Novel Toxin Assays Implicate *Mycoplasma pneumoniae* in Prolonged Ventilator Course and Hypoxemia

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Background: Community-acquired respiratory distress syndrome (CARDS) toxin is a unique *Mycoplasma pneumoniae* virulence factor. Molecular assays targeting this toxin are more sensitive than existing diagnostics, but these assays have not been used to investigate the role of *M pneumoniae* as a nosocomial infection in critical illness. We sought to determine the incidence of *M pneumoniae* among mechanically ventilated subjects using these novel assays and to investigate the impact of this pathogen on pulmonary outcomes.

Methods: We conducted a prospective observational study enrolling subjects with suspected ventilator-associated pneumonia (VAP) undergoing BAL in the surgical trauma ICU at a level I trauma center. Lavage fluid and serum samples were tested for *M pneumoniae* using assays to detect CARDS toxin gene sequences, protein, or antitoxin antibodies.

Results: We collected samples from 37 subjects, with 41% (15 of 37) testing positive using these assays. The positive and negative groups did not differ significantly in baseline demographic characteristics, including age, sex, injury severity, or number of ventilator days before bronchoscopy. The positive group had significantly fewer ventilator-free days ($P = .04$) and lower average oxygenation ($P = .02$). These differences were most pronounced among subjects with ARDS.

Conclusions: Evidence is provided that *M pneumoniae* is present in a substantial number of subjects with suspected VAP. Subjects testing positive experience a significantly longer ventilator course and worse oxygenation compared with subjects testing negative. *CHEST* 2011; 139(2):305–310

Abbreviations: APACHE = Acute Physiology and Chronic Health Evaluation; CARDS = community-acquired respiratory distress syndrome; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; P:F ratio = ratio of PaO_2 to FiO_2 ; VAP = ventilator-associated pneumonia; VFD = ventilator-free day

Mycoplasma pneumoniae is responsible for 20% to 30% of all cases of community-acquired pneumonia and has been implicated in a number of other acute and chronic airway diseases (including tracheobronchitis and asthma), as well as a range of extrapulmonary manifestations.^{1–3} Community-

acquired respiratory distress syndrome (CARDS) toxin is a recently identified 68-kDa protein unique to *M pneumoniae*. CARDS toxin possesses adenosine diphosphate-ribosyltransferase activity similar to the *Bordetella pertussis* toxin and shares limited amino acid sequence homology with the pertussis toxin S1 subunit.^{4,5} CARDS toxin is also highly immunogenic, resulting in dramatic seroconversion.⁴

We have subsequently developed molecular assays using CARDS toxin to detect *M pneumoniae*, providing novel diagnostic techniques for rapid detection of this organism. We used polymerase chain reaction

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(PCR) for unique CARDS toxin nucleotide sequences, enzyme-linked immunosorbent assay (ELISA) for anti-CARDS toxin antibodies, and antigen capture for CARDS toxin protein. CARDS toxin gene sequences are more sensitive for the detection of *M pneumoniae* than other gene sequences using PCR amplification, including the P1 adhesin molecule and the ATPase gene.^{6,7} Likewise, anti-CARDS toxin antibodies were present at a much higher titer than anti-P1 antibodies in a cohort with asthma. Absence of immunologic cross-reactivity of whole-cell lysates from other mycoplasma species, *Escherichia coli*, or *Bacillus subtilis* against anti-CARDS toxin antibodies reveals the specificity of CARDS toxin for *M pneumoniae*. Human sera with high anti-CARDS toxin antibody titers lacked immunoreactivity against the *B pertussis* holotoxin (T. Kannan, PhD and J. Baseman, PhD, unpublished data, September 2009). Comparison of the CARDS toxin nucleotide sequence with genome databases reveals no homologous genes. These data demonstrate the highly sensitive and specific nature of CARDS toxin assays for the detection of *M pneumoniae*.

Published reports of the role of *M pneumoniae* in the ICU are quite limited.⁸⁻¹⁰ Only one prospective study has evaluated the incidence of *M pneumoniae* in patients with suspected ventilator-associated pneumonia (VAP), finding positive results in 3% of patients.¹⁰ Of note, none of these studies used the more sensitive CARDS toxin assays, so the true impact of *M pneumoniae* in this setting may be underestimated. Preliminary results from our institution suggest this to be the case, and demonstrate *M pneumoniae* to be associated with worse oxygenation in ventilator-dependent patients.¹¹ *M pneumoniae* is not susceptible to the antibiotic classes typically used for empiric broad-spectrum therapy of suspected VAP, so understanding the true incidence of this organism in the ICU may have immediate clinical implications. Our aims were to use CARDS toxin assays to determine the incidence of *M pneumoniae* among ICU patients undergoing BAL and to correlate the presence of this organism with pulmonary outcomes.

MATERIALS AND METHODS

Patients and Samples

We conducted a prospective observational study enrolling a convenience sample of subjects undergoing fiberoptic bronchoscopy with BAL in the surgical trauma ICU at a level I trauma center. A sample of BAL fluid (2-20 mL) was collected and centrifuged, the supernatants were discarded, and the pellets were resuspended in 2 mL of saline. The samples were frozen and stored at -80°C . At the time of bronchoscopy, whole blood samples (5-7 mL) were collected in gold-top Serum Separator Tubes (BD Vacutainer; Franklin Lakes, New Jersey). The serum was separated from the whole blood and stored at -80°C .

This study was approved by the institutional review board of the University of Texas Health Science Center at San Antonio (IORG# 0000312). Written informed consent was obtained from the legally authorized representative of each subject, and whenever possible, informed consent was subsequently obtained from the subject.

Detection of CARDS Toxin Gene Sequences

BAL and serum samples (200 μL aliquots) were subjected to DNA extraction using the QIAmp DNA Mini Kit (Qiagen; Valencia, California). BAL samples were homogenized prior to extraction using dithiothreitol, a mucolytic agent. The presence or absence of *M pneumoniae* was determined by analysis with real-time PCR for CARDS toxin nucleotide sequences. Primers specific for CARDS toxin were designed using Primer Express software 2.1 (Applied Biosystems; Foster City, California). The generation of internal inhibition control subjects was conducted according to a protocol described by Ursi et al.¹² Amplifications were performed with the ABI Prism HT7900 Sequence Detection System (Applied Biosystems), and Universal Master Mix (Applied Biosystems) was used in all reactions.

Detection of CARDS Toxin Protein and Anti-CARDS Toxin Antibodies

CARDS toxin protein in the BAL samples was detected and quantified using established "antigen capture" ELISA methods.¹³ The detection of antibodies against CARDS toxin in subjects' serum was also performed using ELISA methods.⁴ In parallel, we detected the presence of serum antibodies against P1 adhesin using a similar protocol but substituting ultrapure P1 recombinant immunodominant carboxy domain for recombinant CARDS toxin protein.^{4,14}

Clinical Data

We collected clinical data on each subject, including demographic characteristics, baseline clinical characteristics at the time of bronchoscopy, results of laboratory and imaging studies, vital signs, length of ICU stay and ventilator course, and mortality. Quantitative microbiologic culture results of BAL fluid were obtained from the clinical laboratory. We also collected data on pulmonary dysfunction, including oxygenation and presence of ARDS. We defined ARDS as a ratio of PaO_2 to FiO_2 (the P:F ratio) < 200 , acute onset of bilateral infiltrates on chest radiograph, and the absence of a known history or clinical evidence of left-sided heart failure. Overall oxygenation of each subject was tabulated using the lowest daily value for the P:F ratio from arterial blood gas.

Statistical Analysis

All statistical analysis was performed using SAS 9.1.3 (SAS Institute; Cary, North Carolina). The Wilcoxon rank-sum test was used to analyze continuous data, and Fisher exact test was used to analyze categorical data.¹⁵ Repeated-measures analysis of variance, with adjustments for the APACHE (Acute Physiology and Chronic Health Evaluation) II score, the Clinical Pulmonary Infection score, and the incidence of VAP and ARDS, was used to test for significant differences between the average cumulative values for the P:F ratio.¹⁶

RESULTS

BAL and serum samples were obtained from 37 subjects; 15 of the 37 (41%) tested positive for *M pneumoniae* with one or more of the CARDS

toxin-based assays. Of these 15 subjects testing positive, nine of them were positive by PCR (five of those nine were also positive by serology, and we were unable to obtain blood to test for serology in another). Three subjects were positive by antigen capture (with one positive by serology, one negative by serology, and one without a blood sample). Finally, three subjects were positive by serology alone. In addition to these CARDS toxin assays, three of these subjects also tested positive based on the presence of P1 adhesin IgM antibodies in serum, but no subjects who were negative for all CARDS toxin-based assays were positive for P1 adhesin antibodies. In the case of four of the positive subjects, sequential serum samples were collected at 2-week intervals during their ICU course, allowing a determination of longitudinal serologic response. Three of the four subjects had an increasing antibody response with each subsequent serum collection, with the increase ranging from 1.5 to 3.3-fold over 2 to 4 weeks. The remaining subject initially had a very high anti-CARDS toxin IgM antibody response that decreased nearly ninefold over the ensuing 6 weeks.

Table 1 summarizes the characteristics of each group at the time of bronchoscopy. The only one of these characteristics that was significantly different between the positive and negative groups was the percentage of subjects with VAP with polymicrobial culture results (77% for positive vs 25% for negative, $P = .009$). Four subjects (27%) in the positive group had received antibiotics with activity against *M pneumoniae* prior to the time of bronchoscopy, yet still tested positive for *M pneumoniae* CARDS toxin. A similar percentage of subjects in the negative group had also received one of these antibiotics (32%, $P = 1.0$).

The positive group ($n = 15$) had significantly fewer ventilator-free days (VFDs) during the 30 days immediately following bronchoscopy compared with the negative group (17.2 ± 10.1 vs 22.4 ± 8.0 days, $P = .04$). The distribution of VFDs by *M pneumoniae* status is depicted in Figure 1. Comparison of VFD by diagnostic category (PCR-positive vs serology/antigen capture-positive) revealed that a significant decrease in VFDs was still observed in the serology/antigen capture-positive subjects, but not in PCR-positive subjects. However, significant overlap occurred between PCR and serology-positive subjects (five of nine PCR-positive subjects were serology positive), preventing direct correlations between serology positivity alone and VFDs. There was one death in each group (all positive and negative), and one subject in each group continued to require mechanical ventilation after 30 days. Oxygenation (as assessed by mean group P:F ratios) was also significantly worse in the positive group than in the negative group

Table 1—Baseline Characteristics at the Time of Bronchoscopy

Characteristic	Negative (−) (n = 22)	Positive (+) ^a (n = 15)	P Value
Age, y, mean ± SD	50.3 ± 20.9	52.7 ± 12.3	.85
Sex, male, No. (%)	16 (72.7)	14 (93.3)	.20
APACHE II score, mean ± SD	26.9 ± 8.6	29.0 ± 8.2	.35
CPIS, mean ± SD	5.3 ± 2.1	6.5 ± 2.0	.08
ARDS, No. (%)	5 (22.7)	5 (33.3)	.71
Ventilator days before BAL, median (IQR)	4.5 (2-5)	6.0 (3-16)	.08
Maximum temperature, °C, mean ± SD	38.5 ± 1.0	38.7 ± 0.9	.73
Leukocyte count, 10 ³ cells/mL, mean ± SD	12.1 ± 4.0	14.0 ± 5.3	.26
Antibiotics with activity against <i>Mycoplasma pneumoniae</i> , No. (%)	7 (32)	4 (27)	1.0
VAP (> 10 ⁴ organisms/mL), No. (%)	16 (72.7)	13 (86.7)	.43
Polymicrobial VAP, No. (%)	4 (25)	10 (77)	.009

APACHE = Acute Physiology and Chronic Health Evaluation; CARDS = community-acquired respiratory distress syndrome; CPIS = Clinical Pulmonary Infection score; IQR = interquartile range; VAP = ventilator-associated pneumonia.

^aPositive designation was based on CARDS toxin-related assays as described in "Materials and Methods."

(224.8 ± 77.4 vs 251.5 ± 83.8 ; $P = .02$). When the data for both VFDs and oxygenation was stratified according to the presence or absence of ARDS, statistically significant differences were noted in patients with ARDS. There was no difference in VFDs or oxygenation in those subjects without ARDS (Table 2).

FIGURE 1. Ventilator-free days (VFDs) by *Mycoplasma pneumoniae* and ARDS status. The positive group had significantly fewer VFDs in the 30-day period following bronchoscopy than the negative group (17.2 ± 10.1 vs 22.4 ± 8.0 days, $P = .04$). When VFDs were further stratified by assay type, PCR-positive subjects were not statistically different from the negative group (20.9 ± 9.6), but the serology/antigen capture-positive group was (15.9 ± 8.9 , $P = .01$). PCR = polymerase chain reaction.

DISCUSSION

The results of this study demonstrate that *M pneumoniae* is often present in critically ill ventilator-dependent patients and can be detected with the CARDS toxin assays described previously. In this cohort, 41% of the 37 subjects tested positive for *M pneumoniae* using one or more of these assays. PCR was the most frequently positive assay, although it revealed no predictive value relative to VFD (Fig 1) and had considerable overlap with serologic testing. There are significant data demonstrating a high degree of variability in the human serologic response to acute *M pneumoniae* infection. Factors that influence the degree and type of immunologic response include patient age and previous exposure to mycoplasma, and this likely explains imperfect between-test correlation regarding PCR and serology.^{3,17,18} Subjects who tested positive had significantly fewer VFD and a lower mean P:F ratio during their ventilator course. Recent experiments in mice and baboons demonstrate that recombinant CARDS toxin results in significant pulmonary inflammation, release of proinflammatory cytokines, and airway dysfunction.¹⁹ These data reveal potential mechanisms to explain our finding that *M pneumoniae* is associated with underlying respiratory failure in critically ill subjects.

The differences in both VFD and oxygenation were confined to those subjects with ARDS, suggesting a relationship between *M pneumoniae* and coexisting pulmonary dysfunction. This may have important implications for the care of critically ill patients, since the reported incidence of ARDS in the ICU ranges from 2.4% to 7.5% and is as high as 23% for mechanically ventilated patients.²⁰⁻²² Mortality rates among adults with ARDS have declined from upwards of 50% initially to between 35% and 40%, but reducing mortality below this threshold has continued to elude clinicians.²³⁻²⁷ The ability to detect a treatable bacte-

rial organism contributing to pulmonary dysfunction in mechanically ventilated patients, particularly those with ARDS, could have a substantial impact on outcomes in this population. Further study in ventilator-dependent patients will be necessary to determine whether the association between *M pneumoniae* and pulmonary dysfunction is isolated to those patients with ARDS or is also present in other populations.

The significantly higher percentage of subjects with polymicrobial pneumonias in the positive group is intriguing and may be due to the ability of *M pneumoniae* to potentiate additional pulmonary bacterial infections, either because of impaired airway immunity or altered respiratory tract flora.²⁸ The fact that 27% of subjects in the positive group had previously received antibiotics with activity against *M pneumoniae* yet still tested positive may be explained by the ability of this organism to survive in an intracellular state following prolonged antibiotic treatment, or perhaps by the recent emergence of *M pneumoniae* strains with resistance to macrolide antibiotics.²⁹⁻³¹ This finding may also represent inadequate duration of treatment, as these antibiotics were administered incidentally and not expressly for the purpose of treating *M pneumoniae* pneumonia.

The high incidence of *M pneumoniae* in our cohort is a unique finding, given the limited number of published reports on this subject. However, this result is consistent with findings from a recent cohort with asthma showing that PCR targeting P1 adhesin is less sensitive than PCR targeting CARDS toxin gene sequences.⁶ In that study, approximately 4% of subjects tested positive for *M pneumoniae* with PCR for P1 adhesin, but 42% of subjects tested positive with PCR for CARDS toxin. This 10-fold increase in the detection of *M pneumoniae* with CARDS toxin assays compared with P1 adhesin assays is the same as that seen in this population (41% in our study compared with 3% in a previous prospective ICU study using PCR for P1 adhesin).¹⁰

Some limitations of this study merit further discussion. The observational nature of this study prohibits a determination of causality. It is possible that infection with *M pneumoniae* results in worse oxygenation and a prolonged ventilator course, in which case treating this pathogen may significantly alter the outcome in these patients. But it is also possible that the high incidence of *M pneumoniae* in this set of subjects is the result of low oxygen tension, prolonged ventilator support, or concomitant VAP culminating in reactivation of a latent *M pneumoniae* infection. The effects of eradicating *M pneumoniae* in these patients will depend largely on this sequence of cause and effect. Another limitation of this study is uncertainty regarding the relative clinical significance of each of the assays used. These tests include PCR,

Table 2—Mean Ventilator-Free Days and Oxygenation by Mycoplasma pneumoniae Status and Presence or Absence of ARDS

Ventilator Course	Negative (–) (n = 22)	Positive (+) ^a (n = 15)	P Value
Ventilator-free days			
Total	22.4 ± 8.0	17.2 ± 10.1	.04
ARDS	24.2 ± 3.7	12.2 ± 10.4	.04
No ARDS	21.9 ± 8.9	19.7 ± 9.5	.54
Mean P:F ratio			
Total	251.5 ± 83.8	224.8 ± 77.4	.02
ARDS	232.1 ± 80.5	179.3 ± 60.8	.004
No ARDS	257.7 ± 84.2	258.2 ± 71.3	.65

P:F ratio = ratio of PaO₂ to FiO₂. See Table 1 for expansion of abbreviation.

^aPositive designation was based on CARDS toxin-related assays as described in “Materials and Methods.”

antigen capture, and ELISA testing for the presence of CARDS toxin DNA, protein, or antibodies, respectively. It may be that each of these techniques has a role in the diagnosis of *M pneumoniae* infections, but further research is needed to elucidate the most effective clinical applications of these assays.

CONCLUSIONS

The results of this study confirm that *M pneumoniae* is commonly present in the lungs of ICU patients at the time of bronchoscopy and can be detected with assays targeting CARDS toxin. The finding that approximately 40% of subjects in this study tested positive for *M pneumoniae* using CARDS toxin assays is consistent with accumulating data from cohorts of subjects with asthma.⁶ Our findings also demonstrate that those subjects who tested positive for *M pneumoniae*, particularly those subjects with ARDS, experienced fewer VFD and worse oxygenation during their ventilator course. The possibility of improving outcomes among mechanically ventilated ICU patients by treating for *M pneumoniae* is a provocative idea. The antibiotic classes with in vivo activity against this pathogen (macrolides, fluoroquinolones, and tetracyclines) are inexpensive and widely available but are not typically used as first-line empiric treatment of pulmonary infections in the ICU or for the prolonged treatment periods required for elimination of *M pneumoniae*. A randomized controlled trial will be essential to determine if these findings truly represent a unique opportunity to improve outcomes in critically ill ventilator-dependent patients.

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Dr Muir: contributed to the study concept and design, the analysis and interpretation of data, and the drafting and critical revision of the manuscript.

Dr Cohn: contributed to the study concept and design, the analysis and interpretation of data, and the drafting and critical revision of the manuscript.

Mr Loudon: contributed to the statistical analysis and interpretation of data and the drafting and critical revision of the manuscript.

Dr Kannan: contributed to the study concept and design, the analysis and interpretation of data, and the drafting and critical revision of the manuscript.

Dr Baseman: contributed to the study concept and design, the analysis and interpretation of data, and the drafting and critical revision of the manuscript.

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