

The Role of Mycoplasma pneumoniae  
in Acute Bronchitis

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## Introduction

According to an Abbott Laboratories pamphlet (1991), physicians diagnose 25 million cases of bronchitis per year. It ranks among the top ten most frequently made diagnoses and costs Americans an estimated 200-300 million dollars each year (Dunlay et al. 1987). Even with the high clinical frequency, there is much debate over the best method of treatment. The disagreement stems from the causes of the disease and the length of time necessary to determine the cause.

This particular study hoped to determine a scheme of clinical predictors, such as a cough, a fever, or crackles in the lungs, which would specifically indicate the presence of acute bronchitis, as induced by an infection by Mycoplasma pneumoniae. The formulation of the scheme necessitated the collection clinical specimens and the completion of a patient/physician questionnaire, inventorying the history and present status of the illness. A culture of the specimen and the correlation of the questionnaire data occurred for specimens positive for Mycoplasma pneumoniae.

The determination of clinical predictors specific for Mycoplasma pneumoniae could enable the physician to quickly isolate the patient and prescribe the appropriate antibiotic, such as erythromycin. Currently this procedure costs less and takes less time than do some of the laboratory analysis.

## Acute Bronchitis

Science provides several definitions for acute bronchitis. A compilation indicates it as an inflammation of the tracheobronchial tree, with a productive cough with no clinical signs of pneumonia, either by chest x-ray or auscultatory examination (Billas 1990; Rodnick 1988; Dunlay et al 1987). A cough and sputum production along with upper respiratory infection (URI) and systemic manifestations characterize acute bronchitis (Williamson 1984). Several microorganisms induce the symptoms and manifestations found in acute bronchitis, and each responds to a different drug therapy, if any therapy at all.

Almost 50% of all cases of bronchitis result from a viral infection. Over 180 viruses cause URI's including rhinovirus, adenovirus, coronavirus, coxsackievirus, respiratory syncytial virus (rsv), parainfluenza virus, and influenzas A and B (Billas 1990; Rodnick 1988). One study determined RSV as the most common isolate in children (Denny and Clyde 1986). Another found that the inhalation of the rhinovirus, by volunteers, does induce acute bronchitis (Cate et al 1964). Still 50% need accounting for.

Haemophilus influenzae, Streptococcus pneumoniae, and Branhamella catarrhalis are the most common bacterial causes of acute bronchitis, but their actual role is not well defined. Sputum cultures grown from patients indicate the presence of normal flora as well as the pathogen. This leads some to believe that the organism becomes pathogenic under the appropriate conditions. However, other microorganisms possess the ability to induce the

disease.

Chlamydia psittaci-TWAR, which causes Taiwan Acute Respiratory disease accounts for 5% of acute bronchitis (Rodnick 1988). The lack of a quick and definitive test for Chlamydia psittaci-TWAR has made diagnosis difficult; however, recent literature indicates the possible development of a test in Europe.

Our organism of focus, Mycoplasma pneumoniae, accounts for 10%-20% of the cases of bronchitis (Rodnick 1988) in the general population, but may show percentages as high as 50 (Cassell et al 1985) in closed populations such as military installations, boarding schools and college campuses, where prolonged and repeated contact is more likely. The slow and fastidious growth of the organism makes detection by standard culture impractical. Other tests such as the cold agglutinins often read falsely negative early on in the course of the disease (Rodnick 1988). It is because of these problems that the organism's role in acute bronchitis has been ignored.

What has made the treatment of the disease extremely difficult is that not one antibiotic can effect all of the organisms. Five randomized and controlled studies have attempted to define the role of specific antibiotics in acute bronchitis treatment (Brickfield et al 1986; Dunlay et al 1987; Franks and Gleiner 1984; Stott and West 1976; Williamson 1984).

Three different medications were tested in five studies; two tested erythromycin, two doxycycline, and one a combination of trimethoprim-sulfamthoxazole (TMP-SMX). The TMP-SMX study revealed

that over a one week course of therapy, the patients symptoms lessened, the patients took fewer over-the-counter drugs and missed fewer days of work (Franks and Gleiner 1984). Of the organisms mentioned, the three common bacteria respond to TMP-SMX therapy (Rodnick 1988). The doxycycline studies indicated the opposite of the TMP-SMX study: the patient on doxycycline returned to health as quickly as the patient not taking doxycycline (Stott and West 1976; Williamson 1984). Doxycycline inhibits S. pneumoniae, H. influenzae, and M. pneumoniae (Abbott Laboratories 1991). The final two studies indicated that a course of antibiotic does lessen patient symptoms (Brickfield et al 1986; Dunlay et al 1987). Erythromycin effectively manages all of the bacteria except H. influenzae, and treats M. pneumoniae and Chlamydia TWAR. These disparate results seem to indicate that similar organisms did not operate, as the infectious agent, in all five studies.

#### Mycoplasma pneumoniae

Eaton and colleagues first isolated Mycoplasma pneumoniae from patients with primary atypical pneumonia in 1944 and dubbed it the Eaton agent (Cherry 1987). Initially thought of as a virus, it was not until 1961 when Marmion and Goodburn pointed out its similarities to the pleuropneumonia like organisms (PPLO), was it recognized as such (Cherry 1987).

The Mycoplasma pneumoniae cell ranges in size between 0.3 and 0.8 microns, appearing as small cocci, pear shaped or as filaments with bulbous ends; the shape seems to vary with the age of the cell (Kreig and Holt 1984; Kenny 1985; Cherry 1987). One can interpret

the pleomorphism as a result of the lack of a cell wall, a characteristic common to all members of the class Mollicutes. A terminal structure plays a role in the organism-host cell attachment and the gliding motility usually observed after reproduction (Kreig and Holt 1984). The organism multiplies by binary fission. Cytoplasmic division must, however, be fully synchronized with genome replication and when this does not occur multi-genome filaments often appear (Kreig and Holt 1984). Cytokinesis will eventually lead to chains of cells and then single cells.

Colonies of M. pneumoniae range in size from 50-100 microns and seem to display two distinct morphologies. The classic morphology looks like a "fried egg"- with a dense inner nucleus and a light outer periphery, and the other appears as a mulberry showing only the dense, rough inner center. The colonial appearance does depend upon the age of the culture, the growth conditions, the agar concentration and such other variables (Kreig and Holt 1984).

The limited biosynthetic capabilities of M. pneumoniae require the presence of a complex growth media containing beef heart infusion, peptone, yeast extract and serum. The serum provides the necessary sterols and fatty acids. The organism grows best between 35-37° C and in a 5%-10% CO<sub>2</sub> environment.

Mycoplasma pneumoniae infection occurs world wide and year-year-round. An endemic occurs most commonly in the larger urban areas. Epidemics follow a 3-7 year cycle, last 12-30 months and happen most readily in less populated areas (Cherry 1987). A

relationship exists between the degree of susceptibility of an individual and their age (Cherry 1987). Infection results from the contact of respiratory secretions, either small particle aerosols or large droplet secretions. These droplets must contact the respiratory epithelium and usually more than one contact is necessary.

In 1962, scientists successfully cultured the organism in a cell free environment and identified it as an etiologic agent in primary atypical pneumonia (Cherry 1987). Through the research of pneumonia much of the pathogenic capabilities of the organism are known. In almost 100% of the patients evaluated, Mycoplasma induces a fever and a cough and potential complaints include a headache and malaise (Cherry 1987). However, this does provide some insight in the possible modus operandi of the organism in the body.

M. pneumoniae utilizes the neuraminic acid cell receptors for cell-organism attachment (Cherry 1987). Hydrogen peroxide becomes increasingly toxic to the host cell because of the inhibition of the catalase activity. The destruction of the epithelial cells leads to the many problems associated with respiratory ailments.

The desquamation and ulceration resulting from the attachment leaves debris within the bronchi, causes the invasion by macrophages, lymphocytes and plasma cells, and leads to edema and possible hemorrhage of the lung areas (Cherry 1987). A host of other manifestations occur elsewhere in the body including vesiculopusticular skin lesions, mesenteric lymphadenitis, focal hepatic necrosis, and acute myocarditis (Cherry 1987). Failure to



detect the organisms presence can lead to serious health problems for the individual.

## **Methods and Materials**

### **Subject Selection**

Consecutive, consenting patients who presented themselves to the Divine Providence Emergency Department, Williamsport, Pa., between January and April 1992 were admitted to the study. Their signature on a written consent form (Appendix A) indicated the individual's agreement to participate. Prior to admission the patients had to meet the established research criteria:

- 1) be between the ages of 18-60,
- 2) have had a cough for less than three weeks,
- 3) have no known underlying lung disorder, and
- 4) have not received an antibiotic with in the last week.

The patients and attending physician were then asked to fill out a questionnaire (Appendix A). The questionnaire provided space for answers, for both the patient and the physician. The patient portion requested information regarding the history of the illness. The physician portion requested information regarding the present state of the condition. After both completed the questionnaire, the doctor took a throat swab using a dacron tipped swab. The doctor then broke the swab off into a vial of transport media, prepared at Lycoming College.

### **Media Preparation**

#### **Transport Media**

The study chose to use a transport media described in Kenny

(1985) and Kenny et al (1990). The preparation included Trypticase soy broth (Becton Dickinson, Cockeysville, Md), bovine serum fraction V albumin (Sigma Chemical Corp, St.Louis, Mo), and penicillin G (Sigma Chemical Corp, St. Louis, Mo), combined in the following manner:

- 1) Dissolved, by heating, 3 grams of Trypticase Soy Broth in 100 ml of reverse osmosis (r/o) water.
- 2) Sterilized solution by autoclaving at 121° C for 15 minutes.
- 3) Aseptically added 0.5 ml of bovine serum albumin to the sterile solution.
- 4) Added the appropriate concentration of Acrodisc filtered penicillin G to the sterile mixture to final concentration of 200 units/ml
- 5) Pipetted 2 ml aliquots to 4 ml flint glass, screw cap vials (Thomas Scientific, Swedesboro, N.J.)

The prepared vials were incubated overnight to determine the presence of any contaminants. The next day the two vials each, for a total of 4, were inoculated with a genetically defined Escherichia coli MG 1655 penicillin resistant bacteria, and a an Escherichia coli MG 1655 penicillin susceptible bacteria, respectively.

#### Culture Media

The study chose to use for the culture media, Mycoplasma agar media (Becton Dickinson, Cockeysville, Md.). The media required the addition of penicillin G (Sigma Chemical Corp, St. Louis, Mo.). To the base media Mycoplasma Enrichment without penicillin was added. The enrichment contained horse serum, yeast extract, and thallium acetate. The media was prepared as follows:

- 1) Dissolved 2.38 g powdered media per 70 ml of r/o water.
- 2) Warmed to complete the solution. Provide agitation

- via a magnetic stir bar. Boiled for 1 minute.
- 3) Sterilized solution by autoclaving at 121° C for 15 minutes
  - 4) Cooled the solution to 50-55° C, and add the enrichment and 500 units/ml of Acrodisc filtered penicillin G.
  - 5) Poured the media into 35 x 10 mm petri dishes.

#### Penicillin preparation

The penicillin was prepared using Penicillin-G (benzylpenicillin), a potassium salt form (Sigma Chemical Co, St Louis, Mo.). The transport and the culture media each utilized different concentrations of penicillin; the transport media had a final concentration of 200 units/ml and the culture media had a final concentration of 500 units/ml. In each case the solution was prepared as follows:

- 1) The appropriate conversion was made from units to milligrams, for each concentration. The final milligram value was doubled for the stock solution.
- 2) The appropriate mass of penicillin was added to 1.0 ml of reverse osmosis water.
- 3) Sterilization occurred by filtration using the Acrodisc filter into a sterile receiving vial.

To check the performance of the media, i.e. determine if it could sustain the growth of Mycoplasma, a pure culture was purchased from American Type Culture Collection (ATCC) #15531. The pure culture arrived as a pellet and required reconstitution. The reconstitution process occurred via the preparation of a broth media from the agar media. This was accomplished by filter sterilization of the agar media and adding the appropriate amount of enrichment. No penicillin was added to the broth media. The reconstituted culture was incubated and examined a week and a half later for growth. Several additional broth cultures were made from

the initial one. Once growth occurred in the new broths, 0.1 ml aliquots were inoculated onto two plates which were labelled and dated. These plates were incubated under the same conditions as the specimens from the hospital.

When the vials arrived from the hospital they were plated as soon as possible. Cultures received from the hospital were plated in 0.10-0.15 ml aliquots per plate, for a total of two plates, per vial. Occasionally only enough media was readily available for the plating of one plate per vial, and in this case, one was plated immediately. The vials were retained to have a second plate made when the new media was prepared. Only after the plating were the questionnaires examined.

The plates were labelled with the date and sample number and stored right side up for twenty four hours so that the media could absorb the excess liquid, insuring that visible colonies would form on the media not in the suspended liquid. The study incorporated the use of a Lunaire incubator (Lunair Co., Williamsport Pa) which provided a moist atmosphere, a CO<sub>2</sub> concentration of 5% and maintained the temperature of 36° C.

After 24 hours the plates were inverted in the incubator and examined every 48-72 hours for growth. The study utilized stereomicroscopes and compound microscopes for examination of the colonies. All potential Mycoplasma specimens were evaluated under both the stereoscope and phase contrast microscope. If a fried egg colony appeared it was examined to confirm the presence of Mycoplasma. The mulberry configuration was ignored if a fried egg

colony was also present.

### Results

The inoculation of the transport media and the culture media with the MG 1655, genetically altered for resistance to penicillin, and MG 1655, the penicillin susceptible strain resulted in the growth of both strains on both media. From this it was determined that an error had been made in the final penicillin concentration. After the adjustment, only MG 1655, penicillin resistant strain, grew.

Upon examination of the plates it was noted that crystals precipitated out of the media. This was seen only in plates that had been held on refrigeration after their 3 week incubation period was over. The crystals appeared white and as snowflakes without the aid of a stereomicroscope. The opacity of the crystal made it appear black under the stereoscope, however, finer detail was evidenced; the main spokes on the crystal had finer small spokes.

The examination of the first broth culture, by phase contrast microscope, yielded some possible Mycoplasma and some crystal artifacts. The Mycoplasma appeared pear shaped and somewhat filamentous with a slight outgrowth on the end. The initial Mycoplasma culture plate showed a swirling effect around some dense yellow-tan bodies. These were determined not to be Mycoplasma.

The second set of broth cultures did show growth. The second set of culture plates did show Mycoplasma. Examination by stereomicroscope demonstrated the presence of the traditional fried-egg. Examination by phase contrast microscope verified the

presence of Mycoplasma.

The study detected 2 infections by Mycoplasma, leading to a final percentage of 5.7%. Their presence was also confirmed through the use of a stereomicroscope; they showed the traditional fried egg as well as some resembling the mulberry.

The study needed to eliminate seven patients because four were over the age limit, two had lung disorders, one of which was also under age, and the final person was also under age. The overall data collected from all of the patients can be seen in Table one.

Table 1: Patient Personal Data

Average Age (in years)	30.8
% Female	66.7
% Male	33.3
% smoke	40.0

\* note that two persons did not report their sex

As mentioned earlier, two clinical specimens grew up Mycoplasma. These patients did share some similar historical and physical examination data, illustrated in Table 4. They both produced phlegm, experienced sore throats and runny noses and the attending physician noted rhonchi and an inflamed throat.

Table 4: Patient Comparison

<u>Sign</u>	<u>1</u>	<u>2</u>
Phlegm	yellow	white
sweating	Y	N
Fever	N	Y
Sore Throat	Y	Y
Runny nose	Y	Y
Too sick...	Y	N
Cough	night	both
Runny/Cough which 1st?	same time	Runny nose
Headache	Y	N
Rhonchi	Y	Y
Inflamed throat	Y	Y

### Discussion

Unfortunately the number of specimens gathered was not large enough to generate sufficient results, in which to run statistical analysis and hence determine the existence of any clinical predictors. Had a larger number of specimens, positive for Mycoplasma been obtained, the positive culture would have been correlated against the questionnaire using specificity, sensitivity, and negative and positive predictive values. Instead, here, the data was analyzed by percentages. The results generated were low when compared with other studies noted previously, but this may be accounted for in several ways.

Some literature indicates that the highest infections by Mycoplasma pneumoniae occur in the summer months. We conducted this study from January 1992 to April 1992. Studies by Cassell et al (1985) and others indicate that the greatest incidence exists in

closed populations. Obviously a random sample from an open and general population may not provide the maximum amount of possible specimens. Rodnick (1988) indicates that M. pneumoniae is the most common cause of acute bronchitis in college aged students. Therefore it may have been better to conduct the study at the Lycoming College health services.

The benefits of conducting the study at Lycoming College include the closed population and easier monitoring of specimen collection. Cherry (1987) cited that the "most common source outbreak" results from an intense eight hour exposure at a party; a most common situation here at Lycoming College.

We initially decided against Lycoming because of the potentially limited amount of specimens; few students seem to use the health services for illnesses and they will most likely have exceeded the limits of our study. Also this would require permission from the College, particularly the administration and have the agreement of the nurse on duty, the family physician, participating students, and parents if the student was under the age of 18. We felt these weighed heavier than the possible advantages.

The age of the specimen is important. If the hospital did not store the specimen properly, the organisms present in the media may have died. Storage of Mycoplasma may take place at -4° C for 48 hours and -70° C indefinitely, however not at -20° C (Cherry 1987: Kreig and Holt 1984). This may have effected the recovery of potential organisms.



We did not begin to collect data until almost the end of January and specimens did not arrive until the fifth of February. The collection of data could have been enhanced by receiving permission for the study from the Hospital institutional review board during the first semester of study. This slight modification might have increased the number of specimens collected.

Several techniques for identification could have been explored. Had the decision to purchase a pure culture occurred during the first semester, the agar could have been tested and modified appropriately for the isolation of the organism. We could have added indicators for M. pneumoniae. Tetrazolium salts act as indicators in the presence of M. pneumoniae. A tetrazolium salt, such as 2,3,5-triphenyltetrazolium chloride could have been added. M. pneumoniae has the ability to reduce tetrazolium to a pink colored formazan compound (Kraybill and Crawford 1965).

Other tests include the organism's ability to haemadsorb and haemolysize guinea pig or sheep erythrocytes. It is believed that this capability relates to the organism's pathogenic properties. According to Edward (1954) and Freundt (1958) M. pneumoniae will produce a pearly or iridescent film and dark spots in a solid media with 20% horse serum. The film results from the degradation of lipids (Kreig and Holt 1984). Fermentation tests could have also been used to determine the presence of Mycoplasma.

With the short duration of the study, it became difficult to evaluate each media separately. The culture media should have inhibited the presence of Gram negative organisms because of the

thallium acetate. The one test strain which was a genetically defined strain of Escherichia coli, for penicillin resistance, did grow up on the media. Since each culture media preparation used a different enrichment bottle for each individual batch of the culture media, the thallium acetate may have not functioned properly in each batch of culture media. The culture media preparation called for a penicillin concentration of 500 units/ml, whereas other studies indicted values above 1000 units/ml. After a week the penicillin susceptible strain managed to grow as well. An increase in the penicillin concentration may have decreased the number of organisms that grew on the plates.

The age of the media have had an effect on the ability of the media to sustain life. Mycoplasma specimens may have been lost because of the media's age. As compared with the media that sat unused longer, the fresher media grew fewer contaminants. The initial pure culture was plated onto a relatively older media than that of the second plate, which was plated the same day as the media was prepared.

Time seemed to be the overall problem of the study. The general basis has been laid however for future research. Improvements can be made by using the facilities available through the Health Services and trying identification techniques, besides those of the microscopes.

## **Apendices**

**I. Consent Form for Voluntary Participation**

**II. Patient/Physician Questionnaire**

CONSENT FOR VOLUNTARY PARTICIPATION THE CLINICAL STUDY;  
CLINICAL PREDICTORS OF *Mycoplasma pneumoniae* AS THE  
PATHOGEN IN ACUTE BRONCHITIS IN ADULTS

I, \_\_\_\_\_, have been asked to voluntarily participate in this study designed to find out if the things I tell my doctor about my bronchitis (chest cold) can be used to identify people who have a special type of bronchitis caused by a bacteria called "mycoplasma". My doctor will send a special throat culture to the Lycoming College Microbiology Laboratory. Since this special throat culture for "mycoplasma" will take almost a month, it will have NO effect at all on my doctor's treatment. My doctor will send a copy of my answers to the questions. The answers will be compared to my culture results. My participation in this study will make it possible for doctors to treat bronchitis better in the future. There are NO additional charges for participation in this research study. The experimental part of this study is the questionnaire and the special throat culture. I have asked my doctor, \_\_\_\_\_ all the questions I have about the study.

The risks are the same as any throat culture such as a "strep culture". I understand and accept these risks.

I understand that the data collected may be published to further medical knowledge. I specifically give my permission for publication of my data. I understand that in all publications or presentations, I will not be able to be identified by name or otherwise.

I understand that my participation in this study is voluntary and if I do refuse to enroll I will incur no penalty. If I do enroll, I may withdraw at any time without penalty. To withdraw I will notify my doctor.

Any injury will be evaluated and treated in keeping with the benefits or care to which I am entitled. I understand that compensation is not available for any injury I sustain through participation in this study.

If I have any questions about the medical aspects of this study, I may contact the Principal Investigator, Dr. Lew Logan at 326-8111. If I have concerns about ethical conduct of this study, I may contact Dr. Warren Robinson, Chairman, Institutional Review Board at 326-8426.

I received a copy of this consent \_\_\_\_\_ (initial).

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Patient

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Investigator

-----  
Witness

CLINICAL PREDICTORS OF *Mycoplasma pneumoniae* AS THE  
PRIMARY PATHOGEN IN ACUTE BRONCHITIS IN ADULTS

Dear Participant,

Thank you for your cooperation in this program to learn more about bronchitis. If you would complete the following questions and give this form to your doctor, he will obtain the throat swab, complete the form and send it in to Lycoming College for the study. Circle your answer.

How long have you had this cough? \_\_\_\_\_

Do you have any history of lung or breathing problems? Y/N  
If yes, explain \_\_\_\_\_

Do you currently smoke cigarettes? Y/N

Do you cough stuff up? Y/N  
If yes, what color? clear? white? yellow/green?

Have you noticed a fever? Y/N ...sweating? Y/N

Are you short of breath? Y/N

Do you have a sore throat? Y/N ...runny nose? Y/N

Are you too sick to work or do your usual activities? Y/N

Do you cough more during the... day? ...night? ...both?

If you have both a runny nose and cough, did they start...  
...at the same time? ...cough first? ...runny nose first?

Do you have a headache? Y/N ...general muscle aches?

---

Dear Doctor,

Please inoculate the transport media with a dacron throat swab and answer the following questions.

Throat swab number \_\_\_\_\_.

Age: \_\_\_\_ years Sex: M/F

Temperature: \_\_\_\_\_ F

Any clinical evidence of pneumonia? Y/N

Ronchi heard? Y/N Wheezes heard? Y/N

Throat inflamed? Y/N Otitis media? Y/N

Thank you.

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Jennifer Likar

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Lew Logan, M.D.

CONSENT FOR VOLUNTARY PARTICIPATION THE CLINICAL STUDY  
CLINICAL PREDICTORS OF *Mycoplasma pneumoniae* AS THE  
PATHOGEN IN ACUTE BRONCHITIS IN ADULTS

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I received a copy of this consent \_\_\_\_\_ (initial).

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Patient

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Investigator

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Witness

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## WORKS CONSULTED

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