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FULL PAPER

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. anthina* (genomovar VIII), *B. arboris (BCC3), B. cepacia* (genomovar I), *B. cenocepacia* (genomovar III), *B. contaminans* (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. seminalis* (BCC7), *B. stabilis* (genomovar IV), *B. ubonensis* (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).

Key words:
Cystic fibrosis, *Burkholderia cepacia complex, recA*, Genomovar, Molecular typing, *Burkholderia cenocepacia.*

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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; Nikaido *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" (Kalferstova *et al.,* 2015; Nash *et al.,* 2010). De Soyza and co-workers confirmed this, but showed excellent long-term survival in a non-*B. cenocepacia* Bcc group (De Soyza *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.

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 (Mahenthiralingam *et al.,* 2000; Payne *et al.,* 2005): BCR1:TGACCGCCGAGAAGAGCAA and BCR2:CTCTTCTTCGTCCATCGCCTC.

The PCR reaction was set up with (final volume =25 µl): Buffer PCR 5X (EuroClone), dNTPs Mix (Promega) 10 mM, $MgCl_2$ (EuroClone) 25 mM, Primer Forward BCR1 10 µM, Primer Reverse BCR2 10 µM, Red-Taq (EuroClone) Polymerase, Water, DNA sample 30 ng/µl. PCR programme 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 order to see if the amplification occurred correctly. The genomic material was purified and then a second PCR was set up, consisting of an amplification of single strand DNA using the primer BCR1 Forward and Reverse primer BCR2 (Boucher R. *et al.,* 2004) with: Water + DNA 7.7 µl, Buffer 5X, BigDye Terminator v. 1.1 Cycle Sequencing kit (Applied Biosystem), Primer BCR1 forward 10 µM and Primer BCR2 Reverse 10 µM. PCR programme was: 96°C 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 Alignement 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 select 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

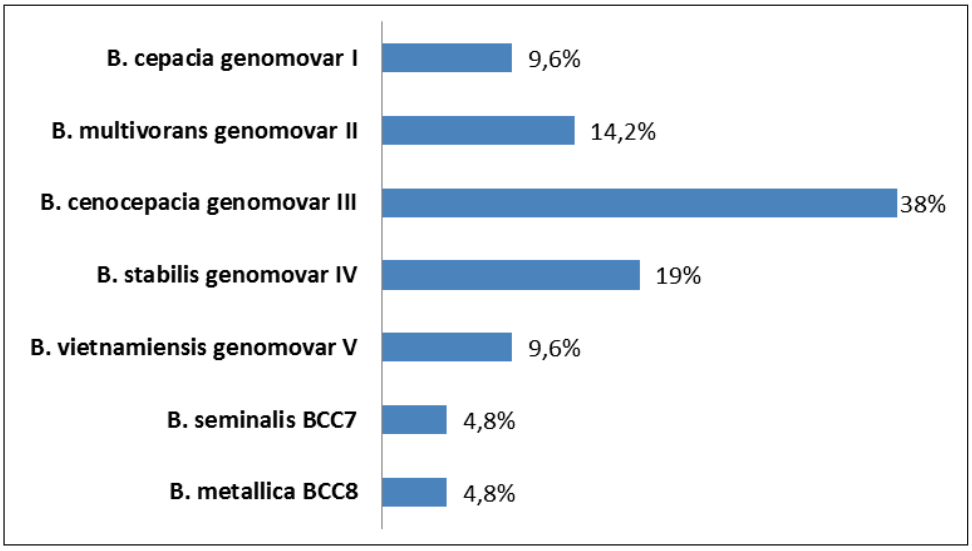

Figure 1 - *Distribution of* Burkholderia cepacia *complex genomovars (identified by their* recA *gene) according to their clinical origin, among 151 strains in 21 patients.*

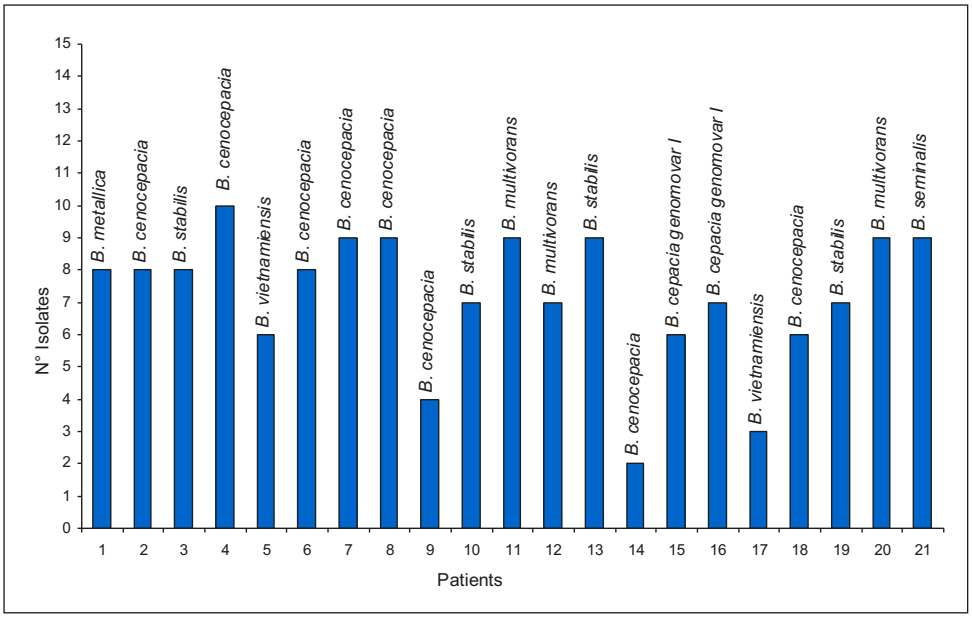

Figure 2 - *Number of isolates typed per year for each patient.*

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 colonization (*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. cenocepacia* genomovar III can be an exclusion criterion since post-transplant recidivism and consequent occurrence of "cepacia syndrome" with fatal outcome have been described (De Soyza *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

Bach E., Hayashi F., Sant'Anna, Magrich J.F. dos Passos, Balsanelli E., de Baura V.A. (2017). Detection of misidentifications of species from the *Burkholderia cepacia* complex and description of a new member, the soil bacterium *Burkholderia catarinensis* sp. nov. *Pathogens and Disease.* **75**.

Baldwin A., Mahenthiralingam E., Drevinek P., Vandamme P., Govan J.R., Waine D.J., et al. (2007). And Dowson C.G. *Burkholderia cepacia* Complex Isolates from Human Infections. *Emerg Infect Dis. Environmental.* **13**, 458-461.

Boucher R. (2004). New concepts of the pathogenesis of cystic fibrosis lung disease. *Eur. Respir. J.* **23**, 146-158.

De Smet B., Mayo M., Peeters C., Zlosnik J.E., Spilker T., Hird T.J., et al. (2015). *Burkholderia stagnalis* sp. nov. and *Burkholderia territorii* sp. nov., two novel *Burkholderia cepacian* complex species from environmental and human sources. Int J Syst Evol Microbiol. **65**, 2265-2271.

De Soyza A., Meachery G., Hester K.L., et al. (2010). Lung trans-plantation for patients with cystic fibrosis and *Burkholderia cepacia* complex infection: a single-center experience. *J Heart Lung Transplant.* **29**, 1395 e 1404.

Detsika M.G., Corkill J.E., Magalha˜es M., Glendinning K.J., Hart C.A., Winstanley C. (2003). Molecular Typing of, and Distribution of Genetic Markers among, *Burkholderia cepacia* Complex Isolates from Brazil. *Journal of Clinical Microbiology.* 4148-4153.

Frangolias D.D., Mahenthiralingam E., Rae S., Raboud J.M., Davidson A.G., Wittmann R., et al. (1999). *Burkholderia cepacia* in cystic fibrosis. Variable disease course. *Am J Respir Crit Care Med.* **160** (5 Pt 1): 1572.

Jones A.M., Dodd M.E., Govan J.R.W., Barcus V., Doherty C.J., Morris J., et al. (2004). *Burkholderia cenocepacia* and *Burkholderia multivorans*: influence on survival in cystic fibrosis. *Thorax.* **59**, 948.

Kalferstova L., Kolar M., Fila L., Vavrova J., Drevineka P. (2015). Gene Expression Profiling of *Burkholderia cenocepacia* at the Time of Cepacia Syndrome: Loss of Motility as a Marker of Poor Prognosis? *J Clin Microbiol.* **53**, 1515-1522.

Kidd T.J., Bell S.C., Coulter C. (2003). Genomovar Diversity Amongst *Burkholderia cepacia* Complex Isolates From an Australian Adult Cystic Fibrosis Unit. *Eur J Clin Microbiol Infect Dis.* **22**, 434-437.

Ku N.S., Han S.H., Kim C.O., Baek J.H., Jeong S.J., et al. (2011). Risk factors for mortality in patients with *Burkholderia cepacia* complex bacteraemia. *Scand - J Infect Dis.* **43**, 792-797.

Lain J. Abbott, MBBS, Anton Y. Peleg. MBBS, PhD, MPH, FRACP. (2015). *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and Non Mieloid Boukholderia Species: Antimicrobial Resistance and Therapeutic Strategies. *Semin Respir Crit Care Med.* **36**, 99-110.

Ledson M.J., Gallagher M.J., Jackson M., Hart C.A., Walshaw M.J. (2002). Outcome of *Burkholderia cepacia* colonisation in an adult cystic fibrosis centre. *Thorax.* **57**, 142.

Lipuma J., Spilker T., Gill L.H., Campbell P.W., Liu L., Mahenthiralingam E. (2001). Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine.* **164**.

Mahenthiralingam E., Bischof J., Byrne S.K., et al. (2000). DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis* And *Burkholderia cepacia* genomovars I and II. *J Clin Microbiol.* **38**, 3165-3173.

Mahenthiralingam E., Urban T.A., Goldberg J.B. (2005). The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat Rev Microbiol.* **3**, 144-156.

Magalhaes M., De Britto M.C.A., Vandamme P. (2002). Burkholderia cepacia genomovar III and *Burkholderia vietnamiensis* double infection in a cystic fibrosis child. *Journal of Cystic Fibrosis.* **1**, 292-294.

Matthew T.G. Holden, Helena M.B. Seth-Smith, Lisa C. (2009). Crossman, Mohammed Sebaihia, Stephen D. Bentley, Ana M. Cerden˜o-Ta´rraga, et all. The Genome of Burkholderia cenocepacia J2315, an Epidemic Pathogen of Cystic Fibrosis Patients. *Journal of Bacteriology*. 261-277.

Nash E.F., Coonar A., Kremer R., Tullis E., Hutcheon M., Singer L.G., Keshavjee S., Chaparro C. (2010). Survival of Burkhol deria cepacia sepsis following lung transplantation in recipients with cystic fibrosis. *Transpl Infect Dis.* **12**, 551-554.

Nikaido H., Pages J.M. (2012). Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol Rev.* **36**, 340-363.

Ong Kuan Shion , Yoong Kit Aw, Learn Han Lee, Catherine M. Yule, Yuen Lin Cheow, Sui Mae Lee. (2016). *Burkholderia paludism* sp. nov., an Antibiotic-Siderophore Producing Novel *Burkholderia cepacian* Complex Species, Isolated from Malaysian Tropical Peat Swamp Soil. *Front Microbiol.* **7**, 2046.

Payne G.W, Vandamme P., Morgan S.H., LiPuma J.J., Coenye T., Weightman A.J., et al. (2005). Development of a recA Gene-Based Identification Approach for the Entire *Burkholderia* Genus. *Applied And Enviromental Microbiology.* 3917-3927.

Pope C.E., Short P., Carter P.E. (2010). Species distribution of *Burkholderia cepacia* complex isolates in cystic fibrosis and non-cystic fibrosis patients in New Zealand. *Journal of Cystic Fibrosis.* **9**, 442-446.

Rodrigo Mendes, Aline A. Pizzirani-Kleiner, Welington L. Araujo, Jos M. Raaijmakers. (2007). Diversity of Cultivated Endophytic Bacteria from Sugarcane: Genetic and Biochemical Characterization of *Burkholderia cepacia* Complex Isolates. *Applied and Enviromental Microbiology.* 7259-7267.

Rushton L., Sass A., Baldwin A., Dowson C.G., Donoghue D., et al. (2013). Key role for efflux in the preservative susceptibility and adaptive resistance of *Burkholderia cepacia* complex bacteria. *Antimicrob Agents Chemother.* **57**, 2972-2980.

Samano M.N., Pêgo-Fernandes P.M., Fonseca Ribeiro A.K., Turaça K., Abdalla L.G., Fernandes L.M., et al. (2013). Lung transplantation in patients with cystic fibrosis. *Transplant Proc.* **45**, 1137-1141.

Siddiqui A.H., Mulligan M.E., Mahenthiralingam E., Hebden J., Brewrink J., et al. (2001). An episodic outbreak of genetically related *Burkholderia cepacia* Among non-cystic fibrosis patients at a university hospital. *Infect Control Hosp Epidemiol.* **22**, 419-422.

Smyth A.R., Scott C., Bojcind S., Bryone M., Duff A., Flumeg P., et al. (2014). European Cystic Fibrosis Society Standards of Care: Best Practice guidelines. *Journal of Cystic Fibrosis.* **13**, (Suppl. 1), S23-S42.

Spicuzza L., Sciuto C., Vitaliti G., Di Dio G., Leonardi S., La Rosa M. (2009). Emerging pathogens in cystic fibrosis: ten years of follow-up in a cohort of patients. *Eur J Clin Microbiol Infect Dis.* **28**, 191-195.

Soltan Dallal M.M., C.F. teleFian, M. Hajia, et al. (2014). Identification and molecular epidemiology of nosocomial outbreaks due to *Burkholderia cepacia* in cystic fibrosis patients of Masih Daneshvary Hospital, Iran. *J Prev Med Hyg.* **55**, 27-30.

Vanlaere E., LiPuma J.J, Baldwin A., Henry D., De Brandt E., Mahenthiralingam E., et al. (2008). *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. Nov. *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. Nov. novel species within the *Burkholderia cepacia* complex. International *Journal of Systematic and Evolutionary Microbiology*. **58**, 1580-1590.

Vandamme P., Dawyndt P. (2011). Classification and identification of the *Burkholderia cepacia* complex: Past, present and future. *Systematic and Applied Microbiology*. **34**, 87-95.

Vandamme P., Holmes B., Vancanneyet M., Coenye T., Hoste B., Coopman R., Revets H., et al. (1997). Occurrence of 246 multiple genomovars of Burkholderia cepacia in cystic fibrosis patients and proposal of Burkholderia multivorans sp. 247 nov. Int. J. Syst. Bacteriol. **47**, 1188-1120.