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Sequential *Burkholderia cenocepacia* Isolates from Siblings with Cystic Fibrosis Show Increased Lung Cell Attachment

To the Editor:

Burkholderia cepacia complex (Bcc) is a group of 20 genetically distinct bacterial species (1) that has a severe impact on the quality of life of people with cystic fibrosis (CF) and is associated with a more rapid decline of lung function than *Pseudomonas aeruginosa* (2). *B. cenocepacia* is the most virulent species within the Bcc and is most frequently associated with septicemia, although other Bcc species have also been linked to bloodstream infections (BSI) (2, 3).

Many pathogens alter their phenotype during chronic infection in response to changing selection pressures, coinfecting species, and antimicrobial therapies (4, 5). Studies on bacterial adaptation in the CF context have predominantly focused on *P. aeruginosa*; however, the adaptive strategies of *B. cenocepacia* isolates have also been examined (4, 6, 7). Antimicrobial resistance, loss of motility, tolerance of iron limitation, and increased virulence to host cells over time of chronic infection were reported. In contrast, *P. aeruginosa* and another member of the Bcc, *B. multivorans*, showed reduced virulence over time of infection (5, 8). We have examined two series of sequential isolates from two adult male siblings with CF (referred to as P1 and P2). Both patients became infected with Bcc during their teens and were chronically infected when transitioning to adult care.

Patient 1

P1 has chronic *B. cenocepacia* infection confined to the lung and experienced reduced FEV_1 values over 5 years, indicative of disease progression (Figures 1A and 1B). P1 was diagnosed with CF lung disease, pancreatic insufficiency, osteopenia, distal intestinal obstruction syndrome, and CF-related liver disease in childhood. Bcc was always grown in combination with *Candida* species from his sputum specimens. After transferring from the pediatric center, initial sputum specimens of P1 were also intermittently positive for *Staphylococcus aureus* and *P. aeruginosa*.

Patient 2

P2 suffered from CF lung disease, pancreatic insufficiency, CF-related diabetes, and CF-related liver disease with splenomegaly. P2 also had chronic *B. cenocepacia* lung colonization and developed Bcc BSI on three occasions (Figure 1A). Bcc was grown from blood cultures taken from central lines and peripherally, and the BSI was considered catheter related rather than by direct invasion from the lung. On these occasions, P2 had been admitted with respiratory exacerbations of CF lung disease and started intravenous antibiotic treatment. Once blood cultures became positive with Bcc, antibiotic treatment was altered and the source of infection, port-a-cath (second BSI) or central venous catheter (final BSI), was removed. All sputum specimens taken over the 3-year period in P2 yielded a heavy Bcc growth in combination with *Candida* species. *S. aureus* and *P. aeruginosa* were only grown intermittently from sputum. Low FEV_1 values (16–27%) were observed (Figure 1B), indicative of late-stage obstructive disease. He died of respiratory failure 3 years after his first *B. cenocepacia* blood culture isolation.

These sequential isolates provide a rare opportunity to examine the alterations that *B. cenocepacia* undergoes during the course of chronic lung infection and recurrent bacteremia. Five sputum isolates spanning 90 months were randomly selected from all P1 isolates designated P1S1 to P1S5. The first and third blood peripheral isolates (P2B1 and P2B3) together with three sputum isolates (P2S1 to P2S3) were selected from P2 isolates for investigation. The isolates were shown to be clonal by pulsed-field gel electrophoresis. Multilocus sequence typing analysis of the *B. cenocepacia* isolates was compared with the *Burkholderia cepacia* complex Multi Locus Sequence Typing website (http://pubmlst.org/bcc/), developed by Keith Jolley and sited at the University of Oxford (9). The isolates from both individuals shared the same unique sequence type on the basis of a new *trpB* allele 405 and were designated ST867. This novel sequence type clusters with *B. cenocepacia*, *recA* group IIIA.

We investigated whether the sequential isolates from both siblings differed in their ability to colonize lung epithelia by examining attachment to CF epithelial cells, $CFBE41o^-$ (multiplicity of infection, 50:1), as previously described (10). The sputum isolates from P1 had an increased ability to adhere to $CFBE41o^-$ cells over time of colonization ($P = 0.0097$), showing increased potential for host epithelial attachment (Figure 2A). P1S5 was the most adherent of all isolates examined. Although isolates from P2 were more than twofold less adherent than isolates from P1 ($P = 0.003$), the P2 sequential isolates also showed more attachment to $CFBE41o^-$ cells with increasing time of colonization ($P = 0.0012$), regardless of origin of the isolate. The P2 isolates examined showed comparable epithelial cell attachment to positive controls, *B. cenocepacia* BC7 and K56-2, which are both *recA* group IIIA strains. The increased host cell attachment of a selection of both siblings' isolates over time of colonization was confirmed by confocal microscopy (Figure 2B). When bacteria were counted (10 fields per sample), attachment increased from 2.75 ± 0.07 (mean $\pm$ SD) to 27.8 ± 23.8 bacteria/100 host cells in the case of P1S1 and P1S4 isolates, respectively, and from 2.29 ± 0.08 to 8.79 ± 1.4 bacteria/100 host cells for P2B1 and P2B3 isolates, respectively ($P < 0.01$).

This is the first demonstration of an increased ability of sequential *B. cenocepacia* isolates to adhere to CF epithelial cells over time of infection. The increased attachment of blood isolates to epithelial cells over time is likely to be a consequence of bloodstream isolates being previously adapted lung isolates, and the phenotype being maintained during bloodstream infection. The increased epithelial attachment of *B. cenocepacia* ST867

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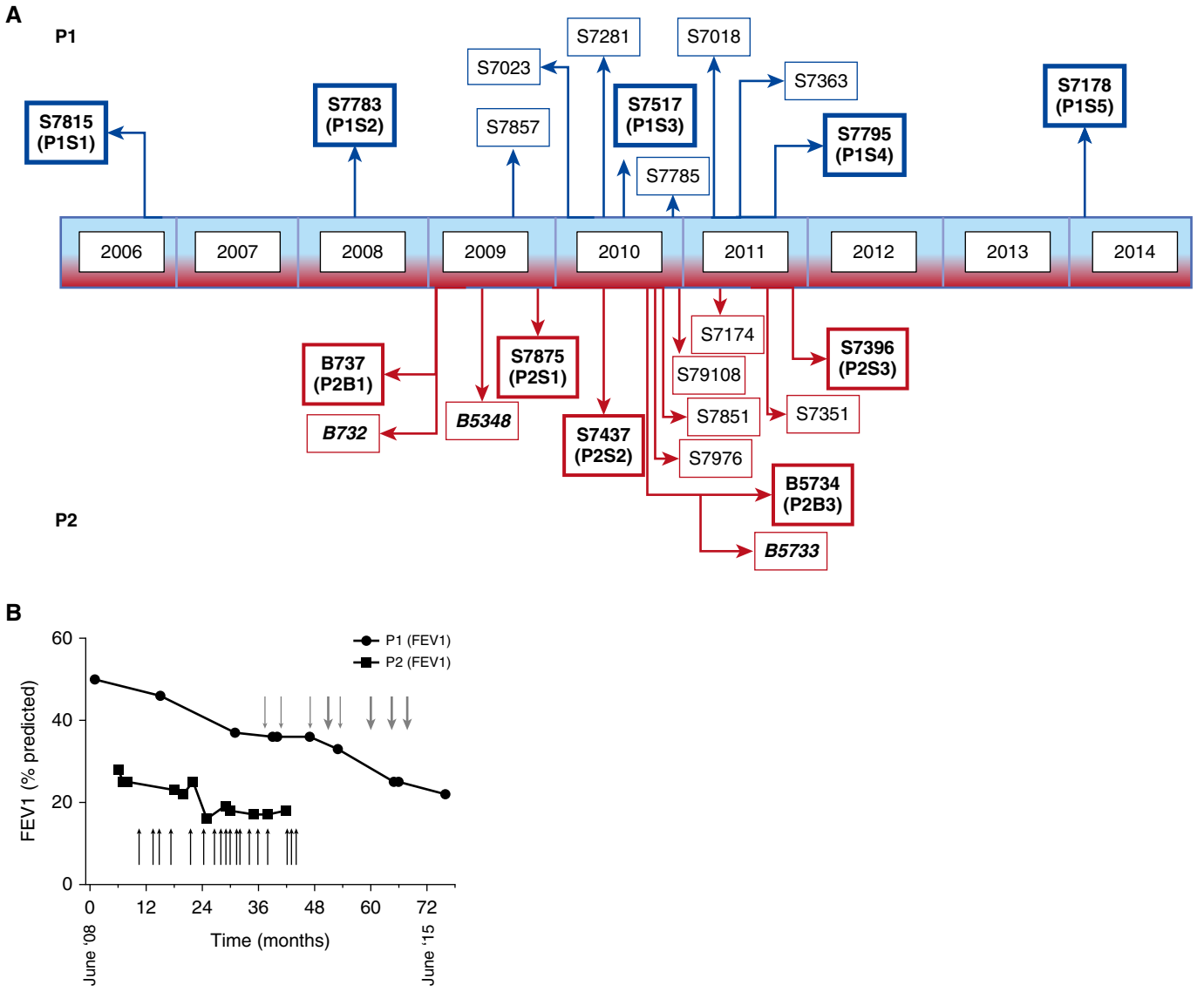

Figure 1. (*A*) Timeline for sequential *Burkholderia cenocepacia* isolates indicating time of isolation from both patients. Isolates from patients P1 and P2 are *above* and *below* the timeline, respectively. Sputum and blood isolates are prefixed with S and B, respectively. The 10 isolates we have examined in this study are highlighted in *bold* with *thick-bordered boxes*. (*B*) Alteration in FEV_1 % predicted in both patients together with their inpatient admissions: *gray arrows* signifying P1 inpatient admissions; *black arrows* signifying P2; *broader arrows* indicating inpatient stays of more than 1 month. P1S1 to P1S5 are five sputum isolates spanning 90 months randomly selected from all P1 isolates. P2B1 and P2B3 are first and third blood peripheral isolates, and P2S1 to P2S3 are three sputum isolates selected from P2 for investigation.

in both sets of isolates suggest that this may be a general strategy by which these isolates avoid clearance by the host and may contribute to the challenges of eradication of *B. cenocepacia* once chronic infection is established. Bcc can switch from a mucoid to nonmucoid phenotype, which correlates with a more rapid decline in FEV_1 (11); however, these isolates were predominantly nonmucoid (unpublished). Increased virulence and alterations in proteomic profile have previously been shown in sequential *B. cenocepacia* isolates (7). It is likely that these isolates alter their surface protein expression to improve host cell attachment.

A detailed examination of the time-dependent alterations across both patients' isolates leading to this increased attachment may provide a mechanism by which *B. cenocepacia* chronically persists, and more studies are required to determine whether enhanced attachment is common among larger cohorts of Bcc patient isolates. The mechanisms behind the phenotypic differences in bacterial isolates at a given time point can be complex, but understanding the key adaptations that differentiate Bcc blood and sputum isolate types should contribute to the prevention of potentially life-threatening BSI in these patients. Overall, the increased ability to attach to host cells is likely to

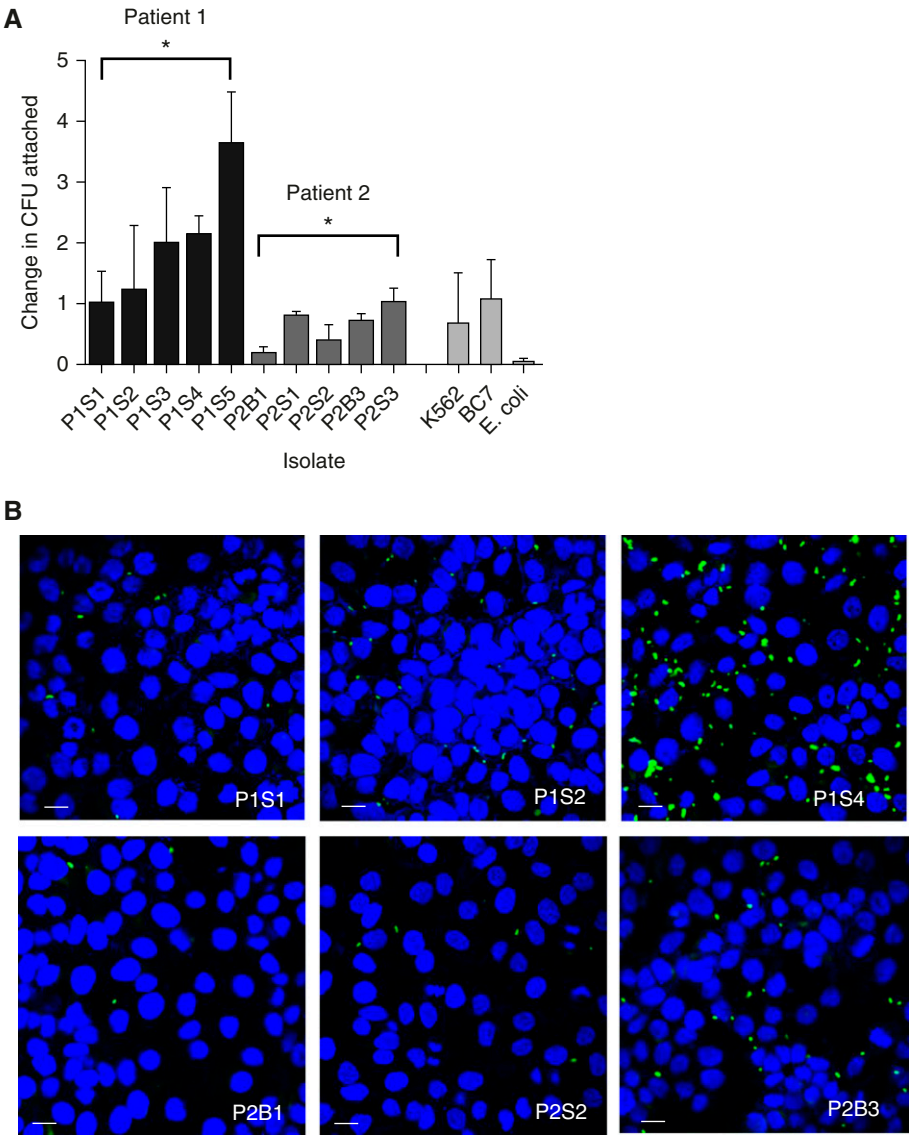

Figure 2. Adhesion of the sequential *Burkholderia cenocepacia* isolates to cystic fibrosis epithelial cells as determined by microbiological plating (*A*) or confocal microscopy (*B*). (*A*) Mean CFU adhered per well to CFBE41o$^-$ cells, as determined on at least three independent occasions. *Error bars* represent standard error. *Statistically significant difference relative to earliest time point (P1S1 or P2B1, respectively) as determined by one-way analysis of variance with Brown-Forsythe test. (*B*) Representative images of *B. cenocepacia* isolates adhering to CFBE41o$^-$ cells over time of colonization as observed by confocal microscopy. Ten fields were counted per sample in each of two independent experiments. Epithelial cell nuclei were stained with 4',6-diamidino-2-phenylindole; *B. cenocepacia* isolates are fluorescein isothiocyanate labeled. *Scale bars* = 20 μm. P1S1 to P1S5 are five sputum isolates spanning 90 months randomly selected from all P1 isolates. P2B1 and P2B3 are first and third blood peripheral isolates, and P2S1 to P2S3 are three sputum isolates selected from P2 for investigation. CFU = colony-forming unit.

confer a bacterial survival advantage during infection, contributing to bacterial survival as a whole during chronic infection in CF. ■

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The ILD-India Registry: *Ignoratio Elenchi*

To the Editor:

We read with great interest the study by Singh and colleagues (1) [this issue, pp. 801–813], wherein the authors have described the profile of interstitial lung diseases (ILDs) in 1,084 subjects from 27 centers across India. We congratulate the authors for conducting this study, which reflects the largest data set on ILDs from India. However, there are certain limitations, consequent to which the results do not reflect the true picture of ILDs in India.

Most important, in this study, hypersensitivity pneumonitis (HP) accounted for about 47.3% of all ILDs. This frequency is higher than in any study on the spectrum of ILDs ever reported from India or elsewhere (2, 3). There may be several reasons for this discrepant finding. Consecutive subjects were not enrolled in this study (only consenting eligible patients who could afford the costs of the evaluations were enrolled), which might have introduced a selection bias in the study right at the time of enrollment. Further, it is well known that a large proportion of patients in India who have sarcoidosis are misdiagnosed as having tuberculosis, based on chest radiographic findings (4). Sarcoidosis is still considered a rare disorder, despite evidence to the contrary. Such patients would not be referred for enrollment into an ILD registry. Further, the biopsy rate in this study was extremely low (7.5%); in fact, it was the lowest ever reported in any large ILD profile study. The diagnosis was completely dependent on radiologic appearances on high-resolution computed tomography (HRCT) in almost all cases. A recent study has shown that the agreement between multidisciplinary teams on diagnostic likelihoods was good for idiopathic pulmonary fibrosis ($\kappa w = 0.71$) and connective tissue disease–related ILD ($\kappa w = 0.73$), moderate for nonspecific interstitial pneumonia ($\kappa w = 0.42$), but poor for HP ($\kappa w = 0.29$) (5). Thus, even multidisciplinary teams may not be congruent on a diagnosis of HP in a large proportion of cases, even with all clinical, radiological, and histopathological data at their disposal. One can only imagine how flawed the diagnosis of HP may get, in the absence of a lung biopsy. We fear that several of the subjects diagnosed with HP might have had other disease entities such as nonspecific interstitial pneumonia, idiopathic pulmonary fibrosis, and sarcoidosis. A recent study showed that 95% of subjects having an HRCT pattern inconsistent with usual interstitial pneumonia (which included patients with mosaic attenuation, a radiologic characteristic of HP) were ultimately found to have definite or probable usual interstitial pneumonia on lung biopsy (6). Finally, peribronchovascular thickening on HRCT was considered a criterion for diagnosing chronic HP, a finding commonly seen in sarcoidosis. This might have led to patients with stage III and IV sarcoidosis being misdiagnosed as HP in the absence of a lung biopsy.

To add to the confusion, in a large proportion (48.1%) of patients with HP, the disease was attributed to exposure to air coolers. These data translate to a finding that about 23% of all