Antimicrobial susceptibility of Bcc isolates from Cystic Fibrosis patients.

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Bacteria belonging to *Burkholderia cepacia* complex (Bcc) are opportunistic pathogens causing a variety of infections. Bcc are highly resistant to multiple antibiotics, and individuals with cystic fibrosis (CF), a lethal autosomal-recessive disease, contracting nosocomial infections of Bcc are particularly vulnerable and difficult to treat.

The treatment of CF patients chronically infected with Bcc often requires an extensive and aggressive antibiotic therapy, exposing these bacteria to prolonged antibiotic selective pressure which may play a fundamental role in the acquisition of antimicrobial resistance.

Keywords Cystic Fibrosis, Bcc, antibiotic resistance

Cystic fibrosis (CF) is the most widespread genetic disease in the Caucasian population. The genetic defect causes an error in the synthesis of the CFTR protein responsible for ionic changes through the cell membrane. Despite a considerable diversity of clinical situations, the presence of thicker and more viscous secretions than normal in lungs, with progressive damage to the respiratory tract is a common clinical manifestation (or feature). The clinical history of CF patients is characterized by frequent episodes of pneumonia and bronchial pneumonia [1]. The abnormal composition and functions of mucous, the defective airway submucosal gland secretion, the defective ingestion and clearing of pathogens by epithelial cells, abnormal mucous pH which leads to a decrease in the mucociliary clearance, and increased availability of bacterial receptors predisposes CF patients to infections of respiratory tract [2]. Mucus stagnation, infections and inflammatory reactions tend to perpetuate a vicious circle that leads to progressive damage to the pulmonary parenchyma, deterioration of pulmonary function and, eventually, respiratory insufficiency in the more advanced cases. Apparently, infections caused by bacterias can either cause an acute infection, growing and spreading rapidly in the host, or, alternatively, adopting a chronic, biofilm infection strategy [3]. A relatively limited number of pathogens, such as bacteria belonging to *Burkholderia cepacia* complex (Bcc), *Pseudomonas aeruginosa* and, to a lesser extent, *Haemophilus influenza*, *Staphylococcus aureus* and *Stenotrophomonas maltophilia* are responsible for these infections [4].

Most of these pathogens are acquired from environmental reservoirs, but patient-to-patient transmission also occurs. The first evidence that suggested the possibility of Bcc transmission appeared in the early 1970s, from a case of a CF patient who became infected with a Bcc strain after having close contact with another CF patient who had a Bcc airway infection for more than 18 months following attendance at a CF summer camp [5, 6]. Molecular investigations using ribotyping subsequently showed that the Bcc strains from the two patients were identical suggesting transmission occurred from one to another [6]. A decade later a report of clinical complications associated with Bcc pneumonia and septicaemia in a 17-year-old patient were registered [7]. The report of septicaemia is noteworthy, since systemic spread of other pathogens from infected lungs is rare in patients with CF. In the early 1980s, the seminal papers of [8] and Thomassen and colleges [9] drew attention to an increase in culture rates for Bcc in two CF centres is USA which forecast the emergence of Bcc as a major threat to the CF community. Today, is it proven that Bcc bacteria acquisition is associated with hospitalization and cross-infection by social contact of CF patients [10-12] and that Bcc strains can spread between CF patients [13,14]. Currently, the segregation policy does not discriminate between Bcc species, although epidemiological studies consistently indicate a disproportionate distribution of individual species amongst CF isolates [15]. These control measures for infections had a strong psycosocial impact on the lives of CF patients [16,17]. Several cases of pseudoepidemics and nosocomial infections have also been reported [18]. Anterior reports illustrated that hospital outbreaks of Bcc might occur from a variety of contaminated sources such as disinfectants, ultrasound gel, nebulized medication, nasal spray, lipid emulsion and anaesthetic solutions where Bcc survives and may persist for long periods of time [12, 19, 21,22].

Burkholderia cepacia complex (Bcc). is a group of Gram-negative bacilli composed of at least 17 species. A term "genomovar" is commonly used with the Bcc to denote species which are phenotypically indistinguishable, but have sufficiently genetic distinctiveness [23]. To date, 17 genomovars have been identified and the complex currently consists of *B. cepacia*, *B. multivorans, B. cenocepacia, B. stabilis, B. vietnamiensis, B. dolosa, B. ambifaria, B. anthina, B. pyrrocinia, B. ubonensis, B. latens, B. diffusa, B. arboris, B. seminalis, B. metallica, B. contaminans,* and *B. lata*[24-26].

Several Bcc species have been shown to be transmissible from one CF patient to another and are able to cause epidemic outbreaks. However, it has been reported that *B. cenocepacia* and *B. multivorans* are the species that predominate in CF patients, are found in significantly higher percentage of respiratory specimens [27-29]. Indeed, two species together account for approximately 85-97% of all Bcc infections in CF patients. Infection with *B. cenocepacia* is associated with the worse outcome, named "cepacia syndrome", a condition occurring in 30 % of CF patients

characterized by fever, pneumonia and bacteraemia. However, the infection with other species although the effect of the less commonly isolated species on prognosis is unclear. In contrast, the prevalence of *B. cepacia*, *B. stabilis*, *B. anthina* and *B. pyrrocinia* [28, 30] among CF patients is low, while *B. vietanmensis* and *B. ambifaria* are rarely found [28, 29].

The identification of virulence factors employed by the Bcc to establish infections in CF patients continues to elude researchers. Some of the potential virulence factors currently under investigation are the cable pili, proteases, lipases, phospholipase C (PLC), hemolysin, a melanin-like pigment, flagella, and the siderophores, salicylic acid, pyochelin, cepabactin, and ornibactin. These potential factors function in a variety of ways that synergise to stablish an infection in the lungs of a CF patient. The cable pili of Bcc bind the 55-kDa epithelial cell protein cytokeratin 13, which could be important for the initial stages of an infection [31, 32]. found that some mutated strains of *B. cepacia* with reduced protease production are less persistent in rat lungs than the wild-type strains, which implies that this protease may play a role in Bcc's pathogenesis. The lipase of the Bcc reduces its phagocytosis by rat pulmonary alveolar macrophages, suggesting that it might contribute to evade the host immune response [33-35]. The PLC can hydrolyze the phosphatidylcholine, a major lipid component of lung surfactant, and consequently may cause a considerable amount of damage to the lung tissues [36]. Some strains of Bcc also produce a hemolysin that will either induce apoptosis in mammalian neutrophils or cause degranulation of the neutrophils. Both are mechanisms to evade the host immune response [35]. A melanin-like pigment also seems to protect Bcc from the host immune response by scavenging the superoxide anions that are produced during the respiratory burst [37]. The flagella appears to mediate the invasion of *B. cepacia* into the host epithelial cells, subepithelial tissues and blood stream. Indeed, Tomich and colleagues found that Bcc mutants lacking a functional flagella were no longer able to invade the respiratory epithelial cell line A549 [38]. Lewenza discovered that Bcc has quorum sensing genes, *cepRI*, that regulate the production of its siderophores [39]. Quorum sensing, also known as autoinduction, is a mechanism used to produce and detect signalling molecules (N-acylhomoserine lactones) in a cell culture. These signalling molecules, in turn, control the expression of certain genes including virulence factors such as siderophores. In agreement with this, clinical isolates of Bcc with reduced siderophore production generally lead to mild infections whereas isolates with high siderophore production lead to severe infections [40-42]. While characterizing a non-hemolytic phospholipase C (PLC-N), Weingart determined that quorum sensing mechanisms appear to play a role in the regulation of the expression of PLC-N, protease and lipase of the Bcc [43]. Bcc form a biofilm that can facilitate the regulation of virulence factor expression through quorum sensing, and also provide protection for the microbe. The majority of the cells in the biofilm are shielded from the damage inflicted by the immune responses and, to some extend by the antibiotics. It is also of interest to point out that CF patients are rarely colonized by *B. cepacia* alone. Although exopolysaccharide (EPS) production by Bcc was initially considered as a rare phenomenon, different studies showed that about 80% of the Bcc clinical isolates from Portuguese CF patients contained variable amounts of a specific EPS, named cepacian[44, 45]. Sousa *et al*., using gp91 $^{phox-/-}$ mice as an infection model demonstrated that cepacian is a virulence factor, and in a survey of 560 Bcc clinical isolates from 100 CF patients, Zlosnik *et al*., showed that all Bcc species represented were able to express the mucoid phenotype due to exopolysaccharide production [46, 47].

Another virulence mechanism that Bcc strains may use to establish an infection is intracellular survival preceded by cellular invasion. Indeed, Bcc isolates may survive intracellularly within amoebae, respiratory epithelial cells and macrophages [48]. Martin and Mohr conducted a study with a highly pathogenic clinical isolate of Bcc, and observed that this strain not only invaded macrophage and epithelial cell lines, but also replicated intracellularly within membrane-bound vacuoles [49]. When they examined an environmental isolate of Bcc, they showed its capacity to invaded cell lines, but failed to survive and replicate within these cells. Intracellular replication may therefore distinguish highly transmissible and pathogenic strains of Bcc from strains unable to establish infections in the lungs of CF patients. As mentioned above, a remarkable characteristic of Bcc is their innate resistance to a wide variety of antimicrobial compounds. It is assumed that clinical isolates are more resistant to antibiotics than environmental isolates because of previous antibiotic exposure, and an antibiotic therapy may play a fundamental role in the acquisition of antimicrobial resistance by Bcc bacteria, however this has not been proven experimentally [50, 51]. Cunha *et al.,* showed results clearly indicating that phenotypic variants of Bcc bacteria exhibiting differences in the antimicrobial susceptibility patterns can be regularly isolated from deteriorated CF patients during chronic lung infection [52]. Remarkably, the isolation of antimicrobial resistant variants was associated with long-term chronic infection as well as with more severely compromised lung function and periods of antibiotic therapy [52].

Although poorly understood, the antibiotic resistance mechanism in Bcc strains has been attributed to a specific efflux pump and reduced outer membrane (OM) permeability, innate decreased permeability of the bacterial membrane is believed to be responsible for resistance to aminoglycosides, polymyxins, β-lactamase and chloramphenicol, trimethoprim and fluoroquinolones [53]. A substancial outer membrane hydrophilic permeability barrier protects the bacillus from all hydrophilic antimicrobial agents [54]. Outer membrane proteins (OMPs) include integral membrane proteins and lipoproteins that have B-barrel structures and are often arranged so form channels or pores through which small molecules can pass. Small channel sizes in Bcc pores were thought to play a role in decreasing diffusion of β-lactam antibiotics while a lack of cation-binding sites in the PLS provided Bcc resistance against polycations such as aminoglycosides and polymixin. Bcc resistance to chloramphenicol, trimethoprim, and ciproflocacin was ascribed to an OM lipoprotein that is part of an antibiotic efflux pump with homology to the *P. aeruginosa* multiple antibiotic

resistance operon *mexA-mexB-oprM.* [55]. another characteristic of Bcc OMP is an iron-regulated OM receptor for the siderophore ornibactin. Inactivation of the gene encoding this receptor resulted in attenuated virulence in a murine model of chronic respiratory infection, indicating the importance of siderophore uptake and utilization in Bcc pathogenesis. [56]. Resistance to β-lactams among *Burkholderia* spp. is attributable to expression of β-lactamases (e.g., PenA in Bcc and PenI in Bpm) [57]. It has been shown that some Bcc used penicillin as a sole carbon source and could potentially grow in the presence of the penicillin G [58]. Other mechanism of resistence reported in the literature include production of modifying enzymes other than β-lactamases and alteration of antibiotic targets [59, 29]. In addition, biofilm-associated cells are often more tolerant to antimicrobials than planktonic cells due to reduced drug penetration in the biofilm, the lower growth rate of sessile cells and/or the expression of specific resistance genes [60-62]. Even when patients are treated with high doses of antibiotics, known to be effective against Bcc species *in vitro*, it is often impossible to clear the infection [63].

A major aspect of CF patient care has been centred on antibiotic therapy to improve the effects of acute pulmonary infections/exacerbations in these patients. The objectives of the treatment in CF are therefore not a long-term eradication of the organism but rather a control of the infection in order to minimize inflammation and damage, and thus slow the decline of pulmonary function. Controlling infection and minimizing inflammation can be achieved by antimicrobial therapy that reduces the numbers of pathogenic organisms [64, 65]. In addition to their bactericidal activity, antibiotics have also been shown to suppress bacterial synthesis of pathogenic factors that are potent inciters of inflammation within CF airways and antibiotics can exert antioxidant effects by neutralizing myeloperoxidase release from polymorphonuclear cells in CF sputum [66]. Morbidity and mortality in patients with CF is mainly due to a chronic relapsing bronchopulmonary infection caused by Bcc. Therefore CF patients often require life-long repeated courses of intravenous antibiotic treatment. During chronic colonization of the airways of CF patients, Bcc bacteria experience changing selection pressures, in particular those resulting from challenges of the immune defences, antimicrobial therapy, nutrient availability and oxygen limitation [67-69]. The adaptive responses occurring in clinical isolates of *Pseudomonas aeruginosa*, another major respiratory pathogen of CF patients, have been the focus of recent studies but equivalent studies on Bcc bacteria are still lacking. Apart from intrinsic mechanism, acquired resistance has also been observed against commonly used antibiotics for infections caused by Bcc in CF patients, and been ascribed mainly to the emergence of hypermutable bacteria [70, 71]. High mutation rate can lead to rapid adaptation, and bacteria with higher spontaneous mutation rate than the majority of the population are more likely to gain resistance via mutation in chromosomal genes. Acquired resistance may be realized during therapy or through induction of cross-resistance among different classes of antibiotics [72, 73].

Effective therapy of CF pulmonary infection is severely limited by the broad-spectrum antimicrobial resistance exhibited by these species, which are among the most drug-resistant bacteria encountered in human infection. The site of infection in CF presents another important obstacle to effective therapy. Infecting bacteria reside primarily within the airway lumen in sputum, the airway epithelial surface fluid, and the bronchial mucosa.

Studies published during the past decade have provided evidence that complete elimination of the organism is usually impossible because of the complex interrelationships between host defenses in patients with CF and these microbes [74].

Consequently, the concomitant use of two or more antibiotics is a common practice in the treatment of CF patients infected with Bcc. In this context, however, convincing data supporting the use of combination therapy are lacking. Published results from trials in pulmonary exacerbations of CF comparing monotherapy and combination therapy are limited, controversial, and include only small numbers of patients. Aaron and colleagues reported that multiple combination bactericidal antibiotic testing (MCBT), a method, which combines antibiotics that together have in vitro bactericidal activity, has become available for use since 2000, did not result in better clinical and bacteriological outcomes in CF pulmonary infection compared to therapy directed by conventional culture and sensitivity testing [74, 75]. Ceftazidime and co-trimoxazole are suggested as drugs of choice. As combination therapy did not have lower mortality rates, it was not considered necessary and the suggestion is prescribed ceftazidime or piperacillin as the preferable empirical treatment. However, a more recent study suggests administration of meropenem in combination with at least one of the following: minocycline, amikacin or ceftazidime.

Several reports confirm that nebulized shows great promising the treatment of Bcc infected CF patients: for example, Weidmann and colleagues recently described the complete eradication of Bcc organisms from the lungs of CF patients by using a combination of nebulized tobramycin and amiloride In addition, a combination therapy with nebulized and intravenous meropenem and tobramycin also resulted in the successful treatment of a female CF patient suffering from cepacia syndrome, although the sputum samples of the latter patient remained positive for *B. cenocepacia* [76].

The suggested treatment options for Bcc infection are generally limited. Although there are alternatives since the advent of new antibiotics, no studies were conducted to compare other potent antimicrobial agents against this group of pathogens.

As an alternative strategy toward finding subsitute methods of treatment for antibiotic resistant bacteria, bacteriophages (or phages) have been suggested by Parisien [77]. The applications of phage therapy are numerous and many of these have been and are currently being evaluated, including the treatment of thermal injury infection [78, 79] and systemic infections [80, 81] including those that are antibiotic resistant [82].

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