnosis of infection

Diagnosis of mycoplasmal infections by isolation of the organism is difficult because the agent is fastidious and grows slowly, whereas most bacterial pathogens may be cultivated quickly. As a result, cultivation services are offered in few laboratories. Therefore in routine laboratory diagnostics, serology is the principal method used to diagnose *M. pneumoniae* infections. 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. Paired sera are required to confirm reinfection by *M. pneumoniae*, which is demonstrated by a four-fold rise in titer in IgG antibodies (22). This can be observed only when the first serum sample is taken within 10 days after the onset of disease (51). In the absence of IgM antibodies in patients with reinfections, IgA measurement in a single serum specimen can be used for diagnosis (22). However, serodiagnostic methods for diagnosis of this organism are much less advanced because of the antigenic complexity and variation.

1. The Complement Fixation Test (CF)

CF is the most widely used serological assay for diagnosis of *M. pneumoniae*. The test relies on the ability of antigen-antibody complexes to fix complement. The sensitivity of this assay depends on whether the first serum sample is collected early or late after the onset of illness and on the availability of paired sera collected with an interval of 2 to 3 weeks (70). A rise in *M. pneumoniae* CF antibody titer of more than four fold in paired serum samples is usually thought to be recent infection. Due to the common occurrence of infections and reinfections by *M. pneumoniae* in the general population, study of a single serum has no diagnostic value because the presence of antibodies could be from an illness prior to the one under consideration (86). The false-positive reactions have been found because the lipids antigen were found to relate with other lipids (70,86). There are cautions in interpreting data from unusual syndromes in persons from whom *M. pneumoniae* has not been isolated. The most notable example is the

antibody increase by lipid CF seen in persons with meningitis caused by ordinary bacteria (87,88).

CF for *M. pneumoniae* antibody is now usually performed with the standard microtitration equipment, requiring only 25 µl 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 (89). The lipid antigen is complex 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 (67), the CF titers of paired test serums are examined. Test results are available after 18 to 24 hrs 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. The titer 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.

2. Cold Agglutination Test (CA)

Cold agglutinins are usually the first antibodies detected (first or second week of illness, peaking at the third week), the first to disappear (by six week) (86). It has been demonstrated that the frequency and the height of the cold agglutinin response are related directly to severity of pneumonic involvement (20,90). This is the best considered to be a nonspecific test for screening *M. pneumoniae* infection(20).

The procedures of this test were described(90), sera were diluted from 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 an minimum of 2 hrs For reading the result, the tubes were tapped once to dislodge any sediment red cells; any agglutination pattern 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 occurred, resulting in disappearance of the agglutination pattern. A titer of >1:64, although non-specific, was taken as suggestive of recent or current *M. pneumoniae* infection.

3. Indirect Immunofluorescence Test (IFA)

The indirect immunofluorescence test is used to detect antibodies in patient sera. The slide test with *M. pneumoniae* suspensions as antigen, as described by Wreghitt and Sillis (91), is used to assess *M. pneumoniae*-specific IgM, IgA, and IgG in patients'sera tested which are twofold-diluted at 1:4 to 1:128. The binding of these antibodies is detected with a fluorescein-conjugated antiserum against the bound immunoglobulins. The presence of a specific IgM titer of >1:4, or a specific IgA titer >1:8, or fourfold rise or fall in the latter, indicates recent or current *M. pneumoniae* infection. However, the procedures of this test is difficult. Recently, the study of Dorigo-Zetsma, et al (92) presented diagnosis of *M. pneumoniae* infection by compared two rapid tests, PCR and IgM IFA commercial kit with culture and the complement fixation kit test, but it found that the rapid IgM IFA has a low positive predictive value.

4. The Enzyme-Linked Immunosorbent Assay (ELISA)

The technology of ELISA was first applied to mycoplasmal serology by Busolo et al. (93). These first ELISA test were base on sonicated whole-cell antigen preparation or even on microtiter plate-cultured and formaline-fixed mycoplasmas. The next generation of ELISA was based on detergent preparation antigen for lowering the content of cross-reacting glycolpids in antigen preparation (20).

The test were performed essentially as described by Voller and Bidwell (94), using a unit volume of 100 µl. *M. pneumoniae* antigen, purchased from Orion Diagnostica, was obtained after tween-ether treatment of the aqueous phase from chloroform-methanol treated *M. pneumoniae* saline suspension. It contain 140 µg of protein in stock preparation. Goat antihuman IgG (H+L chain specific) and antihuman IgM (FC_5 fragment specific) sera, conjugated to alkaline phosphatase, were obtained by Dynatech Laboratory. Optimal dilution of reagent were previous determined by chessboard titration. Antigen, diluted at 1/48 in coating buffer, was incubated overnight at room temperature in each well of microtiter plate. All serum samples were dilute at 1:20 with diluent and distributed in duplicated in the well. Anti-IgG and anti-IgM conjugates were used at 1/1500 and 1/400 respectively. Sera and conjugates were each incubated for 90 min at 37°C. The following controls were included in each plated: a known negative serum, a positive reference serum with a CF titer to *M. pneumoniae* of 512 and an *M. pneumoniae*-specific IgM CF titer of 64 after serum fractionation by sucrose density gradient. After washing conjugated, the substrate was added and incubated for 30 min at 37°C. The reaction was stopped with stop solution and color reaction was measured at 405 nm in a Titertek Multiskan ELISA reader. Sera that gave a photometer reading for IgG, as well as for IgM, of 0.3 (twice the background level) or grater werc regarded as positive. Their antibody titer were calculated in conjunction with the standard curve obtained from serial twofold dilutions of positive reference serum whose titration in 10 separation trials gave result for IgG and IgM which did not vary more than twofold (23).

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 1:10240, which yielded final dilution of 1:40 to 1:20480, were prepared in U-bottom microwell plates. Twenty-five microliter of each serum dilution were added with one drop (25 μl) 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 carefully mixed and then incubated room temperature for 3 hrs. Results were evaluated if the unsensitized particles had settled to form a compact center point. If the particles had not settled completely after 3 hrs, 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 (95).

- DNA Probe for diagnosis of infection

A major shortcoming in the laboratory diagnosis of *M. pneumoniae* infections has been the lack of a rapid, sensitive, and specific diagnostic test. Serological procedures, which are the most widely used, not only lack sensitivity and specificity but require a comparison of antibody titers in acute and convalescent phase sera. Culture identification is fastidious, labor intensive and requires time consuming for recovery of the organism. The development of DNA probes specific to *M. pneumoniae* was expected to provide a solution to diagnostic problems (96). DNA probes should enable rapid detection of the pathogen directly from the clinical specimen in sufficient time for the care and treatment of the patient. The procedures were described, (32,96) 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 transport medium consisting of tryptic soy broth, 0.5% bovine serum albumin, and 500 IU of penicillinG per ml and sent immediately to the laboratory on wet ice for assay within 72 hrs. According to the instructions of the manufacturer, 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 hr 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 a gamma counter. A ratio of sample counts to negative control counts

greater than or equal to 3.0 was taken as positive for *M. pneumoniae* infection.

- Polymerase Chain Reaction for Diagnosis of Infection

Recently, PCR has been investigated as a method for the rapid detection of *M. pneumoniae* in clinical specimens because of its high sensitivity and specificity (35,36). Several procedures for sample preparation and different targets have been described (35-40). 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. In different studies, the use of 16S rDNA sequences has been described for the detection of several microorganisms such as mycobacteria, *Helicobacter pylori*, and mycoplasma (40,97,98). The using of 16S rRNA sequences is advantage the high degree of conservation of the target in the cell, which are available as templates for the PCR after reverse transcription (RT) to DNA (RT-PCR). Although an RT-PCR is disadvantage, since it needs the RNA purification, making it less suitable for routine procedures. However, the success of direct PCR, PCR directly on clinical material, after a sample pretreatment , in many laboratories provide the solution of RT-PCR for the detection of microorganism (29,99,100).

In 1992, Buck, G.E. et al (38) have used the MP-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 (39) have also reported the use of DNA amplification method to detect *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. In 1996, Ieven, M. et al (101) have presented the evaluation of PCR for the diagnosis of respiratory *M. pneumoniae* infections, comparing two procedures of sample preparation (freeze-boiling and guanidine isothiocyanate followed by phenol-chloroform extraction) and the use of two sets of primers (P1 adhesion gene and 16S rRNA gene) in two laboratories to define the procedure best suited for the clinical diagnosis laboratory. The result showed that in the same laboratory, sensitivity of guanidine method was better than freeze-boiling and 16S rRNA gene was less in sensitivity than P1 adhesion gene.

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 (29,35,36).

Detection of the amplification product is usually performed by hybridization with a specific probe (78,102), which is difficult. In 1998, Abele-Horn, M. et al (103) have evaluated the use of a culture-enhanced PCR assay for the detection of *M. pneumoniae*, followed by hybridization with specific probe (MP-HPCR) or without hybridization (MP-PCR), and the use of a nested PCR (MP-NPCR). The sensitivities were 19 CFU for MP-PCR, 1.9 CFU for MP-HPCR, and 0.019 CFU for MP-NPCR.