

Epidemiology of *Burkholderia cepacia* complex species recovered from cystic fibrosis patients: issues related to patient segregation

Andrew McDowell,¹ Eshwar Mahenthalingam,² Kerstin E. A. Dunbar,¹ John E. Moore,¹ Mary Crowe¹ and J. Stuart Elborn³

Correspondence

Andrew McDowell
a.mcdowell@qub.ac.uk

^{1,3}Department of Bacteriology, Northern Ireland Public Health Laboratory¹ and Northern Ireland Regional Adult Cystic Fibrosis Centre³, Belfast City Hospital, Belfast BT9 7AB, UK

²Cardiff School of Biosciences, Cardiff University, Cardiff CF1 3US, UK

Studies of the prevalence of *Burkholderia cepacia* complex species amongst cystic fibrosis (CF) patients in different geographical regions, and the association between cross-infection and putative transmissibility markers, will further our understanding of these organisms and help to address infection-control issues. In this study, *B. cepacia* complex isolates from CF patients in different regions of Europe were analysed. Isolates were examined for *B. cepacia* complex species and putative transmissibility markers [cable pilin subunit gene (*cbIA*) and the *B. cepacia* epidemic strain marker (BCESM)]. Sporadic and cross-infective strains were identified by random amplification of polymorphic DNA (RAPD). In total, 79% of patients were infected with *Burkholderia cenocepacia* (genomovar III), 18% with *Burkholderia multivorans* (genomovar II) and less than 5% of patients with *B. cepacia* (genomovar I), *Burkholderia stabilis* (genomovar IV) or *Burkholderia vietnamiensis* (genomovar V). The *cbIA* and BCESM transmissibility markers were only detected in strains of *B. cenocepacia*. The BCESM was a more sensitive marker for transmissible *B. cenocepacia* strains than *cbIA*, although sporadic *B. cenocepacia* strains containing the BCESM, but lacking *cbIA*, were also observed. Furthermore, clusters of cross-infection with transmissibility marker-negative strains of *B. multivorans* were identified. In conclusion, *B. cenocepacia* was the greatest cause of cross-infection, and the most widely distributed *B. cepacia* complex species, within these CF populations. However, cross-infection was not exclusive to *B. cenocepacia* and *cbIA* and the BCESM were not absolute markers for transmissible *B. cenocepacia*, or other *B. cepacia* complex strains. It is therefore suggested that CF centres cohort patients based on the presence or absence of *B. cepacia* complex infection and not on the basis of transmissibility marker-positive *B. cenocepacia* as previously suggested.

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INTRODUCTION

Burkholderia cepacia has emerged as a life-threatening cause of infection in patients with cystic fibrosis (CF). Strains of *B. cepacia* can be highly transmissible, resulting in patient-to-patient spread within and between CF centres (Govan *et al.*, 1993). To date, the most effective strategy to combat *B. cepacia* infection has been the strict segregation of infected patients. Although successful, the introduction of such a policy has proved controversial and unpopular with many CF patients. Furthermore, segregation will not prevent sporadic acquisition of *B. cepacia* complex organisms from natural environments (LiPuma *et al.*, 2002).

To complicate the clinical problems associated with *B. cepacia* infection, polyphasic taxonomic studies have now discovered that *B. cepacia* is a complex of nine genetically distinct species, all capable of causing CF-related infections (Mahenthalingam *et al.*, 2002). Putative transmissibility markers for *B. cepacia* complex bacteria have now also been identified. These markers include the cable pilin subunit gene (*cbIA*), which encodes a giant cable-like pilus that facilitates adherence to respiratory mucins (Sajjan *et al.*, 1995), and the *B. cepacia* epidemic strain marker (BCESM), a 1.4-kb open reading frame with homology to several negative transcriptional regulatory genes (Mahenthalingam *et al.*, 1997).

Initial studies by Vandamme *et al.* (1997) revealed that *Burkholderia cenocepacia* (formerly genomovar III) was the predominant species amongst their CF-related *B. cepacia* complex isolates. Further investigations by Clode *et al.*

Abbreviations: BCESM, *Burkholderia cepacia* epidemic strain marker; CF, cystic fibrosis; RAPD, random amplification of polymorphic DNA.

(2000) also found that *B. cenocepacia* was the prevalent species amongst 114 *B. cepacia* complex isolates recovered from CF patients in the UK. In addition, their investigations found that putative transmissibility markers, most specifically the *cblA* gene, were associated with epidemic spread of *B. cenocepacia*. This led to the suggestion that, in the UK at least, only patients colonized with transmissibility marker-positive *B. cenocepacia* strains should be strictly segregated from patients colonized with other transmissibility marker-negative *B. cepacia* complex strains, who themselves may not require segregation. Relaxation of segregation is an attractive proposal for CF centres since it would undoubtedly reduce the psychological impact upon the CF patient population, as well as the burden placed upon centres to provide and maintain this tedious and socially difficult policy. However, extensive multi-centre information on the distribution of *B. cepacia* complex species amongst CF patients, and continued data on the relationship between putative transmissibility markers and cross-infective strains, is required before such a recommendation could be seriously considered. Such studies are especially urgent since more recent data from the USA suggest that *cblA* and the BCESM may not be sufficient markers for transmissibility or indeed virulence of *B. cenocepacia* (LiPuma *et al.*, 2001; Sajjan *et al.*, 2002).

In this current study, we investigated the distribution of *B. cepacia* complex species, as well as the association between putative transmissibility markers and cross-infective strains, amongst *B. cepacia* complex organisms isolated from 131 patients receiving treatment in 14 CF centres in different regions of Europe.

METHODS

***B. cepacia* complex isolates.** For this study, *B. cepacia* complex sputum isolates were recovered from 131 CF patients receiving treatment in different regions of Europe between 1997 and 2001. Patients were from four centres in Northern and Southern Ireland (50 isolates), nine centres in Great Britain (46 isolates) and one centre in mainland Europe (35 isolates). Samples were received either as pure bacterial isolates or as expectorated sputum. *B. cepacia* complex organisms in sputum samples were immediately isolated by culture on MAST selective agar (MAST Diagnostics). Isolates were initially analysed using the API 20NE phenotypic identification system (bioMérieux) following the manufacturer's instructions. As in the study of LiPuma *et al.* (2001), when multiple isolates from one patient were received, only the first was included in the study so that the best distribution of *B. cepacia* complex species could be assessed.

Molecular identification of *B. cepacia* complex species. Genomic template DNA for molecular analysis was prepared from all isolates as described previously (McDowell *et al.*, 2001). *B. cepacia* complex species were identified by 16S and 16S–23S rRNA-based PCR, as well as *recA*-based methods (LiPuma *et al.*, 1999; Mahenthiralingam *et al.*, 2000). Our *recA*-based diagnostic scheme for *B. cepacia* complex species identification consisted of RFLP analysis (with *Hae*III and *Mnl*I) of the *recA* gene, followed by confirmation with species-specific *recA* primers. Analysis of PCR and RFLP products by agarose electrophoresis was as described before (McDowell *et al.*, 2001). Nucleotide sequence analysis of *recA* amplicons was performed on isolates with novel RFLP patterns to confirm species identification. Sequences were obtained using an ALF Express II DNA sequencer (Amersham-Pharmacia).

Multiple sequence alignments (CLUSTAL W) and phylogenetic analysis were performed as described previously (Mahenthiralingam *et al.*, 2000). In addition to culture-based analyses, PCR-based detection and identification of *B. cepacia* complex species directly from patients' sputum samples (where applicable) was also carried out in parallel, as described previously (McDowell *et al.*, 2001).

Genotyping. All *B. cepacia* complex isolates were genetically typed by RAPD, using the 10-base oligonucleotide primer 272, as described previously (Mahenthiralingam *et al.*, 1996). RAPD fingerprints were compared visually and with the aid of computer analysis (GelCompar version 3.10 for Windows). Strains presumptively identified as ET-12 were confirmed by macrorestriction of whole genomic DNA with the restriction enzyme *Spe*I, followed by PFGE, as described previously (Vandamme *et al.*, 2000). Profiles were compared with the J2315 Edinburgh index strain for the ET-12 lineage. PFGE was carried out with separation conditions of two cycles of 6 V cm⁻¹, with cycle 1 pulsing from 1 to 40 s over 8 h, and cycle 2 from 30 to 90 s over 16 h.

Detection of putative transmissibility markers. PCR-based detection of the *cblA* gene and the BCESM were as described previously (Sajjan *et al.*, 1995; Mahenthiralingam *et al.*, 1997). To assess the presence and stability of the BCESM and *cblA* for cross-infective isolates identified by genotyping and epidemiological data, sensitivity and specificity values were determined. Sensitivity was calculated as the number of cross-infective isolates positive for each marker, expressed as a percentage of the total number of cross-infective isolates. Specificity was calculated as the number of sporadic isolates negative for each marker, expressed as a percentage of the total number of sporadic isolates.

RESULTS

Prevalence of *B. cepacia* complex species

Table 1 summarizes the total distribution of *B. cepacia* complex species amongst isolates recovered from 131 CF patients attending a total of 14 CF centres in different regions of Europe. Using a combination of rRNA and *recA*-based PCR protocols, 79 % of isolates were identified as *B. cenocepacia* (genomovar III), while 18 % of samples were *B. multivorans* (genomovar II). The remaining isolates were identified as *B. cepacia* (genomovar I), *B. stabilis* (genomovar IV) or *B. vietnamiensis* (genomovar V). *recA* analysis of our *B. cenocepacia* isolates revealed that 91 % were *B. cenocepacia recA* lineage III-A, while the remaining 9 % were identified as *B. cenocepacia recA* lineage III-B. Within each geographical region, *B. cenocepacia* was also the predominant *B. cepacia* complex species responsible for infection amongst the patient population (Table 2).

Genotyping and putative transmissibility markers

Cross-infected and sporadic *B. cepacia* complex strains were identified by RAPD, and their relationship with putative transmissibility markers was examined (Table 3). Analysis of the isolates identified the same *B. cenocepacia recA* lineage III-A strain as the cause of infection in 58 (44 %) patients. Of these isolates, a total of 57 (98 %) contained the *cblA* and BCESM putative transmissibility markers, a feature characteristic of the highly transmissible ET-12 (multilocus enzyme electrophoresis type 12) lineage (RAPD group 02).

Table 1. Distribution of the *B. cepacia* complex species and putative transmissibility markers amongst *B. cepacia* complex isolates recovered from 131 CF patients

Numbers in parentheses represent percentages of *B. cepacia* complex-infected patients.

Species	Total number (%)	Transmissibility marker genes	
		<i>cblA</i> -positive (%)	BCESM-positive (%)
<i>B. cepacia</i>	1 (0.76)	0	0
<i>B. multivorans</i>	23 (17.6)	0	0
<i>B. cenocepacia</i> *	103 (78.6)	57 (43.5)	99 (75.6)
<i>B. stabilis</i>	1 (0.76)	0	0
<i>B. vietnamiensis</i>	3 (2.29)	0	0
Total number	131 (100)	57 (43.5)	99 (75.6)

*Ninety-four (91.3 %) *B. cenocepacia* strains belong to *recA* lineage III-A and the remaining nine (8.7 %) are *recA* lineage III-B.

Table 2. Distribution of the *B. cepacia* complex species within CF centres in different geographical regions

B. cepacia complex species are abbreviated as follows: *Bc*, *B. cepacia*; *Bm*, *B. multivorans*; *Bcnp*, *B. cenocepacia*; *Bs*, *B. stabilis*; and *Bv*, *B. vietnamiensis*.

Geographical region	<i>Bc</i>	<i>Bm</i>	<i>Bcnp</i>	<i>Bs</i>	<i>Bv</i>	Total
Ireland	—	10	39	1	—	50
Great Britain	1	13	29	—	3	46
Mainland Europe	—	—	35	—	—	35
Total	1	23	103	1	3	131

Table 3. Distribution of putative transmissibility markers amongst *B. cepacia* complex species recovered from 131 CF patients

B. cepacia complex species are abbreviated as follows: *Bc*, *B. cepacia*; *Bm*, *B. multivorans*; *Bcnp*, *B. cenocepacia*; *Bs*, *B. stabilis*; and *Bv*, *B. vietnamiensis*.

Strains and markers	<i>Bc</i>	<i>Bm</i>	<i>Bcnp</i>	<i>Bs</i>	<i>Bv</i>	Total
Cross-infection	—	6	91	—	—	97
<i>cblA</i>	—	—	57	—	—	57
BCESM	—	—	91	—	—	91
Sporadic	1	17	12	1	3	34
<i>cblA</i>	—	—	—	—	—	—
BCESM	—	—	8	—	—	8

One clonal isolate (2 %) was PCR-negative for the *cblA* gene, but did contain the BCESM. Confirmation that isolates were indeed the ET-12 strain of *B. cenocepacia recA* lineage III-A was achieved by macrorestriction analysis of whole genomic DNA followed by PFGE (Fig. 1). The ET-12 clonal lineage

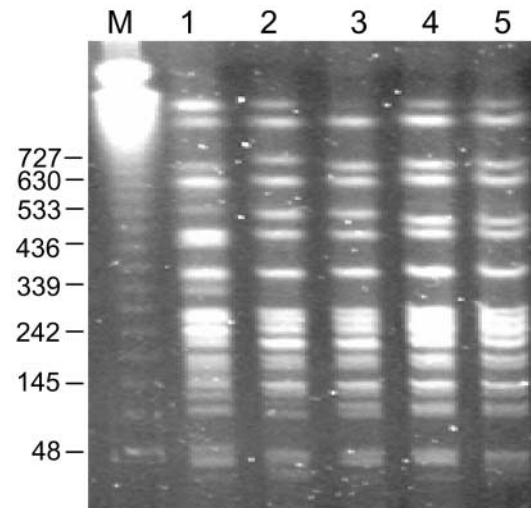


Fig. 1. Macrorestriction fingerprinting of *B. cenocepacia* ET-12 strains recovered from CF patients during this study. Lanes 1–4, clinical isolates of ET-12 strains; 5, J2315 Edinburgh index strain for the ET-12 lineage (Govan *et al.*, 1993); M, molecular size markers, size of relevant bands in kb is indicated.

was found amongst CF patients in Northern Ireland and Great Britain. RAPD analysis of isolates from a mainland European CF centre revealed 33 patients cross-infected with a *B. cenocepacia recA* lineage III-A strain highly distinct from ET-12. This strain contained the BCESM, but not *cblA*. All remaining *B. cenocepacia recA* lineage III-A isolates were genotypically distinct and PCR-positive for the BCESM, but negative for *cblA*. All *B. cenocepacia recA* lineage III-B isolates had unique genetic fingerprints. None of these sporadic strains had *cblA*, but five did contain the BCESM. In addition to the detection of transmissible strains of *B. cenocepacia*, RAPD analysis also identified two separate clusters of *B. multivorans* cross-infection within a CF centre. One cluster

involved two patients and the other a total of four patients. Both strains of *B. multivorans* responsible for these clusters of cross-infection were PCR-negative for *cblA* and the BCESM. All remaining isolates of *B. multivorans*, as well *B. vietnamiensis*, had unique genotypes and were negative for both putative transmissibility markers.

For cross-infective *B. cenocepacia* isolates, the BCESM was found to have a sensitivity of 100 % compared with 63 % for the *cblA* gene. For all isolates implicated in patient-to-patient spread, the BCESM had a sensitivity of 94 % compared with 59 % for the *cblA* gene. The specificity of the BCESM for the detection of cross-infective *B. cenocepacia* was 33 % compared with 100 % for *cblA*. The overall specificity of the BCESM for the detection of cross-infective isolates was 76 % compared with 100 % for *cblA*.

DISCUSSION

The emergence of *B. cepacia* complex organisms as life-threatening pathogens amongst patients with CF has proved a tremendous challenge for the clinician. For patients colonized with bacteria of the *B. cepacia* complex, management of infection under the same principles adopted for patients with chronic *Pseudomonas aeruginosa* is complicated by the highly transmissible nature of certain *B. cepacia* complex strains. In response to this problem, CF centres have now adopted strict infection-control policies that segregate *B. cepacia* complex-infected patients from non-infected individuals. Although highly effective, stringent segregation has proved unpopular with many patients and generated new psychosocial and economic issues for CF centres. PCR-based strategies to refine the segregation of patients based on the properties of the infecting *B. cepacia* complex strain rather than the presence or absence of the organism have been recommended (Clode *et al.*, 2000). In particular, for CF centres in the UK it was suggested that only transmissibility marker-positive *B. cenocepacia*-infected patients need to be segregated. Before such a proposal could be implemented, especially within a broader geographical context, further multi-centre studies on the distribution of *B. cepacia* complex species amongst CF patients in different localities, and the relationship between putative transmissibility markers and cross-infective strains, are required.

In the present study, we examined *B. cepacia* complex isolates recovered from CF patients in different regions of Europe. Most isolates were identified as *B. cenocepacia*, with the majority belonging to the *recA* lineage III-A. Similar results have also been described in Canada (Speert *et al.*, 2002) and Italy (Agodi *et al.*, 2001), while in the USA the majority of *B. cenocepacia* isolates belong to the *recA* lineage III-B (LiPuma *et al.*, 2001). Although the basis of variations between the USA and other geographical regions in terms of the prevalence of *B. cenocepacia recA* lineage is currently unclear, one possibility may be the nature of the transmissible *B. cenocepacia* strains found in these different locations. In our study, the highly transmissible ET-12 strain was identified amongst 56 % of *B. cenocepacia* isolates. This strain was

only recovered from patients attending CF centres in Northern Ireland and Great Britain (UK). The ET-12 strain appears to have first infected patients in Toronto before spreading across Canada, where it is the predominant *B. cenocepacia* lineage (Speert *et al.*, 2002). It is presumed that the ET-12 lineage was first introduced into the UK CF population as a consequence of contact at CF summer camps, with the Edinburgh index case of this strain first observed in 1989 (Govan *et al.*, 1993). Infection with a strain closely related to ET-12 has now also been described amongst CF patients in Italy (Agodi *et al.*, 2001). One characteristic feature of the ET-12 strain is the presence of both *cblA* and the BCESM. However, during our study, we identified one ET-12 isolate that was negative for the *cblA* gene. Although genetic instability of the BCESM has been described (Mahenthalingam *et al.*, 1997; Agodi *et al.*, 2001), instability of the *cblA* gene has only recently been observed (Agodi *et al.*, 2001). Organisms of the *B. cepacia* complex have large and complex genomes with up to three or four replicons, as well as multiple insertion sequences (Lessie *et al.*, 1996). The observation that *cblA* and the BCESM possibly reside on unstable chromosomal regions of the genome raises further important questions regarding the sole use of these putative markers for clinical management of patients within CF centres.

In addition to the detection of the highly transmissible ET-12 strain, we also identified a strain of *B. cenocepacia recA* lineage III-A which was responsible for multiple cross-infections amongst patients attending a CF centre in mainland Europe. This strain, which was genotypically distinct from ET-12, lacked the *cblA* gene but did contain the BCESM. Cross-infection with BCESM-positive *B. cenocepacia* RAPD types other than ET-12 has been observed before, such as the epidemic strain of *B. cenocepacia* responsible for transmission between CF patients in Manchester, UK (Haworth *et al.*, 1997). Studies have now also identified cross-infection amongst CF patients due to *cblA*- and BCESM-negative *B. cenocepacia* strains, such as the epidemic PHDC strain which infects CF patients in the mid-Atlantic region of the USA (Chen *et al.*, 2001). Such studies further highlight the caveats associated with sole reliance on these markers for identification, and infection-control management, of CF patients infected with potentially cross-infective *B. cenocepacia* strains.

Within our CF study population we also observed clusters of cross-infection amongst patients due to *B. multivorans*. None of these strains carried the *cblA* and BCESM putative transmissibility markers. As our understanding of *B. cepacia* complex infection has continued to grow, it has become clear that serious episodes of cross-infection due to *B. cepacia* complex species other than *B. cenocepacia* can arise. Cross-infection due to *B. multivorans* has been reported amongst CF patients in the UK and France (Whiteford *et al.*, 1995; Segonds *et al.*, 1999). Also, the first epidemic strain identified as the cause of patient-to-patient spread of *B. cepacia* has now been identified as *Burkholderia dolosa* (genomovar VI) (LiPuma *et al.*, 2001). Very recently, reports of potential

cross-infection with *B. stabilis* amongst 13 CF patients in the Slovak Republic, and the demonstration of transmission of *B. cepacia* (genomovar I), *B. multivorans* and *B. dolosa*, provides striking evidence of the danger in confining segregation to *B. cenocepacia*-infected patients only (Biddick *et al.*, 2003; Drevinek *et al.*, 2003).

Overall, our sensitivity and specificity calculations revealed that neither the BCESM nor *cblA* were absolute markers for spread amongst CF patients. The BCESM was present in sporadic isolates of *B. cenocepacia*, and absent from cross-infective *B. multivorans* isolates, while *cblA* was only found in the transmissible ET-12 strain of *B. cenocepacia*. However, previous studies have identified the presence of *cblA* gene sequences in a small number of *B. cepacia* (genomovar I) isolates recovered from CF patients in the USA (LiPuma *et al.*, 2001). The majority of these isolates were genotypically distinct and had sequences with high identity (98.2–100 %) to each other, but lower identity (87.8–88.4 %) to the ET-12 *cblA* sequence (Sajjan *et al.*, 2002). Interestingly, electron microscopy revealed the complete absence of pilus expression by these isolates. Therefore, while variant *cblA*-containing sequences have been found amongst a small number of sporadic non-ET-12 strains, the gene appears to be relatively specific for the ET-12 strain of *B. cenocepacia* and not for transmissible *B. cepacia* complex strains in general.

In conclusion, our data demonstrate the prevalence of *B. cenocepacia* amongst *B. cepacia* complex-infected CF patients. Cross-infection was not solely confined to *B. cenocepacia*, and *cblA* and the BCESM were not absolute markers for transmissible strains. We would therefore recommend that CF centres cohort patients based on the presence or absence of *B. cepacia* complex infection, and not on the basis of transmissibility marker-positive *B. cenocepacia*. This is in agreement with the UK CF Trust's guidelines on cross-infection control (<http://www.cftrust.org.uk/index.jsp>). Further segregation of patients infected with *B. multivorans* (and other *B. cepacia* complex species) from those infected with *B. cenocepacia* is also prudent to reduce the risk of cross-infection and superinfection (Mahenthiralingam *et al.*, 2001).

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