Published in final edited form as:

J Heart Lung Transplant. 2012 September ; 31(9): 996–1002. doi:10.1016/j.healun.2012.05.012.

Usefulness of immune monitoring in lung transplantation using adenosine triphosphate production in activated lymphocytes

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Abstract

BACKGROUND—The ImmuKnow (Cylex Inc, Columbia, MD) assay measures the amount of adenosine triphosphate (ATP) produced by helper CD4⁺ cells after stimulation with a T-cell mitogen. We hypothesized that this assay can be used to assess the immune function of lung transplant recipients and identify those at risk of developing acute cellular rejection and respiratory infection.

METHODS—Lung transplant recipients at University of California Los Angeles between January 1, 2006 and December 31, 2009 received a bronchoscopy with bronchoalveolar lavage, transbronchial biopsy and ImmuKnow values drawn at regular intervals as well as during episodes of clinical deterioration. The recipient's clinical condition at each time-point was classified as healthy, acute cellular rejection, or respiratory infection. Mixed-effects models were used to compare the ATP levels among these groups, and odds ratios for rejection and infection were calculated.

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Disclosure statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

RESULTS—The mean ATP level was 431 ± 189 ng/ml for the rejection group vs 377 ± 187 ng/ml for the healthy group ($p = 0.10$). A recipient with an ATP level > 525 ng/ml was 2.1 times more likely to have acute cellular rejection (95% confidence interval [CI] 1.1–3.8). Similarly, the mean ATP level was 323 ± 169 ng/ml for the infection group vs 377 ± 187 ng/ml for the healthy group ($p = 0.03$). A recipient with an ATP level < 225 ng/ml was 1.9 times more likely to have respiratory infection (95% CI, 1.1–3.3). However, the test was associated with poor performance characteristics. It had low sensitivity, specificity with an area under the receiver operating characteristic curve of only 0.61 to diagnose rejection and 0.59 to diagnose infection.

CONCLUSIONS—The ImmuKnow assay appears to have some ability to assess the overall immune function of lung transplant recipients. However, this study does not support its use as a reliable predictor of episodes of acute cellular rejection or respiratory infection.

Keywords

adenosine triphosphate; immune function; immune monitoring; lung transplantation; acute cellular rejection; respiratory infection

Despite advances in donor management, surgical techniques, and perioperative care that have translated into improved early outcomes, the overall mortality associated with lung transplantation has remained disappointingly high. Lung transplantation is associated with one of the highest mortality rates among solid organ transplants: 48% at 5 years and 71% at 10 years.¹ Thirty-six percent of all lung transplant recipients experience an episode of acute rejection during the first year after transplant.^{1,2} Acute rejection is the primary risk factor for the development of chronic allograft dysfunction or bronchiolitis obliterans syndrome (BOS), the leading cause of death after the first year.^{3,4} However, infection remains a major source of morbidity and mortality among lung transplant recipients, accounting for almost 40% of all deaths in the first year.¹ A number of respiratory infections have also recently been implicated in the development of BOS.^{5–11}

In practice, the standard for assessing a recipient's overall immune function and lung allograft status remains the bronchoscopy with transbronchial biopsy, a procedure that carries a small but real risk of morbidity. The ImmuKnow (IK) assay (Cylex Inc, Columbia, MD) measures the amount of adenosine triphosphate (ATP) produced by helper CD4⁺ cells after stimulation with phytohemagglutinin-L, a T-cell mitogen, in vitro. It has received interest as a potential objective measure of overall immune function in solid organ transplant recipients, accounting for the aggregate effect of multiple immune suppressants and other factors that may affect a patient's immunity. In January 2002, the U.S. Food and Drug Administration (FDA) approved the use of the IK assay for the assessment of the overall immune function of patients after solid-organ transplants. We hypothesized that this assay can be used to identify lung transplant recipients at risk of developing episodes of acute cellular rejection and respiratory infection.

Methods

This study was approved the University of California Los Angeles (UCLA) Institutional Review Board.

Study design and patient population

Lung transplant recipients at UCLA received surveillance bronchoscopy with bronchioalveolar lavage (BAL) and transbronchial biopsy (TBB) at 1 week, and at 1, 3, 6, and 12 months. Bronchoscopies were also done for episodes of clinical deterioration, which included new symptoms, such as dyspnea or fatigue, declines in pulmonary function testing or the 6-minute walk test. Induction and maintenance immunosuppression and anti-microbial prophylaxis were administered in accordance with the UCLA lung transplantation protocol and has been described previously.¹⁰ The IK immune function assay was also performed at the time of the bronchoscopies.

Immune function determination

The IK is an FDA-approved assay for T cell function and was performed according to the manufacturer's instructions.¹² Briefly, sodium heparin anti-coagulated whole blood was incubated for 15 to 18 hours with phytohemagglutinin (PHA). CD4⁺ T-cells were selected using paramagnetic particles coated with a monoclonal antibody to the CD4 epitope. The cells were washed and lysed to release intracellular ATP. A luciferin/luciferase mixture was added to the cell lysate, and the bioluminescent product was measured using a luminometer. The amount of light emitted was compared with a calibration curve, and the ATP concentration (ng/ml) was reported. Samples were tested and analyzed at UCLA's Immunogenetics Center.

Determination of the lung allograft status at each surveillance

For the bronchioalveolar lavage, sterile normal saline was instilled into the right middle lobe or lingula. Specimens were retrieved under gentle suction, pooled, and plated onto appropriate agars for culture by the clinical microbiology laboratory. Approximately 6 to 10 TBB specimens were obtained during each bronchoscopy. An experienced lung transplant pathologist examined all TBB specimens for evidence of acute rejection, which were graded according to the standard International Society for Heart and Lung Transplantation (ISHLT) criteria.¹³

For the purposes of this study, the lung allograft status at each surveillance was grouped as respiratory infection, acute cellular rejection, or healthy. Microbiologic cultures, radiographic studies, biopsy results and clinical notes were all reviewed while blinded to the IK scores to determine the allograft status. Respiratory infections were defined as a positive BAL culture with a known respiratory pathogen. Acute rejection was defined strictly by pathologic interpretation of TBB specimens. A healthy allograft was defined by the lack of evidence for respiratory infection or acute rejection.

Inclusion and exclusion criteria

All lung transplant recipients between January 1, 2006, and December 31, 2009, with at least one bronchoscopy with BAL and TBB specimen were eligible for study inclusion. Only the bronchoscopies in which an IK assay was drawn within a 7-day period *before* the bronchoscopy date were included in the analysis. If multiple assays had been drawn within this 7-day window, the most recent IK value was used. The analysis did not include samples

for 18 patients with concurrent infection and rejection, designated as “mixed infection/rejection.”

Statistical analyses

Mixed-effects models were used to compare the mean IK values between the rejection and healthy groups as well as between the infection and healthy groups. The mixed-effects model was used to account for the repeated measurements taken from the same individuals. Similarly, odds ratios (ORs) and 95% confidence intervals (CIs) for rejection and infection were calculated using generalized estimating equations (GEE) models to account for the within-subject clustering effects. ORs for rejection and infection were calculated using the previously published cutoff values of > 525 ng/ml and < 225 ng/ml ATP, respectively. The cutoff value associated with the highest OR was identified by calculating the ORs for rejection and infection for various cutoffs in increments of 50 ng/ml ATP. Receiver operating characteristic (ROC) curves were constructed, and the area under (AU) the ROC (AUROC) was calculated.

The analysis was stratified by whether the bronchoscopy was done for protocol-based surveillance or for clinical deterioration. To account for possible time-dependent effects, the analysis was also stratified by time from transplant (< 30 days, 30–180 days, > 180 days). Mixed-effects models were used to compare the IK values for these stratified analyses.

The association between *changes* in the IK values and episodes of rejection and infection were also evaluated. The *change* in IK value was calculated as the IK value at each of the surveillance intervals minus the IK value during the *first 30 days post-transplant*. The *change* in IK value was also calculated as the IK value at each of the surveillance time points minus the *pre-transplant* IK value.

Results

Patient population

During the 3-year period of data collection, there were 1,015 bronchoscopies with TBB specimens from 175 lung transplant recipients. IK assays were drawn within the 7-day window in 376 of these bronchoscopies, of which 251 were protocol-driven and 125 were due to clinical deterioration. Table 1 lists the baseline characteristics of the transplant recipients.

Allograft status

Acute cellular rejection (grades A1–A3) was present at bronchoscopies (18%). There were 38 grade A1 (10%), 25 grade A2 (7%), and 3 grade A3 (1%) rejection episodes. Respiratory infection was detected in 91 bronchoscopies (24%). Table 2 lists the organisms associated with the respiratory infections. Removed from the analysis were 18 samples that were classified as mixed infection and rejection. The remaining 219 (58%) surveillance bronchoscopy specimens had no evidence of acute rejection or infection and were classified as healthy.

Immune responses over time in healthy recipients

The trend in immune function recovery after lung transplantation for the healthy surveillance bronchoscopies showed a peak in immune function values at 3 months after transplant, followed by a decline for the next 6 months that persisted at 1 year (Figure 1A). The immune function values for ATP (ng/ml) were 405 ± 181 at 1 week, 419 ± 138 at 1 month, 482 ± 181 at 3 months, 299 ± 190 at 6 months, 230 ± 130 at 12 months, and 325 ± 156 after 12 months.

Immune response during allograft rejection

A mixed-effects model was used to compare the mean immune responses between the rejection and healthy groups. The mean IK scores for the rejection (grades A1–A3) and healthy groups were 431 ± 189 vs 377 ± 187 ng/ml ATP ($p = 0.10$; Figure 1B). The IK score in 78 patients (21%) exceeded 525 ng/ml ATP, and 23 (29%) of these patients were diagnosed with acute cellular rejection. As determined by the GEE model, the OR for rejection was 2.1 (95% CI, 1.1–3.8). Figure 2A depicts the ORs associated with different cutoffs values in the IK scores. The ORs for rejection were centered near 2, with no incremental increase using higher IK score cutoffs.

An immune function level > 525 ng/ml ATP was associated with a sensitivity of only 35% and a specificity of 82% to predict episodes of rejection. When the cutoff value was decreased to 425 ng/ml, the sensitivity increased to 53%, but the specificity dropped to 65%. The AUROC was 0.61 (Figure 2B). The immune function cutoff, which maximized Youden's Index [(sensitivity + specificity)/2] for predicting rejection, was 425 ng/ml, with a maximum accuracy of 61%. The difference in the results was not significant when the protocol-driven surveillance bronchoscopies were analyzed separately (data not shown).

Higher rejection grades of A2 were associated with higher immune responses compared with lower rejection grades, 476 ± 201 vs 397 ± 175 ng/ml ATP ($p = 0.01$), respectively (Figure 3). The immune function values during these episodes of higher-grade rejection were also significantly higher than during times of healthy stability, 476 ± 201 vs 380 ± 185 ($p = 0.04$). When an immune function cutoff value > 525 ng/ml ATP was used, the OR for higher-grade rejection was 2.6 (95% CI 1.2–5.8). However, the sensitivity and specificity remained low, at 42% and 81%, respectively. The AUROC curve was 0.67.

No significant difference was found when the analysis was stratified by time from transplant ($p = 0.21$ for IK $\times$ time interaction). There was also no association between *changes* in IK scores and acute rejection. There were 326 sample points from 144 recipients who had IK values drawn in the first 30 days after transplant. The mean change in IK value between the healthy and rejection groups was not significantly different: -41 vs $+6$ ng/ml ($p = 0.21$). There were also 91 sample points from 41 recipients who had IK values drawn before transplant. When the pre-transplant IK score was used as the reference value, there was still no difference in the *mean change* in IK values between the healthy and rejection groups (data not shown).

Immune response during respiratory infections

A mixed-effects model was used to compare the mean immune responses between the infection and healthy groups. The mean IK scores during episodes of respiratory infection were lower than those during times of healthy stability (323 ± 169 vs 377 ± 187 ng/ml ATP; $p = 0.03$). In 68 recipients (22%), the immune response score was < 225 ng/ml ATP, and 27 of these patients (40%) were diagnosed with a respiratory infection, resulting in an OR of 1.9 (95% CI, 1.1–3.3). The ORs associated with different cutoffs in the IK scores (Figure 4A) were centered around 2, with no incremental increase with lower cutoff values. An immune response of < 225 ng/ml ATP was associated with a sensitivity of only 30% and a specificity of 82% to predict episodes of infection. The AUROC curve was 0.59 (Figure 4B). No significant difference was found when the protocol-driven surveillance bronchoscopies were analyzed separately (not shown).

Stratification by time from transplant did not affect the results ($p = 0.40$ for IK $\times$ time interaction). There were also no associations found between *changes* in IK scores and episodes of infection. When the IK value during the first 30 days after transplant was used as the reference value, the mean change in IK score between the infection the healthy groups was not significantly different: -82 vs -41 ng/ml ATP ($p = 0.14$). Similarly, there was no difference in the mean change in IK values between the infection and healthy groups when the pre-transplant IK score was used as the reference value (data not shown).

Immune responses during clinical deterioration

There was no significant difference in the results when the analysis was restricted to the bronchoscopies that were performed for episodes of clinical deterioration. For these clinically indicated bronchoscopies, the mean IK scores for the rejection samples ($n = 22$) and the samples without evidence of rejection or infection ($n = 63$) were 444 ± 230 vs 364 ± 194 ng/ml ATP ($p = 0.35$). With an IK cutoff value of 525 ng/ml, the sensitivity was 41% and specificity was 79%. The AUROC curve was 0.61.

Similarly, during episodes of clinical deterioration, the mean IK score for the 40 samples with infection was similar to the score for the 63 samples without evidence of infection or rejection: 342 ± 186 vs 364 ± 194 ng/ml ATP ($p = 0.94$). At an IK cutoff value of 225 ng/ml, the sensitivity (33%) and specificity (79%) remained low. The AUROC curve decreased slightly to 0.56.

Discussion

We hypothesized that the Cylex IK assay can be used to reliably identify lung transplant recipients at risk for developing acute cellular rejection and respiratory infection. Two prior studies of lung transplant recipients found lower immune function responses during times of respiratory infections.^{14,15} Our study examined the immune function assay during episodes of acute cellular rejection and respiratory infection among lung transplant recipients.

This study was sufficiently powered to detect small differences in ATP levels during episodes of rejection and healthy stability. The *mean* immune scores during episodes of rejection were higher than during periods of healthy stability; however, the difference

detected was small and of questionable clinical utility. Clearly, it would be challenging for a transplant pulmonologist to make immunosuppression adjustments or treatment decisions based on immune function scores of 431 vs 377 ng/ml ATP, the expected values of patients with acute cellular rejection and healthy stability. Similarly, the IK scores during respiratory infection were statistically lower than during healthy stability (323 vs 377 ng/ml ATP), but the difference was small and likely not clinically useful.

The IK assay was associated with low sensitivity and specificity for predicting acute rejection and respiratory infection. An immune monitoring test such as this would probably be most useful with high sensitivity. A positive test result would prompt the clinician to continue with a more definitive invasive test such as TBB. The IK assay however, was associated with very low sensitivity (35%) to detect episodes of rejection. Lowering the cutoff value for rejection to 425 ng/ml ATP would increase the sensitivity to only 53%. Lowering the cutoff to < 425 ng/ml ATP would not be feasible, because the mean immune score of the healthy group is 377. Similarly, the sensitivity of the test to detect episodes of respiratory infection was only 30%.

A few previous studies of other organ transplants found a positive correlation between ATP levels and acute cellular rejection. The study by Thai et al¹⁶ in pancreas transplant recipients found ATP levels of 550 ± 129 and 194 ± 123 ng/ml ATP for the rejection and healthy groups, respectively ($p < 0.05$).¹⁶ Their study, however, was limited by small sample size, with their rejection group representing only 9 samples taken from 4 recipients. Similarly, Hashimoto et al¹⁷ reported higher ATP levels during acute rejection among liver transplant recipients. They found mean ATP levels for the rejection and healthy group were 365 vs 240 ng/ml ($p = 0.001$). However, there were only 11 episodes of rejection from 54 recipients in their study. The meta-analysis by Kowalski et al¹⁸ included 39 episodes of rejection from 504 heart, kidney, kidney-pancreas, liver, and small-bowel transplant recipients. They also found significantly higher ATP levels during episodes of rejection: the mean ATP levels were 488 vs 249 ng/ml for the rejection and healthy groups, respectively ($p < 0.001$).¹⁸

More recently, Torio et al¹⁹ found no association between immune function responses and acute rejection among a cohort of renal transplant patients. The mean ATP levels were 333 ± 132 vs 375 ± 140 ng/ml in the acute rejection and healthy groups, respectively. Their study included 35 samples of acute rejection and 168 samples of healthy stability from 116 renal transplant recipients.¹⁹

Two previous studies examined the utility of immune function monitoring in lung transplant recipients. Bhorade et al¹⁴ found lower immune function scores among lung transplant recipients with acute respiratory infections (111 ± 83 ng/ml ATP) compared with healthy recipients (283 ± 143 ng/ml ATP, $p = 0.0001$). However, there were only 2 recipients with grade A1 rejection in their cohort with no detectable difference in their immune function scores. Husain et al¹⁵ also found lower immune function scores among lung transplant recipients during 129 episodes of respiratory infections. They reported mean IK scores of 93 vs 174 ng ml ATP during bacterial pneumonias and healthy stability, respectively ($p = 0.002$). They did not report any episodes of rejection in their study.¹⁵

Given the possible variation and unclear significance of minimal rejection (grade A1), which have been argued by some,^{20,21} episodes of higher grade rejection (grade A2) were considered separately in our study. These episodes of higher-grade rejection were associated with higher IK scores compared with the healthy group: 476 ± 201 vs 380 ± 185 ng/ml ATP ($p = 0.04$). However, the performance characteristics of the test remained poor, with low sensitivity, specificity, and AUROC curve. The results did not differ significantly when the analysis was stratified by the indication for bronchoscopy (surveillance vs clinical deterioration) or the time from transplantation.

The major limitation of this study is that it is a retrospective observational study and subject to confounding. For example, patients who had known higher levels of immune function scores might have received more frequent TBBs to evaluate for possible rejection. Making a clinical diagnosis of respiratory infection is also difficult to do in a retrospective manner. This study used a broad definition of respiratory infection: a positive BAL culture with a known respiratory pathogen. Although this definition is more objective and avoids the difficulty in making a retrospective diagnosis of infection, it includes episodes of respiratory colonization as well as clinical infection.

The other limitation is the number of missing data points. Of the 1,015 bronchoscopies with TBBs that occurred during this interval, there were only 376 with IK values drawn in the 7 days *before* the bronchoscopy. There were, on average, 2 observation points per transplant recipient.

The IK assay has received FDA approval for the monitoring of the global immune system after solid organ transplants and serves as a tool for overall risk assessment. Although individual IK values do not appear to be clinically useful as a diagnostic tool for episodes of rejection and infection, this study does not assess the efficacy of using this assay for the serial monitoring of the immune system. A prospective randomized study where IK monitoring is used to make immunosuppression adjustments may still be warranted.

In conclusion, immune function values are slightly higher during episodes of acute cellular rejection and lower during respiratory infection compared with times of healthy stability. However, the difference in ATP levels is small and of questionable clinical significance. Furthermore, the test has sub-optimal performance characteristics. It is associated with low sensitivity, specificity, and a ROC curve close to the diagonal for predicting rejection and infection. Although the IK assay appears to have some ability to assess the overall immune function of lung transplant recipients, this study does not support its use as a reliable predictor of episodes of acute cellular rejection or respiratory infection.

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Figure 1.

(A) Adenosine triphosphate (ATP) levels for healthy surveillance bronchoscopies are shown by time from transplant. (B) ATP levels are shown by transplant recipient's clinical status. The horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers mark the maximum and minimum values.

Figure 2.

(A) Odds ratios and 95% confidence intervals (CI) are shown for acute cellular rejection using various adenosine triphosphate (ATP) cutoff values. (B) Receiver operating characteristic (ROC) curve is shown for predicting acute cellular rejection.

Figure 3. Adenosine triphosphate (ATP) levels are shown by rejection grade for transplant recipients with an episode of acute cellular rejection. The horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers mark the maximum and minimum values.

Figure 4.

(A) Odds ratios (OR) and 95% confidence intervals (CI) are shown for respiratory infection using various adenosine triphosphate (ATP) cutoff values. (B) Receiver operating characteristic (ROC) curve is shown for predicting respiratory infection.

Table 1

Baseline Characteristics of Study Participants

Variables ^a	All patients	Healthy	Infection	Rejection
Patients	175 (100)	110 (62.9)	33 (18.9)	32 (18.3)
Age at transplant, years	58	59	55	58
Male sex	111 (63.4)	67 (60.9)	21 (63.6)	23 (71.9)
Organ type				
Double-lung	85 (48.6)	59 (53.6)	22 (66.7)	15 (46.9)
Single-lung	87 (49.7)	48 (43.6)	11 (33.3)	17 (53.1)
Heart and lung	3 (1.7)	3 (2.7)	0 (0.0)	0 (0.0)
Reason for transplant				
Pulmonary fibrosis	89 (50.9)	61 (55.5)	11 (33.3)	17 (53.1)
COPD/emphysema	35 (20.0)	22 (20.0)	8 (24.2)	5 (15.6)
Scleroderma	11 (6.3)	4 (3.6)	4 (12.1)	3 (9.4)
Other ILD	9 (5.1)	5 (4.5)	2 (6.1)	2 (6.3)
Primary PHTN	8 (4.6)	5 (4.5)	0 (0.0)	3 (9.4)
Cystic fibrosis	7 (4.0)	3 (2.7)	3 (9.1)	1 (3.1)
Non-CF bronchiectasis	6 (3.4)	4 (3.6)	2 (6.1)	0 (0.0)
Sarcoidosis	4 (2.3)	2 (1.8)	2 (6.1)	0 (0.0)
LAM	3 (1.7)	3 (2.7)	0 (0.0)	0 (0.0)
BOS	2 (1.1)	1 (0.9)	0 (0.0)	1 (3.1)
A1AT	1 (0.6)	0 (0.0)	1 (3.0)	0 (0.0)
Race				
Caucasian	125 (71.4)	73 (66.4)	25 (75.8)	27 (84.4)
Hispanic	27 (15.4)	20 (18.2)	4 (12.1)	3 (9.4)
African American	10 (5.7)	6 (5.5)	3 (9.1)	1 (3.1)
Asian	8 (4.6)	6 (5.5)	1 (3.0)	1 (3.1)
Other	5 (2.9)	5 (4.5)	0 (0.0)	0 (0.0)
Induction immunosuppression				
Anti-thymocyte globulin	89 (50.9)	55 (50.0)	18 (54.5)	16 (50.0)
Basiliximab	85 (48.6)	54 (49.1)	15 (45.5)	16 (50.0)
Other	1 (0.6)	1 (0.9)	0 (0.0)	0 (0.0)

A1AT, α -1-anti-trypsin deficiency; BOS, bronchiolitis obliterans syndrome; CF cystic fibrosis; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis; PHTN, pulmonary hypertension.

^aCategorical data are presented as number (%).

Table 2Respiratory Infections Detected on Surveillance Bronchoscopy^a

Type of infection	No.
Bacterial	
<i>Pseudomonas aeruginosa</i>	17
<i>Staphylococcus aureus</i>	10
<i>Escherichia coli</i>	6
<i>Klebsiella</i>	4
<i>Stenotrophomonas</i>	2
<i>Serratia</i>	2
<i>Enterococcus</i>	2
<i>Mycoplasma</i>	1
Other gram-negative sp	7
Total	51
Fungal	
<i>Aspergillus fumigatus</i>	16
<i>Aspergillus niger</i>	4
<i>Scedosporium</i>	2
<i>Aspergillus flavus</i>	1
<i>Aspergillus terreus</i>	1
<i>Coccidioidomycosis</i>	1
<i>Cryptococcus</i>	1
<i>Mucor</i> spp	1
Other fungus	7
Total	34
Viral	
Parainfluenza	7
Cytomegalovirus	3
Respiratory syncytial virus	1
Herpes simplex virus	1
Total	12
<i>Mycobacterium</i>	
<i>M avium</i> complex	6
<i>M chelonae</i>	2
<i>M goodii</i>	1
<i>M kansasii</i>	1
<i>M fortuitum</i>	1
<i>M abscessus</i>	1
Other <i>Mycobacterium</i>	1
Total	13

^aMultiple bronchioalveolar lavage samples grew multiple organisms.