Research article

Reliability of cold agglutinin test (CAT) for diagnosis of *Mycoplasma pneumoniae* pneumonia in hospitalized patients

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Abstract

Introduction:
M. pneumoniae is one of the causative agents of primary atypical pneumonia. This infection causes 20-40% of community acquired pneumonia and is associated with an array of extra-pulmonary manifestations. There is a need for a rapid diagnostic test in order to prescribe prompt and appropriate antibiotic therapy. Even though isotype specific antibody testing provides definitive diagnosis, paired sera testing does not help in real time diagnosis. Cold agglutinins detectable by the Cold Agglutination Test (CAT) appear and disappear early in infection compared to long lasting specific antibodies that are detectable by specific immunoassays. Although there are some reports suggesting CAT is unreliable, it is being often used to diagnose *M. pneumoniae* pneumonia in Sri Lankan clinical settings. The aim of the current study was to evaluate the use of CAT as a bed-side screening test for early diagnosis of *M. pneumoniae* pneumonia compared to ELISA for detection of specific antibodies in the Sri Lankan context.

Methods:
Ninety seven clinically and radiologically confirmed patients with pneumonia were enrolled in the study. CAT was performed on acute stage sera. A CAT titer $\geq 1/32$ was considered as positive. Isotype specific *M. pneumoniae* ELISA with paired sera was compared with CAT results.

Results:
Mycoplasma pneumonia was confirmed in 15 of the 97 patients in the study using Mycoplasma specific IgM and 4 fold rise in titre. Of these, 3 were positive by the CAT. The sensitivity and specificity of the CAT compared to IgM/4fold rise in IgG detection were 20% (3/15) and 81.7% (67/82) respectively. Negative and positive predictive values of the CAT compared to ELISA were 84.8% (67/79) and 16.7% (3/18) respectively.

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Conclusion:
CAT is not a reliable screening test compared to specific antibody detection by isotype ELISA for the detection of *M. pneumoniae* pneumonia due to its low sensitivity and positive predictive values.

Keywords: Mycoplasma pneumoniae, pneumonia, cold agglutinin test

Introduction

Pneumonia is defined as inflammation of alveoli that presents as an acute illness, characterized by cough, purulent sputum and fever together with consolidation of the lung.[1] It is the most common acute inflammatory disorder of the lung. Even though use of antibiotics has decreased mortality due to pneumonia, it still remains a potentially dangerous condition. Although most patients with pneumonia can be safely managed at home, hospital admission is required in 20-40% of patients. Hospital death rates of pneumonia fall between 5-10% but may be high as 50%.[2] According to 2014 hospital morbidity statistics in Sri Lanka, inpatient morbidity was noted in 22,815 cases of pneumonia with highest prevalence among the 17- 49 years age group.[3] Pneumonia was the ninth leading cause of hospital mortality accounting for 4.4% of total hospital mortality in Sri Lanka.[3]

Bacteria are responsible for the majority of community acquired pneumonia (CAP) in adults. Most patients present with typical lobar pneumonia which is predominantly caused by *Streptococcus pneumoniae.* However, some may present with an atypical aetiology such as *Mycoplasma pneumoniae*, Influenza A, *Chlamydia pneumoniae* and *Coxiella burnetii*. Atypical pathogens account for almost 1/5 of cases of pneumonia.[1] Amongst the atypical pathogens, *M. pneumoniae* is the commonest and accounts for 50%.[4] It is more common among young adults, accounting for 15-20 % cases of CAP.[5]

Mycoplasmas are the smallest self replicating organisms known and lack a cell wall and thereby show natural resistance to all beta lactam antibiotics. Pneumonias due to atypical pathogens are difficult to diagnose clinically since there are no characteristic clinical features, unlike in the case of typical lobar pneumonia.[1] If appropriate treatment is not instituted early, it can lead to complications and epidemics. *M. pneumoniae* has been associated with extra pulmonary manifestations involving almost all systems of the body. In addition, as antimicrobials are prescribed only in bacterial infections, it is necessary to diagnose a bacterial aetiology including *M. pneumoniae,* in order to minimize irrational antimicrobial use.

Since there is a large range of pathogens that cause pneumonia, routine laboratory tests may not provide the complete range of tests required for aetiological diagnosis. Culture of *M. pneumoniae* is technically demanding, time consuming and requires enriched media. Isolation of the organism from clinical specimens may take several weeks.[6] Even though serology is the mainstay of laboratory diagnosis of *M. pneumoniae*, it has a number of limitations. Specific IgM antibodies are not always produced in adults upon re-infection.[7] Hence, a negative IgM response does not always rule out current infection. Specific IgG levels increase slowly during the course of illness. The need for paired sera makes it less useful in patient management. Molecular

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diagnostic techniques can give rapid results but false positive results may occur due to long term carriage of the organism.[8]

There is a need for rapid diagnostic tests for the diagnosis of *M. pneumoniae* infection in order to prescribe appropriate antibiotic therapy. In some clinical settings in Sri Lanka, the cold agglutination test (CAT) is being used as a rapid test to diagnose *M. pneumoniae* pneumonia.

Cold agglutinins are identified as antibodies that agglutinate human erythrocytes at 4 °C, but not at warm temperature (37 °C).[9] Respiratory infection with *M. pneumoniae* evokes oligoclonal immunoglobulin M (IgM) type antibodies which are cold agglutinins.[9] Cold agglutinins produced in *M. pneumoniae* infection is usually directed against altered "I" antigen on the surface of erythrocytes of *M. pneumoniae* infected patients.[9] High titers of cold agglutinins have been associated with haemolysis, presumably due to activation of complement mediated erythrocyte destruction.[10] Before availability of more advanced serological techniques, detection of cold agglutinins was considered a valuable tool for the diagnosis of *M. pneumoniae* infection. Cold agglutinins have been identified as the first antibody to appear and the first to disappear in *M. pneumoniae* infection.[11] A titer greater than 1:32 is considered as positive.[12] Although this test has several drawbacks, it is still being used in Sri Lanka due to lack of funds to perform more accurate, but costly tests.[13] However, no studies have been carried out to date in Sri Lanka to assess the usefulness of CAT in the diagnosis of *M. pneumoniae* pneumonia. The current study was therefore aimed to assess the efficacy of CAT in the diagnosis of *M. pneumoniae* pneumonia in the Sri Lankan clinical setting.

Methodology

Study sites
Colombo North Teaching Hospital, Ragama and Chest Hospital – Welisara.

Study population
Patients with clinical and/or radiological signs of pneumonia diagnosed by a consultant physician were enrolled in the study. Inclusion criteria for enrollment were adhered as described by Tierney *et al*., 2006.**[14]** Patients with preexisting cardiac or pulmonary diseases were excluded from the study.

Ethical approval, **patients' consent** & **questionnaire**
Ethical approval for the study was obtained from the Ethics Committee, Faculty of Medicine, University of Kelaniya, Ragama. Written consent was obtained from patients. Demographic and clinical data of patients were recorded using hospital administration approved interview administered questionnaire.

Sampling

Five ml of venous blood per patient was drawn during the acute stage of illness. The mean duration of sampling ranged from 10-14 days. One milliliter was aliquoted immediately into an EDTA tube to prevent clot formation. The remaining 4 ml of blood was collected into a plain bottle and immediately kept at 37 °C to facilitate serum separation. The blood sample was transported from the ward to the laboratory in a thermo flask containing water at 37 °C measured using a digital thermometer as shown in Figure 1. In the laboratory, tubes were placed in a 37 °C incubator for 1 hr to facilitate clotting and serum was separated carefully. For samples in which serum separation was difficult, 2-3 minute centrifugation at 4000 rpm was done in a temperature controlled centrifuge. Serum was removed immediately to a new tube and 0.5 ml of serum was placed in a fresh tube.
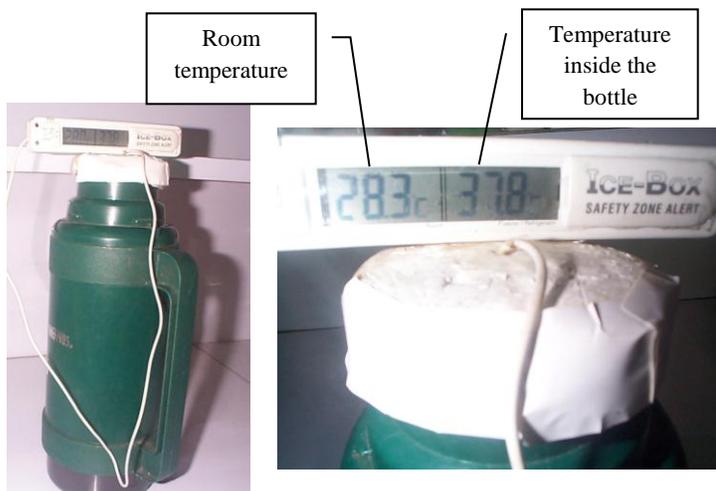

Figure 1: Device used for maintenance of temperature until dispatch to laboratory

Patients' own red cells: EDTA blood was used to obtain 1ml of patients' red cells which was washed three times in normal saline. A 0.1 ml of the RBC suspension was mixed with 4.9 ml of saline to prepare a 2% (v/v) red cell suspension.

Test procedure:

Testing for cold agglutinins was performed as described in "Medical Laboratory Manual for Tropical Countries, Volume 11: Microbiology by Monica Cheesbrough".[12]

Confirmatory test / ELISA: *M. pneumoniae* specific ELISA was performed for *M. pneumoniae* specific IgG and IgM separately using commercially available isotype specific ELISA kits (IBL–Hamburg Company, Germany) as the reference method.

Measurement of IgG seroconversion

The amount of IgG in the sample was calculated using reference IgG concentrations provided by the manufacturer. It was done using the standard curve drawn separately for each IgG test plate. For calculation of the standard curve, optical density (OD) values of controls (y-axis, linear) were plotted against concentration (x-axis, logarithmic) of the standards on a semi-logarithmic graph paper. Each OD value of the sample was converted to antibody concentration against the standard curve of the same plate. IgG seroconversion was determined comparing IgG levels in acute and convalescent phase sera.

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Diagnostic criteria

Patients with a positive IgM response or IgG seroconversion (≥ 4 fold rise in antibody concentration from acute to convalescent stage) against *M. pneumoniae* were considered as positive for the infection.

Results

A positive CAT result was observed for in the "test" sample tube after gentle tilting. It was compared with the negative "control" tube with no clumping (Figure 2). Only 97 patients returned for convalescent stage sampling. Therefore, comparison of ELISA and CAT was done using test data from 97 patients with paired serum samples.

Results of CAT

Negative

Positive

Figure 2: Reading of CAT results

Table 1. Association of results of CAT with ELISA

ELISA		CAT	
		Positive (18)	Negative (79)
Positive (15)	IgM	0	4
	≥4 rise in IgG	3	8
Negative (82)		15	67

Comparison of CAT titers with positive IgM/ ≥4 rise in IgG

CAT results in comparison with ELISA results are shown in Table 1.

A CAT titer of ≥1/32 was considered as positive. Positive CAT results were stratified according to the titre detected. Three patients who were positive with both ELISA & CAT showed 1/32 -1/64 CAT titers and they were diagnosed to have *M. pneumoniae* pneumonia using ≥ 4 rising IgG levels. These 3 patients were negative for *M. pneumoniae* specific IgM antibodies. There was no significant correlation between results of CAT and ELISA (p=0.35).

Sensitivity of the CAT was 20% (3/15) while its specificity was 81.7% (67/82). Negative predictive value was 84.8% (67/79) and positive predictive value was 16.7%(3/18) .

Discussion

The current standard test to diagnose *M. pneumoniae* pneumonia involves demonstration of specific IgM or ≥4 fold rise of specific IgG antibodies. Before arrival of these specific serological tests, CAT was considered as one of the diagnostic tools for *M. pneumoniae* pneumonia. Nevertheless, with time it was identified as a test to be interpreted cautiously although CAT continued to be used, particularly in resource poor countries with less developed laboratory facilities. In the present study, 15 of 97 patients with pneumonia were positive by *M. pneumoniae* specific antibodies. Only 3 of the 15 ELISA positive patients were positive by CAT.

True positives: The sensitivity of CAT in the diagnosis of *M. pneumoniae* infection varies from 33-76%.[12] However, in the current study, only 20% (3/15) of serologically confirmed cases of *M. pneumoniae* could be diagnosed using CAT (Table 1). The present study group was confined to adults. The cold agglutinin that appears after *M. pneumoniae* infection is IgM in type.[9] IgM tends

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to be produced dominantly in children whereas adults show a less prominent IgM response due to multiple previous exposures. Therefore, CAT detection rate could be lower in an adult study group as observed in this study. CAT showed poor sensitivity as not all patients with *M. pneumoniae* pneumonia produced significant cold agglutinin titers, giving rise to negative results in the presence of true *M. pneumoniae* infection.[6] It has also been noted that the cold agglutinin response often correlates directly with the severity of pulmonary involvement. Patients with extensive lobar involvement will usually have a titer of >1:64 whereas those with minimal illness may not develop detectable cold agglutinins.[15] Even though the infection is due to *M. pneumoniae*, if the infection is not severe enough to mount a measurable antibody level, the cold agglutinin test might be negative. The severity of pneumonia was not noted in the current study.

True negatives: The CAT was negative in 67 of 79 (84.8%) patients confirmed as negative for *M. pneumoniae* infection by ELISA. This suggests that a negative predictive value of CAT is strongly associated with true negative results and makes the test a more useful tool to exclude *M. pneumoniae* infection.

False positives: False positive CAT results are well documented and are known to occur in various other infectious and non-infectious diseases. In the present study, 15 of 82 (18%) of patients with negative *M. pneumoniae* antibodies by ELISA, showed positive CAT results. Influenza virus, cytomegalovirus, *Klebsiella pneumoniae* and other atypical pathogens have been shown to induce the production of cold agglutinins leading to a low specificity of CAT.[6] Therefore the test can lead to an inaccurate diagnosis of *M. pneumoniae* pneumonia.

False negatives: Twelve of 15 (80%) patients with confirmed *M. pneumoniae* infection in the present study gave negative CAT results. Cold agglutinins may not be produced by all patients with *M. pneumoniae* pneumonia.[6] As described previously, IgM specific antibodies may not be elicited in adults with reinfection.[15,16,17] Cold agglutinins being an IgM type antibody, false negative CAT could occur. In addition, CAT is a crude test with subjective result interpretation, resulting in additional false negative results, unlike in the objective readout results obtained using ELISA. On the other hand, cold agglutinins appear and disappear early in the infection compared to long standing specific antibodies that are detected by ELISA.[9] Change in the cold agglutinin titer has been observed with time and found that cold agglutinin test titer peaked from 13th to 15th day after onset (89%) and then declines to 33% after the 19th day.[18] In the present study, the duration of illness ranged from 10 -14 days which may account for some of the false negative results.

Among patients who showed positive CAT results, the titre was analyzed to determine the association of positive ELISA results with the CAT titre.   There was no association between a positive CAT titre and a positive result with ELISA ($p = 0.35$). It has also been noted that the height of the cold haemagglutinin response is directly related to severity of pneumonic involvement.[11] This association could not be determined in the current study.

Comparison of CAT results with results of ELISA showed a positive predictive value of only 16.7% which would result in missing the aetiological diagnosis in >80% of patients.

Conclusion

Even in a setting with under-developed laboratory facilities, CAT should not be included as a test to diagnose *M. pneumoniae* pneumonia owing to its extremely poor sensitivity and positive predictive value.

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