Stenotrophomonas maltophilia in cystic fibrosis:

Serologic response and effect on lung disease

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At a Glance Commentary

Scientific Knowledge

Stenotrophomonas maltophilia is one of the more common multi-drug resistant organisms isolated from the respiratory tract of CF patients. Previous epidemiologic studies suggest that it simply colonizes the CF lung and does not contribute to CF lung disease but the effect of chronic S. maltophilia infection is unknown.

What This Study Adds to the Field

This study demonstrates that chronic S. maltophilia infection in CF patients is associated with a specific immune response to this organism and is an independent risk factor for pulmonary exacerbations, suggesting that it may be a true pathogen in some CF patients.
Abstract

Rationale: *Stenotrophomonas maltophilia* is one of the more common multi-drug resistant organisms isolated from the respiratory tract of cystic fibrosis (CF) patients but the effect of chronic *S. maltophilia* infection on CF lung disease is unknown.

Objective: To determine the impact of chronic *S. maltophilia* infection on lung disease in CF.

Methods: We developed a serologic assay specific for *S. maltophilia* and in a cross-sectional study, measured serum antibodies to *S. maltophilia* in CF patients to determine if a definition of chronic *S. maltophilia* isolation based on culture results corresponded to an immunologic response (serologic study). We then used this validated definition to examine the effect of chronic *S. maltophilia* on the severity of lung disease in a retrospective cohort study using the Toronto CF Database from 1997-2008 (cohort study).

Measurements and Main Results: Serum antibody levels to *S. maltophilia* were measured in 179 CF patients. Patients with chronic *S. maltophilia* had significantly higher mean antibody levels to *S. maltophilia* flagellin (p<0.0001) and whole cell (p=0.0004) compared to patients with intermittent or no *S. maltophilia*. The cohort study included 692 patients with an average follow-up of 8.3 years. In an adjusted log linear model, patients with chronic *S. maltophilia* infection had a significantly increased risk of pulmonary exacerbation requiring hospitalization and antibiotics compared to patients who had never had *S. maltophilia* (RR=1.63, p=0.0002).

Conclusions: Chronic *S. maltophilia* infection in CF patients is associated with a specific immune response to this organism and is an independent risk factor for pulmonary exacerbations.

Abstract word count: 250

Key words: cystic fibrosis, *Stenotrophomonas maltophilia*, antibodies, lung function, pulmonary exacerbation
Introduction

Chronic bacterial respiratory infection is the leading cause of death in cystic fibrosis (CF) (1). As CF patients are living longer, respiratory tract colonization and infection with multi-drug resistant pathogens are increasing in frequency. Among these, *Stenotrophomonas maltophilia* is one of the more common, isolated from the respiratory tract of up to 30% of CF patients(2) (3).

It is unclear whether *S. maltophilia* simply colonizes the airway or causes true infection and clinical decline in CF. Epidemiologic studies have not shown any association between *S. maltophilia* and survival or declining lung function in CF(4, 5). However, these studies focused on CF patients who were positive at any point in time for *S. maltophilia* and did not make the distinction between chronic and intermittent isolation of *S. maltophilia* in the respiratory tract. As has been shown with methicillin-resistant *Staphylococcus aureus* (MRSA) and *Aspergillus fumigatus*, chronic infection with *S. maltophilia* may be more likely to cause clinical deterioration in CF(6-8).

The aim of this study was to determine the impact of *S. maltophilia* infection on lung disease in CF. Chronic infection with an organism can be defined by the number or percentage of positive cultures of respiratory specimens in a given year. This strategy has been validated for *Pseudomonas aeruginosa* infection (9). In addition, chronic exposure to an organism can stimulate an immune response and assessing the immune response can help to distinguish between transient colonization and infection. In the first part of this study, we developed a novel serologic assay specific for *S. maltophilia* and measured serum antibodies to *S. maltophilia* in CF patients to determine if a definition of chronic *S. maltophilia* isolation based on culture results corresponded to an immunologic response. In the second part of the study, we used this
validated definition to examine the effect of chronic *S. maltophilia* on the severity of lung disease as measured by pulmonary function tests (PFTs) and number of acute pulmonary exacerbations in a larger retrospective cohort study in CF patients.

**Methods**

**I. Serologic Study**

*Study Design*

This was a cross-sectional study of CF patients followed at the Hospital for Sick Children and St Michael’s Hospital (Toronto, Canada). Patients were excluded if they could not produce sputum, were unable to perform reproducible spirometry or had received a lung transplant. A serum sample was collected for each patient with routine clinical blood sampling in the CF clinic. Pulmonary function testing was performed on the same day as serum specimens were obtained in 94% of patients (within 3 months in remainder).

*Microbiological Classification*

All subjects were classified according to their respiratory culture status in the previous 12 months (8): 1) Chronic *S. maltophilia*: ≥ 2 positive sputum or bronchoalveolar cultures for *S. maltophilia* in a given year, 2) Intermittent *S. maltophilia*: 1 positive culture for *S. maltophilia* in a given year or a previous positive culture, 3) Never *S. maltophilia*: never having a positive culture for *S. maltophilia*. Subjects who never had *S. maltophilia* were divided into those who had chronic *P. aeruginosa* and those without chronic *P. aeruginosa*. These definitions were validated against the serologic results.

*Sera Processing and Serologic Assay*
Serologic responses were measured by an enzyme-linked immunosorbent assay (ELISA) using 3 *S. maltophilia* antigens individually (flagellin(10), protease (11)and whole bacterial cell(12)) (additional details available in online data supplement at www.atsjournals.org). Results were represented as the ratio of the average serum sample optical density (OD) to the average OD value of the 2 negative controls (wells with pooled normal human serum) (Ratio Units: RU).

**Statistical Analysis**

Analysis of variance (ANOVA) was used to assess if there were significant differences between groups. Correlation analysis was done using Pearson’s correlation. Multiple regression analysis was also done on forced expiratory volume in 1 second (FEV$_1$) % predicted and the mean antibody levels adjusting for age, *P. aeruginosa* and *Burkholderia cepacia* complex infection.

**II. Cohort Study**

**Study Design**

This was a retrospective cohort study from 1997 to 2008 using the Toronto CF Database. Patients with chronic *S. maltophilia* were compared to those who had intermittent *S. maltophilia* and those who did not have *S. maltophilia* over the study period. The primary outcome measure was the rate of decline of FEV$_1$ in percent predicted in patients in each of these three groups. The secondary outcome was the number of hospital admissions for pulmonary exacerbations requiring antibiotics per year. The same exclusion criteria and microbiologic classification were used as above.

**Statistical Analysis**
Two separate regression models were generated as used in a previous study (8). In the first model, a hierarchical linear model was generated to look at the effect of chronic *S. maltophilia* on quarterly FEV$_1$ allowing for a random effect and slope for each patient. The second model assessed the effect of chronic *S. maltophilia* on the number of hospitalizations for pulmonary exacerbation requiring antibiotics using Poisson regression. Multivariable analysis was performed to account for potential confounding variables (additional details available in online data supplement at www.atsjournals.org). The Research Ethics Board at the Hospital for Sick Children and St Michael’s Hospital approved the studies.

**Results**

*Serology Patient Characteristics and Antibody Levels*

Sera were obtained and *S. maltophilia* antibody levels were measured for 179 CF patients. Patient characteristics at the time of serologic testing are summarized in Table 1. Patients with chronic *S. maltophilia* isolation were significantly older, had lower mean FEV$_1$ % predicted and were not co-infected with *B. cepacia* complex. Figure 1 illustrates the mean antibody levels to *S. maltophilia* flagellin (Figure 1A), whole cell (Figure 1B) and extracellular protease (Figure 1C) in patients with chronic, intermittent and in those who never had *S. maltophilia* (subdivided into those who had chronic *P. aeruginosa* and those without chronic *P. aeruginosa*). Patients with chronic *S. maltophilia* had significantly higher mean antibody levels to *S. maltophilia* flagellin (p<0.0001) and whole cell (p=0.0004), but not to protease, compared to patients with intermittent or no *S. maltophilia*. Mean antibody levels to flagellin and whole cell were 1.5 times higher in the chronic group compared to the intermittent group and approximately 2 times higher in the chronic group compared to the negative group. There was also no evidence of cross-reactivity of
antibodies to \textit{S. maltophilia} with antibodies to \textit{P. aeruginosa} as mean antibody levels in patients with chronic \textit{P. aeruginosa} infection were similar to those in \textit{S. maltophilia} negative patients.

\textit{Antibody Levels and Pulmonary Function}

The relationship between antibody levels and FEV$_1$ percent predicted was then examined. There was a significant inverse correlation between mean flagellin antibody levels and FEV$_1$ % predicted (p=0.0061) (Figure 2) but not between mean whole cell antibody levels and FEV$_1$ % predicted (p=0.28) (data not shown). A multivariate regression analysis of mean flagellin antibody level and FEV$_1$ % predicted was performed adjusting for age, \textit{P. aeruginosa} infection and \textit{B. cepacia} complex infection. Mean flagellin antibody levels were significantly inversely associated with FEV$_1$ % predicted (p=0.0094).

\textit{Retrospective Cohort Patient Characteristics}

Using this validated definition of chronic infection, we then retrospectively compared CF patients with chronic \textit{S. maltophilia} to those who had intermittent \textit{S. maltophilia} and to those who did not have \textit{S. maltophilia}. Of 692 patients with CF included in the analysis, 49 (7\%) were chronically infected with \textit{S. maltophilia} at baseline. Baseline patient characteristics are summarized by \textit{S. maltophilia} infection status in Table 2. Patients chronically infected with \textit{S. maltophilia} were not significantly different in age, sex, pancreatic status, CFRD or BMI compared with those who had intermittent \textit{S. maltophilia} or never had \textit{S. maltophilia}. There were no significant differences in the percentage of patients in the chronic, intermittent or never \textit{S. maltophilia} groups with respect to mucoid \textit{P. aeruginosa} infection or \textit{B. cepacia} complex infection. A greater percentage of patients with intermittent \textit{S. maltophilia} infection were \textit{P. aeruginosa} positive (67\%) than patients with chronic \textit{S. maltophilia} (46\%) or patients who never
had *S. maltophilia* (36%) (p=0.01). In addition, patients with chronic *S. maltophilia* infection had a significantly lower mean FEV\(_1\) % predicted (47.06%) than patients with intermittent *S. maltophilia* (78.76%) or patients who never had *S. maltophilia* (73.40%) (p=<0.0001).

**Pulmonary Function**

The mean number of years of patient follow up was 8.3 years. In an adjusted model, the rate of decline in FEV\(_1\) % predicted was -1.02% pred/year for patients with chronic *S. maltophilia* (p=0.3 compared to those who never had *S. maltophilia*), -0.94% pred/year for patients with intermittent *S. maltophilia* and -1.06% pred/year for patients who never had *S. maltophilia*. Figure 3 illustrates the adjusted FEV\(_1\) % predicted over time for patients with chronic, intermittent and those who never had *S. maltophilia* infection, demonstrating similar rates of pulmonary decline.

**Pulmonary Exacerbations**

The effect of chronic *S. maltophilia* infection on the number of pulmonary exacerbations requiring hospitalization and antibiotics per year was evaluated using a log linear model. In an unadjusted model, patients with chronic *S. maltophilia* infection (RR=2.5, p<0.0001) and patients with intermittent *S. maltophilia* infection (RR=1.85, p=0.0002) had a significantly increased risk of pulmonary exacerbation compared to patients who had never had *S. maltophilia*. Nonsignificant variables were then removed and a reduced model was generated adjusting for age, pancreatic insufficiency, *P. aeruginosa*, BMI and baseline FEV\(_1\) % predicted. In this model, patients with chronic *S. maltophilia* infection still had a significantly increased risk of pulmonary exacerbation (RR=1.63, p=0.0002) compared to patients who had never had *S. maltophilia* but patients with intermittent *S. maltophilia* infection no longer had a significantly
increased risk (RR=1.18, p=0.28) (Table 3). Patients with pancreatic insufficiency (RR=2.44, p=0.01), *P. aeruginosa* infection (RR=1.61, p<0.0001), lower BMI (RR=1.05, p=0.02) and lower baseline FEV₁ % predicted (RR=1.02, p=<0.0001) also had a significantly increased risk of pulmonary exacerbation.

**Discussion**

To our knowledge, this is the first study demonstrating that CF patients with chronic *S. maltophilia* infection have a specific immune response to *S. maltophilia* that is associated with lower lung function. We have also shown that chronic *S. maltophilia* infection is an independent risk factor for pulmonary exacerbation requiring hospitalization and antibiotic therapy. This association was not observed in patients with intermittent *S. maltophilia* infection. These data suggest that chronic exposure of the respiratory tract of CF patients to *S. maltophilia* may predispose to tissue invasion, resulting in rising antibody levels to *S. maltophilia*, pulmonary inflammation and exacerbations.

Chronic infection can be defined in different ways. Multiple studies have used definitions based on number of positive cultures in a given year and shown that chronic, but not intermittent, MRSA and *A. fumigatus* infection in CF patients are associated with more severe lung disease (6-8). The original definition of chronic *P. aeruginosa* infection in CF patients was based on both the presence of repeated positive respiratory tract cultures and rising *P. aeruginosa* antibody titers (13). The group in Leeds subsequently validated a definition of different stages of *P. aeruginosa* infection based on the percentage of positive cultures and showed that chronic infection was associated with increased disease severity and higher anti-*P. aeruginosa* antibody results(9). Several investigators have developed serologic assays to detect antibodies to *P.
aeruginosa in CF patients and have shown that higher titers are associated with a worse clinical status, validating their own specific definitions of chronic infection.(14),(15). In our study, the microbiologic classification of 2 or more positive cultures in a year as chronic infection was also supported by the selective immune response demonstrated in this group.

The immune response to whole cell S. maltophilia and flagellin in chronically infected patients was approximately two-fold that seen in patients who never had S. maltophilia, suggesting that these patients are truly infected and not simply colonized (16). The serologic response to whole cell S. maltophilia suggests that antibodies may be recognizing epitopes exposed on the bacterial surface (17). However, only the antibody response to flagellin, the main structural component of flagellae, correlated with lower FEV₁ percent predicted. Flagellae are highly immunogenic and are a common, conserved feature of reference and clinical isolates (10). Even though S. maltophilia, like P. aeruginosa, may lose its motility in the CF lung over time(18), antibodies to gene products expressed early in the pathogenesis of infection, such as flagella, persist (13). In addition, S. maltophilia flagellae are antigenically distinct from those of P. aeruginosa which prevented cross-reactivity with antibodies to this common CF pathogen in our study (10).

Unlike serologic assays for P. aeruginosa in CF patients, however, this serologic assay did not detect increased antibody levels to S. maltophilia protease(13) (19). Clinical strains of S. maltophilia are known to produce extracellular alkaline serine protease (11) and protease production may play a role in the development of acute fulminant hemorrhagic pneumonia due to S. maltophilia in immunocompromised patients, for example (20). In CF patients, however, chronic infection with S. maltophilia may be associated with adaptive, phenotypic changes in the bacteria such as loss of protease production(21).
The ability of *S. maltophilia* to cause infection has been well characterized in vitro. Clinical strains of *S. maltophilia* have been shown to adhere to, form biofilm on and invade CF airway epithelial cells, albeit at low levels (22). Clinical *S. maltophilia* strains have direct cytotoxic effects on a variety of cells (23) and are highly immunostimulatory, eliciting significant IL-8 expression by airway epithelial cells, as well as TNF-α production by macrophages (18, 24). In two separate studies using a mouse model of pneumonia, *S. maltophilia*, similar to *P. aeruginosa*, caused pneumonia with a significant associated inflammatory response, mediated primarily by neutrophils (18, 25). In comparison to *P. aeruginosa*, *S. maltophilia* induced substantially more TNF-α in the murine lung. TNF-α, a potent proinflammatory cytokine that induces neutrophil activation, appears to be important in the pathogenesis of *S. maltophilia* infection as significantly fewer TNFR1 null mice compared to wild type mice developed pneumonia and bacteremia (18). Thus, although *S. maltophilia* does not readily invade, causing sepsis and increased mortality (18), it does contribute significantly to airway inflammation.

There are few studies, however, investigating the effect of *S. maltophilia* on clinical outcomes in CF (26-28). The largest epidemiologic study using the CF Foundation National Patient Registry (CFF NPR) found no association between *S. maltophilia* and short-term survival (3 years) or declining lung function (4, 5). However, only short term outcomes were assessed, antibiotic treatment of *S. maltophilia* infection was not taken into account and the effect on pulmonary exacerbations was not determined. In addition, the authors focused primarily on CF patients who were positive at any point in time for *S. maltophilia*. In order to determine the impact of repeated *S. maltophilia* detection on lung function, they defined chronic infection as *S. maltophilia* detected for 2 or more years (not necessarily consecutive). This could include a patient with 1 out of 4 cultures positive one year and 1 out of 4 cultures positive the following year, which is
not a validated definition of chronic infection in CF (9). Our study adjusted for *S. maltophilia* antibiotic therapy, had twice as long a follow up time and used a validated definition of chronic infection. Similar to the study by Goss et al. (4), chronic *S. maltophilia* infection was not associated with an increased rate of decline in FEV$_1$ % predicted in CF patients. The length of patient follow up may still not have been long enough and the rate of decline in FEV$_1$ (approximately 1%/year) may have been too slow to detect the effects of chronic infection on pulmonary function. It is possible that these effects occur predominantly in younger patients before the onset of severe bronchiectasis but our study would be underpowered to detect this due to the small number of pediatric CF patients with chronic *S. maltophilia*. Chronic *S. maltophilia* infection, however, was a risk factor for hospitalization for pulmonary exacerbation requiring antibiotic therapy even after adjusting for other factors associated with increased lung disease severity, such as age, *P. aeruginosa* infection and baseline FEV$_1$ % predicted. This risk was similar to that seen for *P. aeruginosa* infection and suggests that *S. maltophilia* may be playing a role as a pathogen in CF patients.

There are several limitations to this study. Antibodies to whole cell *S. maltophilia* were measured using the laboratory ATCC strain and strain specific immune responses may not have been detected (29). In addition, serologic responses in CF patients were measured at one point in time with corresponding cross-sectional clinical data. It is possible that higher *S. maltophilia* antibody levels are associated with lower FEV$_1$ because chronic *S. maltophilia* occurs more frequently in sicker patients. Similarly, the increased risk of hospitalization for pulmonary exacerbation in patients with chronic *S. maltophilia* may simply reflect an increased intensity of therapy in an already severely affected patient population. Antibiotic use is a known risk factor for the isolation of *S. maltophilia* in the respiratory tract of CF patients (30-33). Increased treatment of pulmonary
exacerbations may select out *S. maltophilia* in the airways of CF patients that are older and have lower lung function. Studies that examine patient cohorts before and after the acquisition of infection are more appropriate in determining the effect of organisms on lung function but require large sample sizes (6, 34). Therefore, to address the question of whether chronic *S. maltophilia* directly results in worsening lung function, prospective longitudinal studies, examining the change in immune responses over time and corresponding clinical outcomes, are needed.

In conclusion, this study shows that CF patients with chronic *S. maltophilia* mount a specific immune response to this organism that is associated with worse lung function, suggesting that this represents a true infection. Whereas previous studies have suggested that *S. maltophilia* simply colonizes the CF airways and does not affect pulmonary function, we have demonstrated for the first time that chronic *S. maltophilia* infection is an independent risk factor for pulmonary exacerbation requiring hospitalization and antibiotics. Further studies are required to define the role of *S. maltophilia* in pulmonary exacerbations in CF patients.
Acknowledgments

We gratefully acknowledge Drs Mary Corey and Annie Dupuis for their statistical support and assistance with the Cystic Fibrosis Database. We also acknowledge Dr Niels Hoiby for his expertise and advice in the development of the serologic assay.
References


Figure Legends

**Figure 1.** Mean antibody levels, with standard error of the mean (SEM), to *S. maltophilia* A) flagellin B) extracellular protease and C) whole bacterial cell in sera samples from patients with Chronic *S. maltophilia* (Chronic SM), Intermittent *S. maltophilia* (Intermittent SM), Never *S. maltophilia* with Chronic *P. aeruginosa* (Never SM with Chronic PA) and Never *S. maltophilia* without Chronic PA (Never SM without Chronic PA). Antibody levels are reported as Ratio Unit (RU) which represents the ratio of the average serum sample OD to the average OD value of the 2 negative controls (wells with pooled normal human serum). * p=0.0004; **p<0.0001 by ANOVA comparing Chronic SM to Intermittent SM, Never SM with Chronic PA and Never SM without Chronic PA.

**Figure 2.** Mean flagellin antibody levels and corresponding FEV$_1$ % predicted. Each dot represents a single patient.

**Figure 3.** Decline in FEV$_1$ % predicted over time for chronic *S. maltophilia* (Chronic), intermittent *S. maltophilia* (Intermittent) and never *S. maltophilia* (Never) based on adjusted model.
Table 1. Serology Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Chronic SM (n=37)</th>
<th>Intermittent SM (n=33)</th>
<th>Never SM (n=109)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Age, mean (range)</td>
<td>25.5 (9.5-58.7)</td>
<td>17.6 (5.4-56.6)</td>
<td>22.0 (6.2-52.5)</td>
<td>0.02</td>
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<td>Sex (male)</td>
<td>21 (57%)</td>
<td>13 (39%)</td>
<td>57 (52%)</td>
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<tr>
<td>Pancreatic insufficient</td>
<td>32 (86%)</td>
<td>31 (94%)</td>
<td>93 (85%)</td>
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<tr>
<td>CFRD</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
<td>2 (2%)</td>
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<tr>
<td>BMI</td>
<td>20.3 (13.5-26.9)</td>
<td>18.7 (13.8-24.6)</td>
<td>19.9 (12.7-27.8)</td>
<td>0.16</td>
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<tr>
<td>FEV₁ mean (range)</td>
<td>58.8 (23.9-97.3)</td>
<td>71.3 (27.3-114.5)</td>
<td>72.3 (27.4-120.5)</td>
<td>0.01</td>
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<td>P. aeruginosa positive</td>
<td>15 (40%)</td>
<td>14 (42%)</td>
<td>59 (54%)</td>
<td>0.25</td>
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<tr>
<td>B. cepacia positive</td>
<td>0 (0%)</td>
<td>3 (9%)</td>
<td>15 (14%)</td>
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Table 2. Baseline Cohort Characteristics

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<th>Chronic S. maltophilia (n=49)</th>
<th>Intermittent S. maltophilia (n=22)</th>
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<td>17.4 (2.4-49.4)</td>
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<td>Pancreatic insufficient</td>
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<td>88.5%</td>
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<td>CFRD</td>
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<td>9.6%</td>
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<td>BMI (range)</td>
<td>18.7 (12.9-27.7)</td>
<td>19.4 (14.1-30.4)</td>
<td>20.1 (12.4-34.8)</td>
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<tr>
<td>FEV₁ mean (range)</td>
<td>53.8 (17.4-102.8)</td>
<td>78.8 (55.6-101.9)</td>
<td>74.3 (20.0-127.0)</td>
<td>&lt;.0001</td>
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<td>P. aeruginosa positive</td>
<td>46.9%</td>
<td>67.3%</td>
<td>36.8%</td>
<td>0.01</td>
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<tr>
<td>Mucoid P. aeruginosa</td>
<td>18.0%</td>
<td>28.8%</td>
<td>21.0%</td>
<td>0.15</td>
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<tr>
<td>B. cepacia positive</td>
<td>0%</td>
<td>1.9%</td>
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Table 3. Adjusted Pulmonary Exacerbation Risk

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<th>Variable</th>
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<th>P value</th>
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<td>Chronic S. maltophilia</td>
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<td>0.0002</td>
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<td>Intermittent S. maltophilia</td>
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<tr>
<td>Pancreatic insufficiency</td>
<td>2.44</td>
<td>0.35</td>
<td>0.01</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>1.61</td>
<td>0.11</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lower BMI</td>
<td>1.05</td>
<td>0.02</td>
<td>0.02</td>
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<tr>
<td>Lower baseline FEV₁ % pred</td>
<td>1.02</td>
<td>0.003</td>
<td>&lt;0.0001</td>
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Figure 1

A.

Ratios of Units (RU)

Chronic SM (n=37)  Intermittent SM (n=33)  Never SM with Chronic PA (n=43)  Never SM without Chronic PA (n=66)

B.

Ratios of Units (RU)

Chronic SM (n=37)  Intermittent SM (n=33)  Never SM with Chronic PA (n=43)  Never SM without Chronic PA (n=66)

C.

Ratios of Units (RU)

Chronic SM (n=37)  Intermittent SM (n=33)  Never SM with Chronic PA (n=43)  Never SM without Chronic PA (n=66)
Figure 2
Figure 3.
Stenotrophomonas maltophilia in cystic fibrosis:

Serologic response and effect on lung disease

Valerie Waters, MD, MSc, Yvonne Yau, MD, Sudha Prasad, PhD, Annie Lu, Eshetu Atenafu, MSc, Ian Crandall, D. Phil., Stephanie Tom, Elizabeth Tullis, MD, Felix Ratjen, MD, PhD.

Online Data Supplement
Methods

I. Serologic Study

Study Design

This was a cross-sectional study of patients with CF followed at the Hospital for Sick Children and St Michael’s Hospital (Toronto, Canada). Patients were included in the study if they had a confirmed diagnosis of CF based on the following: a) the presence of clinical features consistent with CF, or b) a positive family history for CF plus either 2 documented sweat chloride values > 60 mEq/L measured by quantitative pilocarpine iontophoresis test, genetic testing showing 2 CF-causing mutations or a nasal potential difference consistent with CF (1). Patients were excluded if they could not produce sputum, if they were unable to perform reproducible spirometry or if they had received a lung transplant. A serum sample was collected for each patient with routine clinical blood sampling in the CF clinic and tested for *S. maltophilia* antibodies as outlined below. Clinical data and pulmonary function were obtained for each patient. Pulmonary function testing was performed on the same day as serum specimens were obtained in most (94%) patients and was performed within 3 months in the remainder.

This study was approved by the Research Ethics Board at the Hospital for Sick Children and St Michael’s Hospital.

Microbiological Classification

All enrolled subjects were classified, using the microbiology database at the Hospital for Sick Children and St Michael’s Hospital, into the following groups according to their respiratory culture status in the previous 12 months based on previously validated definitions (2): 1) Chronic
S. maltophilia: 2 or more positive sputum or bronchoalveolar cultures for S. maltophilia in a given year, 2) Intermittent S. maltophilia: 1 positive sputum or bronchoalveolar cultures for S. maltophilia in a given year or a previous positive culture, 3) Never S. maltophilia: never having a positive sputum or bronchoalveolar culture for S. maltophilia. For the serologic analysis, subjects who never had S. maltophilia were divided into those who had chronic P. aeruginosa (Never S. maltophilia with Chronic P. aeruginosa) and those without chronic P. aeruginosa (Never S. maltophilia without Chronic P. aeruginosa) as it was important to demonstrate that high antibody levels were not due to cross-reaction with antibodies to P. aeruginosa. These definitions were validated against the serologic results.

Antigen Isolation and Purification

Flagellin

S. maltophilia flagellin was isolated from the ATCC S.maltophilia strain 13637 known to express flagellae using a protocol described by de Oliveira-Garcia (3). Electron microscopy (EM) of the purified product revealed an abundance of flagellae and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified protein revealed a predominant band at 38 kDa, corresponding to the molecular weight of S. maltophilia flagellin. The identity of the purified product was further validated by matrix-assisted laser desorption time-of-flight (MALDI-TOF) peptide mass fingerprinting. The concentration of the flagellin was determined using BCA™ Protein Assay (ThermoScientific, Rockford, IL).

Extracellular Alkaline Serine Protease

To identify a clinical strain that produced extracellular alkaline serine protease, proteolytic activity of clinical strains of S. maltophilia from CF patients was screened using a microassay
with the chromogenic substrate, azoalbumin (Sigma), as described by Plantner et al. (4). The clinical strain CFSM51 was then used in the large scale purification of the protease. The extracellular alkaline serine protease of *S. maltophilia* was purified as previously described (5). Secreted *S. maltophilia* serine protease was purified from cell-free culture supernatant by anion exchange chromatography using DEAE-Sephacel (Sigma). Protein fractions were eluted by a linear gradient of 0 – 1000 mM NaCl at a flow rate of 1 mL/minute. One hundred 6 mL fractions were collected and assayed for serine protease activity with the specific substrate Suc-Ala-Ala-Pro-Phe-pNA (Sigma) (5). A single peak of activity was detected and corresponding fractions were pooled together. Pooled fractions were further fractionated by size exclusion chromatography through a Sephacryl S200HR column (Sigma) at a flow rate of 10 drops/minute. Fractions that demonstrated good serine protease activity as assessed by the Suc-Ala-Ala-Pro-Phe-pNA assay were pooled. Silver stain gel of the preparation revealed a predominant band at 47 kDa that corresponded to the molecular weight of *S. maltophilia* protease previously isolated from a clinical strain of *S. maltophilia* (5). The protein concentration was determined using the Bradford assay (6).

**Whole Bacterial Cell**

*S. maltophilia* whole bacterial cell was prepared using a modification of a previous protocol (7). In brief, the ATCC strain 13637 of *S. maltophilia* was grown overnight at 37°C on a blood agar plate. Colonies were harvested from the plate and resuspended in 10 ml of PBS at an optical density (OD) of 0.08 at 600 nm, corresponding to $10^8$ colony forming units (CFU)/ml. The bacteria were then heat-killed at 65°C for 1 hour which was subsequently verified by inoculating a blood agar plate.
**Sera Processing and Serologic Assay**

Blood samples were centrifuged at 2500 rpm at 4°C for 10 minutes. The sera were then aliquoted and stored at -80°C for batched serologic studies.

Serologic responses were measured by an enzyme-linked immunosorbent assay (ELISA) using each of the 3 antigens (flagellin, protease and whole bacterial cell) individually. We optimized concentrations of the purified antigens and the developing reagent by criss-cross serial dilutional analysis. Wells of high-binding polystyrene 96-well microtiter plates (NUNC, MaxiSorp™) were coated directly with 50 µl/well of purified antigen solution (flagellin: 1 µg/well; protease: 0.1 µg/well and whole bacteria: $10^8$ CFU/ml heat-killed *S. maltophilia* ATCC) by passive absorption overnight at room temperature. Unbound proteins were then removed and residual binding capacity of the plate was blocked by incubating with blocking buffer (phosphate-buffered saline (PBS), 2.5% casein) for 1 hour at room temperature. After 3 washes with PBS, the coated plates were then incubated with dilutions of sera from CF patients in duplicates for 2 hours at room temperature. Based on criss-cross serial dilutional analysis, the sera dilution was 1:2,500 for flagellin, 1:500 for protease and 1:500 for whole bacterial cell. Sera were screened in a blinded fashion. Two wells in each 96-well plate were incubated with pooled normal human serum obtained from more than 50 adults (negative control) (Fisher Scientific, Pittsburgh, PA). In addition, each plate included 2 blank control wells coated with antigen and secondary antibody but with no serum (primary antibody). The plates were then washed 3 times with PBS and incubated with various dilutions of secondary antibody for 2 hours at room temperature. Based on criss-cross serial dilutional analysis, the optimal secondary antibody dilution was 1:5,000 for flagellin, 1:25,000 for protease and 1:5,000 for whole bacterial cell. Alkaline phosphatase-conjugated goat anti-human IgG/IgA/IgM (Jackson ImmunoResearch Laboratories Inc, West
Grove, PA) was used as the secondary antibody. After 3 more washes with PBS and 0.05% Tween20 (Sigma, St Louis, MO) and one additional wash with PBS alone, 75 µl of pNPP (Sigma, St Louis, MO) substrate solution was added to each well and incubated for 1 hour at room temperature. To stop hydrolysis, 25 µl of 3M NaOH was added to each well and OD was measured immediately at 405 nm. Results were represented as the ratio of the average serum sample OD to the average OD value of the 2 negative controls (wells with pooled normal human serum) run on the same plate to account for day to day variation in the ELISA (Ratio Units: RU). The median inter-well variation for ELISA with flagellin, protease and whole bacteria was 7%, 11% and 7% respectively.

Statistical Analysis

Analysis of variance (ANOVA) was used to assess if there was significant difference in mean antibody levels between groups based on the outcome variables. Based on the result, further analysis was performed on the two outcome measures (flagellin and whole bacterial cell) in comparing the chronic group versus all the other groups combined. The correlation between mean antibody levels and forced expiratory volume in 1 second (FEV$_1$) % predicted were done using Pearson’s correlation. Multiple regression analysis was also done on FEV$_1$ % predicted and the mean antibody levels adjusting for age, *P. aeruginosa* and *B. cepacia* complex infection. Statistical analysis was performed using SAS (version 9.1; SAS Institute Inc., Cary, NC). A p-value of 0.05 or less was considered statistically significant.
II. Cohort Study

Study Design

This was a retrospective cohort study of patients with CF followed at the Hospital for Sick Children and St Michael’s Hospital (Toronto, Canada) from 1997 to 2008. Patients with chronic *S. maltophilia* were compared to those who had intermittent *S. maltophilia* and those who did not have *S. maltophilia* over the study period. The primary outcome measure was the rate of decline of FEV$_1$ in percent predicted in patients in each of these three groups. The secondary outcome was the number of hospital admissions for pulmonary exacerbations requiring antibiotics per year.

The data for this study was extracted from the Toronto Cystic Fibrosis Database housed at the Hospital for Sick Children. The database contains information for patients’ sputum microbiology, medications, pulmonary function testing and hospital admissions. Specific information that was unavailable from the database was retrieved through systematic review of hospital health records by one of the study investigators (A.L.). The time period 1997-2008 was chosen because *S. maltophilia* positive respiratory specimen cultures were first recorded in the database in 1997.

Patients were included in the study if they had a confirmed diagnosis of CF (1). Patients were excluded if they could not produce sputum or if they were unable to perform reproducible spirometry. Patients above the age of 50 were excluded as they represent a milder phenotype not typical of the general CF population (8). Patient data was censored at lung transplantation.

Patients were classified as chronic *S. maltophilia*, intermittent *S. maltophilia* and never *S. maltophilia* (as outlined above) for each year of the study period and the effect on FEV$_1$ and
hospitalization for pulmonary exacerbation was examined in the following year(2). Patients could thus move from one microbiological classification to another over the study period.

This study was approved by the Research Ethics Board at the Hospital for Sick Children and St Michael’s Hospital.

Statistical Analysis

Descriptive statistics, frequency distributions and percentages were calculated for the outcome variables and other covariates of interest. ANOVA was used to assess if there was significant difference in average age, body mass index (BMI) and FEV₁ % predicted at one year in the study between chronic groups. Chi-square test based on one year study of the data was also carried out for assessing the baseline association of gender, pancreatic insufficiency, CF related diabetes (CFRD), *P. aeruginosa*, mucoid *P. aeruginosa* and *B. cepacia* among the chronic group. Baseline FEV₁ was defined as the best FEV₁ one year before the first observation in the follow-up period. Two separate regression models were generated as used in a previous study by Amin et al.(2). In the first model, a hierarchical linear model was generated to look at the effect of chronic *S. maltophilia* on quarterly FEV₁ allowing for a random effect and slope for each patient. The second model assessed the effect of chronic *S. maltophilia* on the number of hospitalizations for pulmonary exacerbation requiring antibiotics using Poisson regression. Multivariable analysis was performed to account for age, gender, body mass index (BMI), baseline FEV₁, presence of *Pseudomonas aeruginosa* (current, never, gone), mucoid *P. aeruginosa, Burkholderia cepacia* complex (current, never, gone), use of antibiotic therapy specific for *S. maltophilia*, use of inhaled tobramycin, use of oral antibiotics and use of intravenous antibiotics. Multivariable analysis was also performed to account for pancreatic status and CF-related diabetes (CFRD).
Statistical analysis was performed using SAS (version 9.1; SAS Institute Inc., Cary, NC). A p-value of 0.05 or less was considered statistically significant.
References


Persistent methicillin-resistant *Staphylococcus aureus* and rate of FEV1 decline in cystic fibrosis.