Molecular typing of *Burkholderia cepacia* complex isolated from patients attending an Italian Cystic Fibrosis Centre

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**SUMMARY**

Bacteria from the *Burkholderia cepacia* complex (Bcc) are capable of causing severe infections in patients with cystic fibrosis (CF). Bcc infection is often extremely difficult to treat due to its intrinsic resistance to multiple antibiotics. In addition, it seems to speed up the decline of lung function and is considered a contraindication for lung transplantation in CF. This study investigates the species of the Bcc strains recovered from chronically infected CF subjects by means of: isolation, identification methods and complete recA nucleotide sequences of 151 samples. Molecular typing showed that *B. cenocepacia* III is the dominant strain found in the group of subjects being treated at the Milan CF Centre (Italy) and that the infection is chronically maintained by the same species. Defining species by means of molecular analysis yields important information for the clinician in order to establish the most appropriate therapy and implement correct measures for prevention of transmission among CF subjects.

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**INTRODUCTION**

The *Burkholderia cepacia* complex (Bcc) is a group of strictly aerobic gram negative bacteria routinely isolated from the natural environment where they can exert a range of beneficial properties (Mahenthiralingam et al., 2005; Baldwin et al., 2007; Mendes et al., 2007). Bcc currently includes at least 21 closely related species (formerly called genomovars): *B. ambifaria* (genomovar VII), *B. anithina* (genomovar VIII), *B. arboris* (BCC3), *B. cepacia* (genomovar I), *B. cenocepacia* (genomovar III), *B. contingens* (group K, BCCAT), *B. diffusa* (BCC2), *B. dolosa* (genomovar VI), *B. lata* (group K), *B. latens* (BCC1), *B. metallica* (BCC8), *B. multivorans* (genomovar II), *B. pyrrocinia* (genomovar IX), *B. seminilis* (BCC7), *B. stabilis* (genomovar IV), *B. utonensis* (genomovar X), *B. vietnamiensis* (genomovar V), *B. stagnalis* (BCC B), *B. territorii* (BCC L), *B. pseudomultivorans*, *B. paludis* (Soltan Dallal et al., 2014; Abbott et al., 2015; Vandamme et al., 2011; De Smet et al., 2015, Ong et al., 2016). These species cause infections in vulnerable people and mainly affect patients with underlying chronic diseases, such as Cystic Fibrosis (CF) (Ku et al., 2011; Siddiqui et al., 2001).

CF (MIM#219700) is the most common life-threatening autosomal recessive condition affecting Caucasians. It is characterised by recurrent lower respiratory infections, inflammation and lung damage eventually leading to respiratory failure requiring lung transplantation (Samano et al., 2013). The difficulty in treating *B. cepacia* complex infections is attributed to its intrinsic resistance to multiple antibiotics (Jones et al., 2004; Matthew et al., 2009; Mahenthiralingam et al., 2005; Niihada et al., 2012; Rushton et al., 2013) and chronic Bcc infection, especially by *B. cenocepacia* genomovar III, is associated with a more rapid decline in lung function (Ledson et al., 2002; Magalhaes et al., 2002) with an increased need for antibiotics (Frangolias et al., 1999). Unfortunately, preoperative infection by *B. cenocepacia* is generally associated with poor transplantation outcomes, due to the tendency to develop a form of necrotizing pneumonia, frequently associated with a "cepacia syndrome" (Kollerstova et al., 2015; Nash et al., 2010). De Soyzia and co-workers confirmed this, but showed excellent long-term survival in a non-*B. cenocepacia* Bcc group (De Soyzia et al., 2010).

The aim of the study was to evaluate the prevalence of Bcc species and to study the epidemiology of the patients in care at the CF Centre in Milan, Italy. Furthermore, we aimed to unravel the question of whether chronic infection is sustained over the years by the same genomovar. Increasing knowledge on different *Burkholderia* species would allow better therapeutic management by clinicians and help to deal with the risks on transplantation outcomes posed by this pathogen in CF patients.

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MATERIALS AND METHODS

We carried out a 2005-2015 retrospective survey on the prevalence of Bcc in CF patients by performing isolation, identification and then molecular typing of the Bcc strains in order to assess the possible persistence over the time of the same species. The patients in care at present at the Cystic Fibrosis Centre in Milan number 838 and we focused on 21 patients (mean age 29 years) chronically infected by B. cepacia complex. Three of them have died over the years and one after lung transplantation. In this survey 151 strains (one per year for each colonized patient) were studied and isolated from chronic patients.

Samples
The microbiological evaluation was performed on sputum samples routinely processed and investigated according to standard CF microbiological procedures (Smyth et al., 2014). Samples were collected at least every 3 months over a one year period and were obtained from the lower airways. As concerns the microbiological evaluation, samples were first mixed with Sputasol (Oxoid) at a 1:1 dilution and then incubated at 37°C for 30 minutes to reduce the viscosity of the sputum. Treated samples were spread on agar plates selective for Burkholderia cepacia (BCSA), then they were incubated aerobically at 37°C for 72 hours and growth was assessed. Negative plates were subsequently stored at room temperature for a further five days before re-examination for growth. All presumptive B. cepacia complex colonies were identified by Maldi-ToF spectrometry and by Microscan WalkAway plus System (Beckman Coulter). At the same time the Bcc colonies were spread on Columbia agar plates supplemented with 5% sheep blood and incubated at 37°C for 24 hours and the purified strains were stored at -80°C to allow further examinations and species identification.

Sequencing analysis
The analysis was performed by using the Sanger sequencing method. First of all DNA was extracted using the kit QIAamp cador Pathogen Mini Kit (Qiagen®) and the genomic material was quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fischer®). Then sample dilutions were set up in order to have a concentration of 30 ng/μl. After that, the recA gene of the B. cepacia complex strains was examined, PCR primers used were (Mihenthiralingam et al., 2000; Payne et al., 2005): BCR1:T-GACCGCGAGAAGGCAA and BCR2:CTCTTTCTCGTCATCGCCTC. The PCR reaction was set up with (final volume =25 μl): Buffer PCR 5X (EuroClone), dNTPs Mix (Promega) 10 mM, MgCl2 (EuroClone) 25 mM, Primer Forward BCR1 10 μM, Primer Reverse BCR2 10 μM, Red-Taq (EuroClone) Polymerase, Water, DNA sample 30 ng/μl. PCR program was: 98°C 5 minutes; 98°C 10 seconds, 65.7°C 20 seconds, 72°C 20 seconds (x 35 cycles); 72°C 5 minutes. The PCR products were loaded on agarose gel at 2% in 1 minute; 96°C 10 seconds, 60°C 4 minutes (x 25 cycles); 10°C 10 minutes. Sequencing reactions were prepared with the BigDye Terminator v1.1 Cycle Sequencing Kit, following the manufacturer’s instructions, and were analysed using an ABI PRISM 3100 Genetic Analyzer Capillary Electrophoresis System (Applied Biosystem). Sequences were assembled using BioEdit Sequence Alignment software and were submitted to the Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov/) and GenBank were used to establish gene identities. Analysis of the recA gene of the B. cepacia complex provides a rapid and robust nucleotide sequence-based approach to identify and classify this taxonomically complex group of opportunistic pathogens (Vanlaere et al., 2008; Detsika et al., 2003).

RESULTS

BCC prevalence
Among the 838 patients in care at the CF Centre in Milan at present, annual frequency isolation is 2.5% (year 2015). From the data at our disposal, it was observed that the prevalence of chronic infection has remained constant over the last five years.

Genomovar distribution
The analysis described in Figure 1 shows the distribution of B. cepacia complex species among CF patients treated at CF Centre in Milan, genomovar III is the most prevalent (38%), followed by B. stabilis genomovar IV (19%) and B. multivorans genomovar II (14.2%). B. cepacia genomovar I is present with a frequency of 9.6% and it is notable that the strains were recovered from siblings, suggesting a possible cross-infection. The total number of isolates typed per patient differs because it depends on the age of first colonization.

Interspecies variability
Results shown in Figure 2 highlight that each patient presents the same species over the years, therefore excluding interspecies variability. Thus, every patient has maintained the same species since the first colonization.

DISCUSSION

The number of patients in care at the CF Centre in Milan from 2005 to 2015 increased from 450 to 838. CF is a multi-organ disease, but predominantly affects the respiratory system. Persistent lung infection that causes a progressive damage to pulmonary tissue represents the principal cause of mortality among CF patients. In particular, Bcc infection currently represents a significant challenge for physicians treating CF patients. The colonization/infection prevails in adulthood probably as the result of previous infection by pathogenic bacteria, such as S. aureus, P. aeruginosa, and many others. The constant pressure of antibiotic treatment to eradicate these bacteria selects the most resistant, the so-called Multi Drug Resistant, promoting their adhesion to the lung parenchyma. Lung damage is due to both bacterial pathogenic factors and excessive inflammatory response, with release of soluble mediators (cytokines, chemokines).

Thanks to the introduction of selective media for the growth of Bcc there have been improvements in isolation
techniques and also in the field of the molecular biology, so that molecular typing of the recA gene was introduced as a molecular identification method for Bcc species. Over the period from 2009 to 2015, the prevalence of Bcc infection has remained almost constant (about 2.5%) probably because of the implementation of an internal protocol of “segregation” of Bcc colonized patients (dedicated environments for in and out patients clinic), in order to prevent transmission to uninfected patients (with absence of the germ in at least three successive sputum samples carried out three months apart from each other during a year). This percentage is consistent with previously reported data from other CF centres in Italy (unpublished data). In this study, an epidemiological investigation was also performed to observe the distribution of the different species of Bcc in the CF population of patients referred to the centre. Molecular studies of the recA gene revealed 7 out of 21 genomovars known so far, with a high prevalence of B. cenocepacia genomovar III colonizing 8 out of 21 patients (38%), followed by B. stabilis genomovar VI in 4 out of 21 patients (19%) and B. multivorans genomovar II found in 3 patients (14.2%). In contrast, the prevalence (Figure 1) of B. cepacia genomovar I in 2 patients (9.6%), B. vietnamiensis genomovar V in 2 patients (9.6%), B. seminalis (BCC7) in 1 patient (4.8%) and B. metallica (BCC8) in 1 patient (4.8%) is substantially lower. Even if some studies have mainly reported that B. multivorans has become more common than B. cenocepacia III (Pope et al., 2010, Spicuzza et al., 2009), B. cenocepacia was reported as being the most common species isolated from CF patients since the 1990s (Vandamme et al., 1997). This is also confirmed by the subsequent literature and is in accordance with our data that show the prevalence of the B. cenocepacia followed by B. multivorans in CF infections and a lower occurrence of other Bcc members (Bach Evelise et al., 2017; Lipuma et al., 2001; Kidd, 2003; Mahenthiralingam et al., 2005).

For each chronic patient we analysed one strain per year...
in order to see if first genomovar colonizing the lungs persists or an inter-species variability occurs. Data show that each patient maintained the same genomovar over time after the first colonisation (Figure 2). The isolation of Bcc strains in the respiratory samples of CF patients must be immediately reported by the laboratory to the clinician, in order to ensure adequate therapeutic measures and strategies to prevent the spread of germs and transmission to non-colonized patients. Species characterization is extremely important because in subjects with severe lung disease, transplantation is often the last chance to survive. However, colonization by B. cepacia genomovar III can be an exclusion criterion since post-transplant recidivism and consequent occurrence of “cepacia syndrome” with fatal outcome have been described (De Soysa et al., 2010).

Defining species by means of molecular analysis therefore yields important information for the clinician. This information is pivotal in prognostic evaluations of the patient, to establish the most appropriate therapy and implement correct prevention of transmission among CF subjects.

**Abbreviations**

Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

**References**


