Genotypic and phenotypic characterisation of isolates of *Burkholderia cepacia* from cystic fibrosis

Thesis

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Genotypic and phenotypic characterisation of isolates of *Burkholderia cepacia* from Cystic Fibrosis

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Thesis submitted for the degree of Master of Philosophy

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September 2002

Submission date: 30 September 2002
Award date: 14 July 2003

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Abstract

Burkholderia cepacia has become an important cause of lung infections in cystic fibrosis sufferers of whom approximately 20 % succumb to severe and often fatal necrotizing pneumonia and septicaemia. The factors which cause this are largely unknown as are those factors predisposing to epidemic spread. At present, published data only confirms that certain strains notably the UK epidemic strain ET 12 can spread amongst the CF population.

Another problem facing scientists which also impinges on patients and their carers, is the difficulty in isolating *B. cepacia* in mixed specimens and its subsequent identification and differentiation from closely related species which may also colonise the CF lung. Identification has recently been compounded by the identification of more than one species or genomovar of *B. cepacia.*

This study compared traditional phenotypic methods of identifying *B. cepacia* with PCR methods and also evaluated direct detection of the organism in sputum by PCR. The presence of *B. cepacia* in the natural environment was determined by culturing environmental samples on selective media.
Putative epidemic markers in *B. cepacia* were detected and this was correlated with genomovar group and RAPD fingerprint.
Finally, the distribution of various virulence markers including serum sensitivity and presence of LPS were monitored and were also correlated with genomovar group.

The results of this study showed that PCR methods for the identification of *B. cepacia* are more sensitive and specific than traditional methods and allow for its direct detection in sputa. Only the UK epidemic strain appears to spread epidemically in the UK and this strain is most reliably identified by the presence of the cable pili gene (*cblA*). Serum sensitivity and correlation with strain-type and presence of LPS was significant as was the presence of intracellular enzymes and strain or genomovar group. However the significance of these enzymes in pathogenicity remains uncertain.

Acknowledgements

I would like to thank my supervisor in LHI, Dr Tyrone Pitt for all the help, advice and support which he contributed during this study. I would also like to thank Professor Margaret Hodson who was my supervisor at the Royal Brompton Hospital for all her support, especially in providing clinical specimens, isolates of *Burkholderia cepacia* and clinical information.

I would also like to thank Dr Henrik Chart, the Postgraduate coordinator at CPHL for all his help and advice and the Medical Illustration department at CPHL who helped with many of the figures.

Finally, I would like to thank all my colleagues in LHI for their support and encouragement especially Mrs Polly Kaufmann and Dr Donald Morrison who taught me many of the techniques carried out in this work.

Publications from this thesis

Clode F.E, Kaufmann M.E, Malnick H, Pitt T.L. (1999). Evaluation of three oligonucleotide primers in PCR identification of *Burkholderia cepacia* and their differentiation from *Burkholderia gladioli.* Journal of Clinical Pathology **52** 173-176

Clode F.E, Metherell L.A. Pitt T.L. (1999). Nosocomial acquisition of *Burkholderia gladioli* in patients with cystic fibrosis. American Journal of Respiratory and Critical Care Medicine **160** 374-375.

Clode F.E, Kaufmann M.E, Malnick H, Pitt T.L. (2000). Distribution of genes encoding putative transmissibility factors among epidemic and nonepidemic strains of *Burkholderia cepacia* from cystic fibrosis patients in the United Kingdom. Journal of Clinical Microbiology **38** 1763-1766

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1. Introduction

1.1. Cystic Fibrosis

1.1.1. The disease

Cystic fibrosis (CF) is the most common inherited disorder affecting 1 in 2000 Caucasians (Davies 1991). Approximately 4 to 5% of this racial group may be carriers of the defective gene but only homozygotes acquire the disease. Relatively few of the body's cells are affected by CF but include the epithelial cells lining the lungs, intestine, pancreas and skin (Davies 1991). In normal cells an increase in intracellular cAMP allows chloride and water to move from the cell to the mucosal surface. This liquifies the mucous layer covering the epithelium allowing clearance of particles and bacteria. In CF cells, chloride and water secretion is reduced and subsequently the mucous becomes dehydrated and viscous. The disease itself is characterised by high electrolyte concentrations in sweat, male infertility, pancreatic insufficiency leading to malabsorption and production of thick sticky mucus in the lungs followed by chronic pulmonary infection.

1.1.2. Genetics

In 1989, researchers at the University of Toronto and Michigan located the genetic defect that causes CF on chromosome 7 (Davies 1991). The gene spans about 250 kb of DNA and is divided into 27 protein coding regions or exons (Knight 1992). In epithelial cells this gene codes for a protein known as the Cystic Fibrosis Transmembrane Regulator (CFTR). This was believed to function as a channel allowing chloride ions to enter and leave cells and which was faulty in CF sufferers

resulting in abnormally salty sweat (Davies 1991; Knight 1992). However, instead of an ion channel, the protein more closely resembles a membrane ATP-dependent transporter. Removing a segment of DNA from the middle of the gene (R-domain) led to an uncontrolled flow of chloride ions across cell membranes suggesting that the R-domain codes for a protein that acts as a flap or gate over the pore of the channel (Davies 1991). It was also discovered that sites on the protein are recognised by kinases which add phosphate groups to certain amino acids and it was suggested that these negatively-charged groups cause the flap to retract from the cell membrane thus leading to the opening of the pore. In CF, the flap appears to block the pore preventing transport of chloride ions in the epithelial cells which results in a lack of water movement into the airway passages. This in turn leads to the production of dehydrated sticky mucus in the lungs which is an ideal breeding ground for bacteria. There is, however, more than one genetic defect in the CFTR gene, although most of these effect the structure of the CFTR protein. Approximately 85% of CF are caused by four mutations; the most common being the ΔF508 mutation which is characterised by deletion of phenylalanine at position 508 in the protein chain (Davies 1991; Knight 1992). The remaining 15% are caused by rarer mutations (> 800 to date) with some being found in only one family. Some mutations result in failure of the CFTR protein to be produced. These patients appear to fare better, presumably because there is no blocking of the chloride pore. Other patients have normal copies of the CFTR gene and so the mutation may be located elsewhere on chromosome 7.

One example where the outward symptoms appear to be directly related to the genetic defect is in those CF patients who are homozygous for the ΔF508 mutation. These patients invariably have pancreatic insufficiency (Fiel 1993). The frequency of the

ΔF508 mutation is significantly lower in the pancreatic sufficient group than the pancreatic insufficient group (Santis *et al* 1990).

The discovery of the CF gene has led to therapeutic trials where the normal CFTR gene is introduced into the airways of rats via adenoviruses or liposomes (Davies 1991). Clinical trials of gene therapy are in progress but so far improvements in clinical status have been less dramatic and sustained than expected.

1.1.3. The patients

As recently as 20 years previously, the prognosis for CF patients was poor with up to 10% dying from meconium ileus shortly after birth. Other early problems include intestinal blockages due to dehydrated stools and steatorrhoea may also occur. However, with improvements in the understanding and treatment of the disease, the mean age of survival is now 30 years and beyond and many are able to lead reasonably normal lives. Many go on to higher education and careers and partake in most social activities. Often CF patients socialise as a group and the CF Trust in the UK and the CF Foundation in the US have sponsored summer camps abroad, other social events and meetings and conferences for older patients. Interaction between CF patients at these events where they can discuss mutual issues and problems is considered to be good for their general well being.

The improvements in life expectancy and good health have mainly come about as the result of the setting up of specialised CF clinics. These provide advanced medical and nursing care, physiotherapy to aid lung clearance and dieticians to advise on the necessity of a high energy diet to combat the problems of malabsorption and salt loss. Patients are also treated with pancreatic enzymes, anti-inflammatory drugs to prevent early lung damage, vitamins and nebulised and intravenous antibiotics. Lung

transplants have also been successfully carried out on patients with end-stage lung disease.

However, increased life expectancy has brought about its own problems which mainly involve the respiratory system. Severe haemoptysis (bleeding from the lungs) occurs rarely in older patients but chronic lung infections due to numerous opportunistic bacterial pathogens is common.

1.1.4. Lung infections

Problems of the gastrointestinal tract in CF patients have been adequately managed for some years now but lung deterioration due to chronic bacterial infections remains the major cause of morbidity and mortality. The lungs of CF patients are normal at birth and in theory damage is preventable (Littlewood 1986). Evidence suggests that regular culturing of lung secretions and appropriate antibiotic therapy are important in minimising early lung damage which in children is usually caused by infections with *Staphylococcus aureus.*

***Staphylococcus* aureus.** The reasons why the CF lung is predisposed to infection with *S. aureus* are not entirely clear. May and Roberts (1969) found that sputum, serum and urine from patients with CF support better the growth of *S. aureus* than specimens from controls. They suggested that this may be due to an elevated level of p-hydroxyphenylacetic acid which appears to enhance the growth of *S. aureus.* Schwab *et al* (1993) examined the adherence mechanisms of *S. aureus* in the CF lung to determine whether the airway epithelial cells themselves have specific receptors for *S. aureus* or whether CF isolates of the species have a greater affinity to bind to the airways. They found no evidence for the presence of increased or different *S. aureus*

receptors on CF epithelial cell surfaces but CF isolates did appear to have a greater binding affinity for CF airways.

Haemophilus influenzae. This is a significant pathogen in the lungs of children with CF and may colonise in place of *S. aureus* after the latter is eradicated by antimicrobial therapy. *H. influenzae* was more frequently isolated from the lower respiratory tract of children with CF than those with asthma and a rise in isolation rate preceded the development of acute exacerbations (Rayner *et al* 1990). They also showed that treatment was associated with clinical improvement and eradication of the organism in most cases.

Pseudomonas aeruginosa. This is the most common coloniser of the lungs of older CF children and adults and approximately 80-90% of CF patients develop chronic infections (Pier 1998). The main factor thought to be responsible for its persistence is a switch to a mucoid phenotype which occurs frequently in the CF lung. This mucoid exopolysaccharide (MEP) which is composed of alginate protects the organism from host defences. Studies by Santis *et al* (1990) demonstrated a correlation between the CFTR mutation and susceptibility to *P. aeruginosa* infection in that pancreatic sufficient patients were less likely to have chronic sputum colonisation by *P. aeruginosa* than pancreatic insufficient patients. Colonisation was higher amongst patients homozygous for the ΔF508 mutation and who were pancreatic insufficient (De Braekeleer *et al* 1998). Pier (1998) explained this by suggesting that CFTR is a specific receptor for *P. aeruginosa* on epithelial cells which ingest it and then clear it from the lungs by shedding. Cells with mutant forms of CFTR such as ΔF508 have difficulty in ingesting the organism resulting in chronic infection. However, this is unlikely to be the only reason for *P. aeruginosa* infection in CF but may help to

explain its persistence in some patients. *P. aeruginosa* also produces a wide range of virulence factors which include a potent cytotoxin, exotoxin A, which inhibits protein synthesis, phospholipase C and proteases such as elastase (Wretlind and Pavlovskis 1981).

Other pathogens Numerous bacterial, viral and fungal pathogens may infect the CF lung resulting in varying degrees of morbidity. In some cases bacteria proliferate as apparently "harmless" colonisers. Examples include *Stenotrophomonas maltophilia* which occurs occasionally in CF patients especially after courses of broad-spectrum antibiotics. Persistent colonisation with this organism may lead to deterioration in lung function (Ballestero *et al* 1995). *Pseudomonas* species of rRNA group II which have recently been transferred to the genus *Burkholderia* may also be a problem, especially *B. cepacia* which in recent years has emerged as a very important pathogen in the CF lung.

1.2. Burkholderia cepacia

1.2.1. History

B. cepacia was first described in the 1940s by Burkholder as a cause of sour rot in onions (Burkholder 1950). The rot was found to attack only the outer leaves of the bulb resulting in a sour and slippery skin. Microscopical examination of the tissue revealed numerous bacteria many of which were harmless saprophytes. The actual bacterial pathogen was only present in small numbers amongst the saprophytes and was described as a motile non-sporing gram-negative rod. A water-soluble yellow/green pigment was produced on nutrient agar distinct from the yellow

fluorescent pigment of *P. aeruginosa.* However, other characteristics classified it in the genus *Pseudomonas* and he described it as a new species, *Pseudomonas cepacia* n. sp.

1.2.2. Taxonomy

Stanier *et al* (1966) proposed the name *Pseudomonas multivorans* for a group of aerobic pseudomonads isolated from soil and water which reflected the range of organic compounds they used as carbon and energy sources. All the isolates studied had multitrichous flagella and accumulated poly-β-hydroxybutyrate as a cellular reserve material. Most hydrolysed gelatin and produced lecithinase. All produced a lipase and could use ammonia and nitrate as nitrogen sources. Pigment production was variable and included, yellow, brown, red and purple pigments. Ballard *et al* (1970) examined 18 isolates of *P. cepacia* from plants and were unable to distinguish them phenotypically from *P. multivorans* isolates described by Stanier *et al* (1966). Two of the *P. cepacia* isolates were shown by DNA-DNA hybridization, to share a level of genetic homology with the type strain of *P. multivorans* as high as that of other isolates of this species. The DNA base composition of some of the *P. cepacia* isolates, also fell in the *P. multivorans* range of 67-68 G+C moles %. The main difference was the ability of *P. cepacia* to produce onion rot, although some of Stanier's *P. multivorans* isolates were later shown to have this property. Ballard *et al* (1970) concluded that the two species were synonymous and that the correct designation was *P. cepacia.* Phytopathogenicity was removed as a characteristic of all isolates but many of the phenotypic tests described by Stanier for *P. multivorans* were included. He also found that *P. cepacia, P. marginata* (*gladioli*) and *P. caryophylli* shared many phenotypic properties and showed DNA-DNA hybridisation homology

with *P. pseudomallei* and *P. mallei.* These species subsequently became known as the *Pseudomallei* group.

Jonsson (1970) studied the cultural, biochemical and serological characteristics of *Pseudomonas* isolates described by King as Eugonic Oxidisers Number One (EO-1) which were isolated from a variety of clinical sources. On the basis of these findings, the name *Pseudomonas kingii* n. sp. was proposed. Morphological, biochemical and carbon utilisation tests carried out by Snell *et al* (1972) on *P. cepacia, P. multivorans, P. kingii* and EO-1 strains confirmed that *P. kingii* was a further synonym of *P. cepacia.* Analysis of the fatty acid composition of strains of *P. multivorans* and *P. kingii* by gas-liquid chromatography irrefutably showed that they were identical species (Samuels *et al* 1973). Similar findings were reported by Sinsabaugh and Howard (1975) from a wide range of tests on isolates of *P. cepacia, P. multivorans* and *P. kingii.*

Palleroni *et al* (1973) examined rRNA homologies (rRNA-DNA hybridisations) in the genus *Pseudomonas* and proposed five distinct sub-groups. *P. cepacia* was classified in group II along with *P. marginata* (*gladioli*), *P. caryophylli, P. pickettii,* and *P. solanacearum.*

At the meeting of the Judicial Commission of the International Committee of Systematic Bacteriology, held in Jerusalem in March 1973, an Ad Hoc committee was appointed to review bacterial names (Skerman *et al* 1980). The intention was to retain only names of those taxa which were adequately described and for which a type or reference strain was available. *P. cepacia* lost its validity by exclusion from this Approved List of Bacterial Names and hence lost its standing in bacterial nomenclature. However, Palleroni and Holmes (1981), revived the name *P. cepacia*

with a description of the species and its relationship to other species of *Pseudomonas* in rRNA group II. They also designated ATCC 25416 as the type strain.

Yabuuchi *et al* (1992) proposed that seven species of *Pseudomonas* rRNA group II should be transferred to a new genus, *Burkholderia,* with *B. cepacia* as the type species. The genus also included *B. caryophylli, B. gladioli, B. mallei, B. pseudomallei, B. pickettii* and *B. solanacearum.* Two species, *B. plantarii* and *B. glumae*, were added to the genus on the basis of phenotypic and chemotaxonomic characteristics and they were later joined by *B. vandii* (Urakami *et al* 1994) and *B. cocovenenans* (Zhao *et al* 1995), although recently the latter two have been proposed as junior synonyms of *B. plantarii* and *B. gladioli* respectively (Coenye *et al* 1999). Gillis *et al* (1995) described nitrogen fixing strains of the same rRNA complex as *B. cepacia* and named them *B. vietnamiensis* due to their origin from macerates of rice cultured in Vietnam. They also demonstrated that *B. solanacearum* and *B. pickettii* constituted one rRNA branch of *Burkholderia* whilst the others were segregated into another branch which included *B. andropogonis* comb. nov. Subsequently, the former two species were transferred to the genus *Ralstonia* (Yabuuchi *et al* 1995). Three further pseudomonads (*P. glathei, P. pyrrocinia* and *P. phenazinium*) have also been proposed for reclassification as *Burkholderia* (Vandamme *et al* 1997), (Viallard *et al* 1998). *B. graminis, B. thailandensis, B. caribensis* and *B. uboniae* have recently been proposed as new species (Viallard *et al* 1998) (Brett *et al* 1998), (Achouak *et al* 1999), (Yabuuchi *et al* 2000).

It was becoming increasingly clear to microbiologists that the species *B.cepacia* was not homogeneous but it was the work of Vandamme *et al* (1997) using a polyphasic diagnostic approach that showed that *B.cepacia* isolates comprised at least five

genomovars. The tests that they carried out included SDS-PAGE of whole cell proteins, G+C values, DNA-DNA and DNA-RNA hybridisation experiments, fatty acid methyl ester analysis and biochemical tests. Genomovar I was termed *B. cepacia* and included the type strains and plant isolates. Genomovar II was termed *B. multivorans* but genomovars III and IV were not assigned a species name at this time. Genomovar V was termed *B. vietnamiensis*. The reactions of the genomovars in biochemical tests are shown in table1.

Table 1
Biochemical reactions of the *B. cepacia* complex and *B. gladioli* (Vandamme *et al* 1997)

Biochemical reaction	I	II		III		IV	*B. gladioli*	*B. vietnamiensis*
			a	b	c			
Acid production in ASS								
Glucose	+	+	+	+	-	+	+	+
Adonitol	+	+	+	-	-	+	+	-
Cellobiose	+	+	+	+/-	-	+	+/-	+
Dulcitol	+	+	+	-	-	+	+	+
Fructose	+	+	+	-	+/-	+	+	+
Inositol	+	+	+	+/-	-	+	+	+
Lactose	+	+	+	+	-	+/-	-	+
Maltose	+	+	+-	+/-	-	+	-	+
Mannitol	+	+	+	-	-	+	+	+
Raffinose	+/-	-		-	-	-	-	+/-
Salicin	+/-	+/-	+/-	-	-	-	-	-
sorbiol	+	+	+	-	-	+	+	+
Sucrose	+/-	-	+	+	-	-	-	+
Xylose	+	+	+	+/-	-	+	+	+
Acid from								
Glucose peptone sugar water	+/-	+	+	+/-	-	+	-	+
10% glucose	+	+	+	+	-	+	+/-	+
10% lactose	+	+	+	+	-	+	+/-	+
Alkaline reaction in OF medium	-	-	-	-	+/-	-	-	-
Casein digestion	+/-	-	+/-	+/-	-	+/-	+/-	-
Growth at 42^{0}C	-	+	+	+/-	+	-	-	-
Growth on cetrimide agar	+	+	+	+	+	+	+/-	+
Growth on Simmons' citrate	+	+	+	+	+/-	+	+	+
Malonate utilisation	+	+/-	+/-	+/-	+/-	+	-	+
Pigment on tyrosine	-	-	+/-	+/-	+/-	-	+/-	-
Motility at 37^{0}C	+/-	+	+	+/-	+/-	+	-	+
Nitrate reduction	+/-	+	-	+/-	+/-	+/-	+/-	+/-
Oxidative in OF medium	+	+	+	+	-	+	+/-	+
Urease production	+/-	+	+/-	-	+\-	+/-	+/-	+

+/- Indicates positive or negative reaction

Subsequently, genomovar IV isolates were designated as *B. stabilis* due to the highly conserved nature of their genomes in contrast to other members of the complex (Vandamme *et al* 2000). More recently, genomovar VI has been described as a member of the *B. cepacia* complex (Coenye *et al* 2001) and this group can be distinguished from the other five genomovars (except for *B. multivorans*) by biochemical tests and SDS-PAGE of whole-cell proteins. The main distinguishing features of the genomovars are shown in Table 2.

Table 2
Tests which discriminate between members of the *B. cepacia* complex

Test	Genomovar						
	I	II	III	IV	V	VI	*B. gladioli*
Growth at 42^0C	-	+	+	-	-	+	-
β-Galactosidase activity	+	+	+	-	+	+	+
Decarboxylation of:							
Lysine	+	v	+	+	+	-	+
Ornithine	+	-	+	+	+	-	+
Oxidation of:							
Sucrose	+	-	+	-	+	-	-
Lactose	+	+	v	+	+	+	-
Adonitol	+	+	v	+	+	+	-
Maltose	+	+	v	+	+	+	-

1.2.3. Habitat

B. cepacia is ubiquitous in the environment and can be isolated from soil, rivers, plants and raw milk (Gregory and McNabb 1986). It has the ability to survive in nutritionally limited environments and can use a wide range of organic compounds as energy sources including penicillin G as a sole carbon source (Beckman and Lessie 1979). *B. cepacia* is unusual in that it is both a plant and a human pathogen and it can also suppress other plant pathogens. McLoughlin *et al* (1992) showed that sunflower wilt fungus was suppressed in the presence of *B. cepacia* leading to increased sunflower emergence; although the antimicrobial agents produced by *B. cepacia* were

not the main inhibitors of the fungi. *B. cepacia* has also been found to reduce "damping off" in plants such as corn and pea roots due to the fungus *Pythium* (Hebbar *et al* 1998), (Parke *et al* 1991). These and other studies have led to interest in the use of *B. cepacia* as a biopesticide and plant-growth-promoting bacterium. However, this is controversial due to the importance of *B. cepacia* in human disease (Govan and Vandamme 1998), (Holmes *et al* 1998). Butler *et al* (1995) sampled a large botanical complex including a range of soils, aquatic sites and vegetation and did not recover *B. cepacia* as frequently as would be expected except in moist soil or rhizosphere. In the home *B. cepacia* may be found in tap water and on fresh fruit and vegetables but it does not heavily contaminate the domestic environment in comparison with other *Pseudomonas* species such as *P. aeruginosa, P. putida* and *P. fluorescens* (Mortensen *et al* 1995). In the hospital environment *B. cepacia* may contaminate stored water, distilled water and antiseptic solutions as it can survive for long periods of time in nutritionally limited conditions especially if sources of carbon and nitrogen are present (Geftic *et al* 1979). Prolonged survival in antiseptic solutions is believed to be due to protection of the organism by cellular and extracellular material forming on surfaces (Anderson *et al* 1991).

1.2.4. Appearance

B. cepacia is a pleomorphic, aerobic gram-negative rod of approximately 0.5 x 2.5 μm and motile by means of a tuft of three to eight polar flagella (Gregory and McNabb 1986). It grows on blood agar at 35 ^{0}C to 37 ^{0}C to produce smooth circular non-pigmented colonies of approximately 0.5 to 1.0 mm in diameter after 48 h incubation. Pigment may be produced on some media such as Pseudomonas P agar. There are two main types of pigment which are probably phenazine derivatives. One of these is

diffusible and fluorescent whilst the other is a sulphur-yellow diffusible but non-fluorescent pigment. More rarely seen are brown, red and purple pigments which are usually strain dependent (Gregory and McNabb 1986).

1.2.5. Genetics

B. cepacia possesses an unusually large and complex genome. It contains numerous insertion sequences and consists of approximately three large circular replicons (Cheng and Lessie 1994). This arrangement probably explains the ability of the organism to utilise a wide variety of substrates and to survive in different and often unfavourable environments (Holmes *et al* 1998). It may also account for pathogenic versatility and variable antibiotic sensitivity profiles. A comparison of two clinical isolates with two agricultural isolates showed that all four clustered within the genus *Burkholderia* but there were differences in the 16S rDNA between the two groups (Tabacchioni *et al* 1995). Indeed a recent study has found that all isolates of *B. cepacia* have between two and four large replicons (>500kbp) but clinical isolates were more likely to have three or four replicons than environmental and plant pathogenic isolates (Wigley and Burton 2000).

1.2.6. Disease

General infections. *B. cepacia* rarely causes infections in healthy people and most cases occur in patients with an underlying predisposition to infection. Examples include osteomyelitis and endocarditis in heroin addicts (Smith *et al* 1985) (Noriega *et al* 1975) and brain abscess following chronic otitis media treated with contaminated ear drops (Hobson *et al* 1995). It has been reported as a cause of community-acquired septicaemia as a secondary consequence of pulmonary infection (Reed 1998) and has

been linked to primary pneumonia in a previously healthy individual after heavy exposure to dust (Dailey and Benner 1968). An outbreak of nosocomial infection due to *B. cepacia* amongst ventilated ITU patients in two US hospitals was attributed to contaminated mouthwash (Matrician *et al* 2000). *B. cepacia* may be introduced into normally sterile sites such as the cerebrospinal fluid to cause meningitis and has been implicated in a number of infections including urinary tract infection, endocarditis, peritonitis, pneumonia and conjunctivitis. Contaminated disinfectant solutions and surgical appliances may be responsible in some cases (Bassett *et al* 1973), (Berkelman *et al* 1982), (Speller *et al* 1971), (Hardy *et al* 1970), (Ederer and Matsen 1972).

Trench foot. *B. cepacia* has been shown to colonise the toewebs of troops during training in swamp areas (Taplin *et al* 1971). The swamp waters themselves did not yield large numbers of *B. cepacia* and so it was concluded that damp but otherwise intact skin was a suitable site for multiplication of the organism leading to hyperkeratotic lesions. This condition also provides evidence for the pathogenic potential of environmental isolates.

Chronic granulomatous disease (CGD). This is a hereditrary disease where oxygen-dependent killing of microorganisms in neutrophils fails to function due to an absence of NADPH oxidase to trap oxygen for the hexose-monophosphate shunt. Oxygen-dependent killing of bacteria is brought about by hydrogen peroxide (H_2O_2), free radicals such as superoxide (O^-_2) and singlet oxygen ($^1O_2$). Organisms that produce H_2O_2 (which is the most important oxygen-derived killing agent in neutrophils), contribute to their own death but catalase-producing bacteria such as *P. aeruginosa* and *B. cepacia* actually break down H_2O_2. *P. aeruginosa* is not a problem in CGD as it is sensitive to oxygen-independent killing mechanisms (Speert *et al* 1994). *B.*

cepacia however, is resistant to nonoxidative bactericidal effects and is an important pathogen in CGD patients where it commonly causes pneumonia in previously undamaged lungs (O'Neil *et al* 1986; Speert *et al* 1994). The infections tend to be severe and the slow healing is accompanied by granuloma formation in various organs.

Cystic fibrosis. Although not as common as *P. aeruginosa, B. cepacia* colonisation of the CF lung is the cause of extreme concern amongst patients, their carers and families. This is because 0-20% of those infected succumb to "cepacia syndrome" which is characterised by the onset of a necrotising pneumonia, sometimes with septicaemia and often rapidly fatal.

1.3. Burkholderia cepacia in Cystic Fibrosis

1.3.1. General

As well as concern about "cepacia-syndrome", other anxieties are also raised when caring for *B. cepacia* colonised patients. A major cause of current anxiety is the discrimination of *B. cepacia*-positive patients during selection for lung transplants. They are believed to be inferior candidates due to persistence of the organism after transplant and often have a poor prognosis (Steinbach *et al* 1994), (Snell *et al* 1993). There is also a social stigma attached to colonised patients who are often segregated in hospitals and discouraged from attending summer camps and other events for fear of cross-infection. There are many questions and much debate about aspects of *B. cepacia* acquisition and infection in CF sufferers. The most important question is why is *B. cepacia* attracted to the CF lung and what strain factors cause disease? Is only a

minority of CF strains virulent or does it depend on host factors such as previous infections? Also, what is the reservoir of CF infection: do patients acquire the organism from the environment and/or other patients and are all strains equally transmissible between patients? Some of these problems are discussed in more detail in the following sections and although the emphasis is on isolates from CF patients, much is also applicable to *B. cepacia* infection in other diseases.

1.3.2. Isolation

The isolation of *B. cepacia* from the CF lung and differentiation from organisms such as *P. aeruginosa, S. aureus, H. influenzae* and other gram-negative rods is often difficult. To tackle this problem, selective media containing the polymyxin antibiotic, colomycin to which the genus is constitutively resistant have been developed and two formulations are available commercially: Pseudomonas cepacia (PC) agar (Gilligan *et al* 1985) and Oxidation-fermentation-lactose-polymyxin B (OFPBL) agar (Welch *et al* 1987). These media suppress the growth of the mixed flora of CF sputum and support the growth of *B. cepacia* better than conventional media. An enrichment broth has also been developed which may be more efficient for the isolation of the organism if present in small numbers (Cimolai *et al* 1995). More recently, Henry *et al* (1997), described a medium which was enriched for *B. cepacia* but more selective against other organisms than the media described above. They named this medium *B. cepacia* selective agar (BCSA). Furthermore, the selectivity for *B. cepacia* was increased by the addition of vancomycin.

1.3.3. Identification

The identification of *B. cepacia* is also problematic. This is due to the difficulty of distinguishing *B. cepacia* from other *Burkholderia* and *Pseudomonas* species that may grow on the selective media. As a general rule, colonies which grow on selective agar and prove to be gram-negative rods and producers of oxidase enzyme, oxidise glucose and fail to convert arginine can be presumptively classified as *B. cepacia* subject to further test results. However, some strains from CF sputum may be asaccharolytic and fail to react in sugar-containing media.

Phenotypic methods

Probability matrix. Holmes, *et al* (1986) constructed a computer based probability matrix for the identification of Gram-negative non-fermentative bacteria. This gives the probability of a particular species to produce a positive or negative result in a number of tests. Although some strains of *B. cepacia* were atypical and difficult to identify, the identification rate achieved for the species by this approach was 91.5%. The biochemical tests used to differentiate *B. cepacia* from other species are listed in Table 3.

Table 3
Biochemical reactions used in the identification of *B. cepacia*

Reaction
Acid production in ammonium salt sugars
Reaction in oxidation/fermentation medium
Casein digestion
Growth on cetrimide agar
Growth on Simmon's citrate
Nitrate reduction
Urease production
Oxidase production

Commercial kits. A number of kits including Rapid NF Plus and API RAPID NFT kits are available for the identification of gram-negative rods. These have the advantage over conventional tests in that they are more rapid but have the drawback of misidentifying other closely related species (Kiska *et al* 1996). More recently, Shelly *et al* (2000) found that more than 10% of isolates identified as *B. cepacia* by referring laboratories proved to be other gram-negative species which may colonise the CF lung especially *B. gladioli*. However, conventional tests are not without their problems and if the reactions are weak or the incubation times are shortened, false-negative or positive results may occur which may lead to misidentification of isolates (Burdge *et al* 1995).

Genomovars. As described earlier, *B. cepacia* and closely related species have been classified into genomovars on the basis of the test reactions (section 1.2.2.)

(Vandamme *et al* 1997). Many of the biochemical tests used to define genomovars are also used to identify *B. cepacia* (Holmes *et al* 1986). This method of grouping also enables identification of the closely related species *B. gladioli* and *B. vietnamiensis* which may be misidentified as *B. cepacia* and also allows classification of asaccharolytic variants of *B. cepacia*. These isolates are more likely to be mistaken for alkaline-producing gram-negative rods because of their weak alkaline or negative reaction in Hugh and Leifson's O/F glucose medium. These variants are now classified as *B. cepacia* genomovar III biovar c.

Genotypic methods

A problem with biochemical tests, apart from rapid identification kits, is the need for specific reagents and the time taken to obtain a result may be impracticable for many diagnostic bacteriology laboratories. Also, despite the extended range of phenotypic identification tests, some *B. cepacia* isolates remain difficult to identify unequivocally. Indeed, some isolates of the strain of *B. cepacia* prevalent in outbreaks among CF patients in Scotland and the north of England, has been reported to have phenotypic features indicative of both *B. cepacia* and *B. gladioli* (Simpson *et al* 1994). This has led to research into alternative methods of identification. O'Callaghan *et al* (1994) developed a polymerase chain reaction (PCR) and DNA hybridisation assay based on 16S rRNA sequences for the identification of *B. cepacia* and *B. cepacia* specific 16S rRNA sequences were also exploited by Campbell *et al* (1995). Tyler *et al* (1995) opted for 23S rRNA sequences to generate oligonucleotide primers for amplification of a region specific for *B. cepacia* and utilized sequences from the internal transcribed spacer (ITS) region of 16S-23S rRNA for the identification of *B. gladioli.* More recently, a number of studies have focused on PCR methods for the

identification of the individual genomovars of the *B. cepacia* complex (Bauernfeind *et al* 1999), (LiPuma *et al* 1999), (Vandamme *et al* 2000), (Whitby *et al* 2000), (Mahenthiralingam *et al* 2000).

1.3.4. Epidemiology

The demonstration of transmissible strains of *B. cepacia* amongst CF patients has caused much anxiety during recent years due to their association with morbidity and mortality. Contact with other patients is good for their general well-being and draconian segregation policies introduced both in the hospital and socially has caused both emotional and administrative problems not to mention possible legal challenges of these policies.

Environmental transmission

Many patients harbour sporadic strains which may suggest that their isolates are acquired from environmental sources (Steinbach 1994). However, *B. cepacia* is apparently infrequent in the home and on food (Mortensen *et al* 1995) and the suggestion that it is ubiquitous in the natural environment has been questioned by Butler *et al* (1995). Furthermore, environmental and clinical isolates often show differences in their DNA profile and pathogenic activity for plant tissue (Butler *et al* 1995), (Bevivino *et al* 1994).

Hospital transmission

The survival of *B. cepacia* in antiseptic solutions has already been mentioned (Anderson, *et al* 1991), (Geftic *et al* 1979). This suggests that the hospital environment could be a major source of *B. cepacia* for CF and other susceptible patients. Colonisation and even outbreaks have been attributed to contaminated antiseptic solutions and equipment including nebulisers and spirometry mouthpieces

used by CF patients (Gravel-Tropper *et al* 1996), (Reboli *et al* 1996), (Hutchinson *et al* 1996), (Govan *et al* 1993). Nosocomial acquisition of *B. cepacia* was shown to be related to humidifier and nebuliser treatment but not to antibiotic usage or room sharing and socialising with other patients and *B. cepacia* failed to be recovered in air samples after coughing, or on the hands of patients, nurses and physiotherapists (Burdge *et al* 1993). Doring *et al* (1997), found that *B. cepacia* was less ubiquitous in the hospital environment than *P. aeruginosa* as it was isolated only once from a sink drain in a CF ward. Furthermore, it was never isolated from patients or staff but they were able to demonstrate that both *B. cepacia* and *P. aeruginosa* embedded in sputum survived sufficiently well to be transmissible by hand shaking for up to three hours. *B. cepacia* can also persist in CF patients who have received lung transplants. Steinbach, *et al* (1994) found that CF patients at a transplant centre all had unique strains of *B. cepacia.* They also showed that disseminated infection following lung transplantation was due to the same strain as that isolated preoperatively.

Patient to patient transmission

There is evidence to suggest that some strains of *B. cepacia* are directly transmissible between CF patients and a number of studies have shown that segregation of patients as out- and in-patients in hospital reduces the rate of acquisition of the organism and its spread (Paul *et al* 1998), (Muhdi *et al* 1996), (Thomassen *et al* 1986). Cross infection has also been documented as a result of social contact and this raised the issue as to whether attempts should be made also to segregate patients outside of hospital (LiPuma *et al* 1990), (Govan *et al* 1993), (Smith *et al* 1993), (Smyth *et al* 1993).

Govan *et al* (1993) described an epidemic strain that spread between clinics in Edinburgh and Manchester. This strain had been imported from North America to the UK as a result of CF patients staying at a summer camp in Canada. This is now the predominant strain in the UK CF population and was named ET12 by Johnson *et al* (1994). Some isolates give characteristic dry sticky colonies on agar and produce a melanin-like brown pigment. Some isolates are highly resistant to most antibiotics and may also possess characteristics of both *B. cepacia* and *B. gladioli* (Simpson *et al* 1994). The UK epidemic strain has since been classified in *B. cepacia* genomovar III (Vandamme *et al* 1997).

Typing of *B. cepacia*

A number of phenotypic and genotypic typing systems have been employed to determine the relatedness of *B. cepacia* isolates for epidemiological studies (Rabkin *et al* 1989), (Ouchi *et al* 1995).

Biotyping. *B. cepacia* is fairly uniform in its biochemical characteristics but some test properties are sufficiently variable to be used as biotype markers. Identifying common strains of *B. cepacia* involved in outbreaks by biochemical tests has had variable degrees of success but biotyping is neither as sensitive nor specific as genotypic methods (Rabkin *et al* 1989), (Ouchi *et al* 1995). Strains indistinguishable by genotyping may exhibit marked phenotypic variability and be classified as different biotypes.

Antimicrobial susceptibility. *B. cepacia* is intrinsically resistant to a number of antimicrobial agents and thus the relatedness of isolates cannot be accurately determined by antimicrobial susceptibility. This is due to variabilty in susceptibility

amongst isolates of the same genotype and the instability of susceptibilities of individual colonies within an isolate (Rabkin *et al* 1989), (Pitt *et al* 1996).

Bacteriocin typing. Bacteriocins are proteinaceous substances which are active against strains of the same or closely related species. They are non-replicative but many *Pseudomonas* bacteria have phage tail type bacteriocin structures. Isolates may be typed by the spectrum of production and/or sensitivity to bacteriocins. Both these features have been found to be stable and reproducible in *B. cepacia* and the method has been reported to give good discrimination (Govan and Harris 1985).

Serotyping. *B. cepacia* can be distinguished by somatic antigen and flagellar antigen serotypes. The somatic or O-antigens are lipopolysachharide (LPS) in nature and at least two schemes have been described (Heidt *et al* 1983), (Nakamura *et al* 1986). Further classification of strains is afforded by flagellar H-antigens which may be detected by immobilisation of cells with specific antibodies. Serotyping is a very useful method for epidemiological studies but discrimination is inadequate for strain identification due to the limited number of serotypes.

Ribotyping. This method detects variation in rDNA gene loci. The rRNA operon in bacteria is highly conserved and is a good indicator of phylogenetic relationships between species. Providing there are sufficient copies of the operon and an appropriate restriction endonuclease is used, ribotyping can also distinguish between strain populations. *B. cepacia* chromosomal DNA is digested with the restriction endonuclease *Eco*RI and the resultant fragments are separated on an agarose gel before transferring to nitrocellulose and hybridising with *E.coli* rRNA. Isolates within a ribotype may differ from each other by three or fewer bands whereas those within the same subribotype have identical banding patterns or differ from each other by

single bands. Rabkin *et al* (1989) found this method to be sensitive and specific for *B. cepacia*. Ribotyping has also been exploited by LiPuma *et al* (1988) who demonstrated *B. cepacia* isolates of the same ribotype within CF centres and Pitt *et al* (1996) identified more than 50 ribotype patterns in UK CF isolates and found the epidemic strain (ribotype 1) in 10 different CF centres.

PCR Ribotyping. Traditional ribotyping is time consuming and technically demanding. PCR ribotyping on the other hand is more rapid and simpler to perform. This technique detects length polymorphisms in the intergenic spacer region between 16S and 23S rRNA genes using primers complementary to highly conserved sequences. This method has been shown to give identical patterns for *B. cepacia* strains involved in patient-to-patient transmission but also distinguishes unrelated strains (Kostman *et al* 1992), (Ryley *et al* 1995).

Randomly Amplified Polymorphic DNA (RAPD). This PCR-based DNA fingerprinting method is best suited for a large throughput of isolates. As with PCR ribotyping, it detects length polymorphisms but differs in that it employs random primers. Mahenthiralingam *et al* (1996) evaluated a number of primers and selected one for RAPD fingerprinting of a collection of isolates of *B. cepacia* from CF and non-CF patients and the environment. They found the method to be reproducible and discriminatory and more sensitive than PCR ribotyping. It was more comparable with ribotyping and pulsed-field gel electrophoresis (PFGE) and gave similar strain groupings. The major problem with RAPD is that the results vary from laboratory to laboratory making inter-laboratory comparisons difficult.

Multilocus Enzyme Electrophoresis (MLEE). This technique separates isolates on the basis of differences in enzyme electrophoretic mobilities and is a good indicator of

population structure and phylogenetic relationships. Johnson *et al* (1994) was able to differentiate isolates of *B. cepacia* according to origin by MLEE and found that environmental, CF and other nosocomial isolates clustered in separate groups. Isolates from an individual CF centre often had the same MLEE profile but were sometimes of different ribotypes. This work ascribed individual strains to clonal complexes and notably classified the UK epidemic strain in clone ET12.

Pulsed-Field Gel Electrophoresis (PFGE). This is probably the most discriminatory DNA typing method for a wide range of bacteria and offers restriction enzyme analysis of the entire genome. Rare cutting restriction endonucleases are employed to produce fragments of >50 kb. To achieve separation of such large DNA fragments, the electric field is alternated in two directions in a pulsed current with smaller and larger fragments being separated by shorter and longer pulse times respectively. Some investigations have employed both ribotyping and PFGE for *B. cepacia* outbreaks, with ribotyping forming the primary division and the PFGE pattern discriminating further between strains where possible (Steinbach *et al* 1994), (Ouchi *et al* 1995), (Pitt *et al* 1996).

Epidemic markers

In recent years a number of studies have described the presence of epidemic markers associated with strains of *B. cepacia* linked to outbreaks in the CF population. These markers do not necessarily have an obvious role in transmissibility but their presence may confirm the identification of an epidemic strain.

Cable pili. Sajjan *et al* (1995) described giant fibres known as cable pili which were present on isolates of *B. cepacia* from a Toronto CF centre. These pili are believed to mediate adherence to CF respiratory mucins and this enhances colonisation of lungs

by *B. cepacia*. A 22-kDa adhesin is located along the length of the fibre as well as at the appendage in order to maximise adhesion. The pilus is encoded by the chromosomally located *cblA* gene which can be detected by PCR. Sun *et al* (1995) characterised the genetic relatedness of isolates expressing cable pili from the outbreaks in Toronto and showed by PFGE and ribotyping that they were all grouped in the same lineage. Interestingly, isolates from the Edinburgh CF centre which were epidemic in the UK were also grouped in this lineage.

Insertion sequence hybrid. Tyler *et al* (1996) identified a hybrid of two insertion sequences IS*402* and IS*1356* which was exclusive to epidemic strains from Canada and the UK. Its role in transmission of strains however, is unknown.

***Burkholderia cepacia* epidemic strain marker.** Mahenthiralingam *et al* (1996) using RAPD typing with a 10-base primer (P272), identified a conserved 1.4 kb open reading frame (ORF) in the DNA fingerprint of seven epidemic *B. cepacia* strains including one positive for *cblA*. This sequence was absent in isolates from sporadic infection. The ORF showed homology with a family of negative transcriptional regulators and was termed *B. cepacia* epidemic strain marker (BCESM) (Mahenthiralingam *et al* 1997). As with the insertion sequence hybrid, BCESM has no known role in transmission.

1.3.5. Virulence

B. cepacia is rarely pathogenic in healthy subjects and a suitable animal model has not been identified. However in CF patients clinical outcome varies from symptomless pulmonary carriage to severe necrotising pneumonia and septicaemia. Little is known about the factors which determine outcomes and *B. cepacia* does not appear to produce cytotoxic enzymes equivalent to exotoxin A and exoenzyme S as in

P. aeruginosa (McKevitt and Woods 1984). Indeed, host factors probably dictate whether the organism will simply colonise the lung or progress to extensive pulmonary damage and systemic spread. Nevertheless, a number of virulence factors both structural and chemical have been attributed to *B. cepacia.*

Adherence

The ability of a pathogen to adhere to host mucosal or cell surfaces is often the first step in the infection process (Nelson *et al* 1994). *B. cepacia* from CF patients binds equally efficiently to both CF respiratory mucin and non-CF intestinal mucin (Sajjan *et al* 1992). The receptors are thought to be carbohydrates present on all normal mucins but the quantity produced in CF airways and its accessibility to inhaled organisms may increase the susceptibility of the lung to colonisation. There is also some evidence that isolates which bind to mucin are associated with a poorer prognosis in the patients from which they were isolated. The adhesins present on *B. cepacia* which determine this binding may be expressed as polar or peritrichous fimbriae. Kuehn *et al* (1992) identified a16 kDa protein, which was believed to be a fimbrial subunit of *B. cepacia,* that showed homology with *P. aeruginosa* PAK fimbriae. These fimbriated *B. cepacia* cells showed a much higher level of adherence than non-fimbriated cells. Sajjan and Forstner (1992) however, isolated a 22 kDa protein from mucin-binding *B. cepacia* which was distinguishable from pilin proteins of *Escherichia coli* (type1 pilus) and *P. aeruginosa* (PAK and PA01) fimbriae. As stated earlier, strains with the 22 kDa protein pilus (cable pili) have increased adherence and a greater propensity for epidemic spread (Sajjan *et al* 1995), (Sun *et al* 1995). More recently it has been shown that only cable-piliated isolates bound preferentially to CF airway tissue compared with non-CF tissue (Sajjan *et al* 2000).

The receptor involved was identified as a 55 kDa protein known as cytokeratin 13 which is synthesised in response to epithelial tissue injury and so is normally present in the CF lung.

Invasion

Pathogens which can invade host cells have an advantage over extracellular organisms that are exposed to immunological defences and antibiotics. *B. cepacia* has been shown to invade epithelial cells in the lung by parasite-mediated endocytosis and to replicate intracellularly (Burns *et al* 1996). It may also transcytose the epithelium causing systemic spread which would explain its potential to cause septicaemia. Indeed, patient isolates appear to exhibit a higher capacity to invade and establish a greater number of organisms per epithelial cell than environmental strains (Burns *et al* 1992) (Tipper *et al* 1998).

Serum resistance

It is clearly in the interests of a potential pathogen to be able to protect itself from host defence mechanisms. One way in which this may be achieved is by synthesis of an outer cell layer of capsule or slime. This has already been referred to in relation to *P. aeruginosa* and its production of an alginate capsule in the CF lung. Some strains of *B. cepacia* do produce a capsule but it is chemically distinct from alginate. An outer membrane structure which serves as a defence mechanism in gram-negative bacteria is the long oligosaccharide side (O-side) chain of LPS which in some species may consist of up to 40 repeated units of 3-4 sugar residues. This structure sterically hinders complement molecules from reaching the cell surface to form membrane attack complexes in complement-mediated lysis. However, some bacterial species lose their O-side chains during the infective process as is the case with many isolates of *P.*

aeruginosa from the CF lung. These are referred to as 'rough' phenotypes and are more susceptible to complement-mediated serum killing than 'smooth' phenotypes with complete O-side chains. It is unclear whether other gram-negative species colonising the CF lung undergo phenotypic changes from smooth to rough LPS. Certainly *B. cepacia* has not been shown to do this and many CF isolates exhibit rough LPS phenotypes (Butler, unpublished). The serum resistance of isolates of *B. cepacia* from CF does not correlate strictly with smooth and rough LPS phenotypes and other factors presumably play a role in the susceptibility of a strain to serum killing (Butler *et al* 1994).

Toxins and enzymes

Adherence to tissues, invasion into cells and evasion of host defences assist a colonising pathogen to survive although some damage to host tissues may occur as a result of an exaggerated inflammatory reaction to the bacteria as occurs with *P. aeruginosa* in the CF lung. However, toxins and/or hydrolytic enzymes have a direct pathological effect on host cells and may therefore play an important role in the pathogenesis of the organism.

Lipases. Phospholipids are important components of eukaryotic cell membranes, acting as support structures for membrane proteins and protecting the cell from the external environment. Membrane phospholipids consist of a water soluble head of phosphatidylcholine (lecithin) or phosphorylinositol linked to a hydrocarbon chain (Titball, 1998). As a consequence, a number of bacterial species produce phospholipase enzymes which target specific regions of the phosopholipid. These enzymes may be grouped according to the site of cleavage into phospholipases C, D,

A_1, and A_2 with phospholipase C (a lecithinase) being the best characterised to date. Lysis of erythrocytes by phospholipase C has been demonstrated *in vitro*. Both *P. aeruginosa* and *B. cepacia* have a haemolytic and a non-haemolytic phospholipase C and recently the non-haemolytic phospholipase C of *B. cepacia* was isolated and characterised (Weingart and Hooke 1999). In the lung, lecithinase appears to degrade phosphatidylcholine leading to tissue colonisation (Shortridge *et al* 1992). Lonon *et al* (1988) were unable to detect phospholipase C activity in culture supernates of 10 clinical isolates of *B. cepacia.* They did, however, record lipase activity on polyoxyethylene sorbitans (Tweens). These included Tween 20, 40, 60 and 80 which are esters of lauric, palmitic, stearic and oleic acids respectively. The role of these lipases in pathogenicity is uncertain and may be linked to nutrition. Purified lipase from *B. cepacia* when injected intravenously into mice produced no detrimental effects (Lonon *et al* 1988). Gessner and Mortensen (1990) examined lipase production in CF isolates and control strains and found that most hydrolysed Tween 80 and produced lecithinase activity on solid egg yolk medium. Similar findings were earlier described by McKevitt and Woods (1984).

Proteases. The proteases of pathogenic microorganisms are widely held to be responsible for aiding the spread of the pathogens through destruction of tissues. Gessner and Mortensen (1990), found that most CF and non-CF isolates of *B. cepacia* were proteolytic on various protein substrates, although activity on any substrate was not universal. None of the isolates produced collagenase or elastase. However, an extracellular proteinase from culture supernatants of *B. cepacia* was purified by McKevitt *et al* (1989) who found that it was antigenically similar to the elastase produced by *P. aeruginosa.*

Haemolysin. The ability to lyse erythrocytes may be important in the pathogenicity of a number of bacterial species. Mention has already been made of the haemolytic activity of phospholipase C of *P. aeruginosa* and *B. cepacia.* Nakazawa *et al* (1987) screened isolates of *B. cepacia* for extracellular products and found that although 70% produced protease, lipase and lecithinase, only 4% were haemolytic . The latter isolates also produced the other three enzymes. Characterisation of the haemolysin from one isolate showed that it was most active on human erythrocyes but also lysed horse, sheep, chicken and rabbit erythrocytes. Similar results were reported by Gessner and Mortensen (1990).

Endotoxin. Endotoxins in contrast to exotoxins are heat-stable polysaccharides which are bound to cells and released on cell growth and lysis. In gram-negative bacteria, endotoxins are LPS molecules consisting of lipid A, core oligosaccharide and O-oligosaccharide side-chains. The toxicity of LPS is due entirely to lipid A and therefore rough mutants despite their susceptibility to serum killing, are nonetheless as potentially toxic as their smooth counterparts. Endotoxin activates the pathways of inflammation, blood-clotting, fibrinolysis and complement via the alternative pathway. It is taken up by phagocytes such as neutrophils and Kupffer cells which release their lysosomes and endogenous pyrogens outside of the cell. Endogenous pyrogens are responsible for fever while lysosomes stimulate vasoactive mediators of inflammation. Zughaier *et al* (1999) compared LPS from *B. cepacia, P. aeruginosa* and *S. maltophilia* in stimulating human monocytes to release TNF-α and found that LPS from *B. cepacia* had CD14 dependent TNF-α inducing activity considerably greater than the LPS from the other two pathogens. This may explain why acute lung inflammation is more often experienced in *B. cepacia*-colonised CF patients. They

also found that LPS from *B. cepacia* stimulated monocytes to over-produce superoxide anion which is important in the respiratory burst . The respiratory burst could however be inhibited by the melanin-like pigment produced by some strains and which can be detected on tyrosine agar (Zughaier *et al* 1999).

Other enzymes. Many other enzymes are produced by *B. cepacia* but their role in pathogenicity is unknown. Examples include catalase and oxidase, nitrate reductase and urease. Some are probably not involved in the disease process but are necessary for nutrition. Indeed, the UK epidemic strain often exhibits variants which are asaccharolytic, non-proteolytic, non-haemolytic and non-lipolytic but are equally associated with transmission and a fatal outcome (Wilsher *et al* 1997), (Clode *et al* 1999). Nevertheless, Gessner and Mortensen (1990) noted differences between CF and non-CF isolates of *B. cepacia* for some enzymes such as catalase, urease and nitrate reductase .

Siderophores

To overcome the problem of iron limitation during host colonisation, microorganisms have evolved ways of sequestering iron in a utilisable form. One way is to produce high-affinity iron-binding compounds known as siderophores (*iron bearers*) which chelate ferric iron from the host in a form that can be assimilated. Iron starvation leads to the synthesis of siderophores which are very efficient at scavenging iron when it is in short supply. This is followed by the induction of specific transport systems for ferricsiderophore reception or binding and internalisation (Briat, 1992). There are three classes of siderophores; hydroxamates, catechols and miscellaneous unclassified compounds.

Sokol (1986) screened *B. cepacia* isolates from CF patients for the production of the siderophore pyochelin which is produced by all clinical isolates of *P. aeruginosa.* Approximately half of them produced it and most of these were from patients with severe infections. Of the pyochelin-negative isolates, just over a half were isolated from patients with mild infections. They also showed that pyochelin-negative isolates took up externally supplied pyochelin which increased the dissemination of the isolates in a rat lung model and raised the question of whether synergy occurred between pyochelin-negative *B. cepacia* and *P. aeruginosa* in the CF lung (Sokol and Woods 1988), (Sokol, 1986). Around about the same time, another siderophore produced by *B. cepacia* was discovered in iron-deficient growth conditions (Meyer *et al* 1989). This was named cepabactin and may also be produced by other non-fluorescent pseudomonads. Subsequently, Sokol *et al* (1992) isolated a novel siderophore in ethyl acetate extracts of culture supernatants of *B. cepacia* which had neither the characteristics of hydroxamates nor catechols and so was placed in the miscellaneous class. They named the siderophore azurechelin due to its blue fluorescence under UV light. In a medium containing transferrin, azurechelin was able to compete with the transferrin for iron and promote the growth of two siderophore-negative *B. cepacia* isolates. It was further demonstrated using radiolabelled iron, that azurechelin can only remove iron from transferrin at acid pH such as at sites of inflammation. Azurechelin was later identified by Visca *et al* (1993) as salicylic acid by thin layer chromatography. Stephan *et al* (1993) described novel linear hydroxamate/hydroxycarboxylate siderophores in *B. cepacia* known as ornibactins. These were designated ornibactin -C4, -C6 and -C8 and all possessed the same peptide sequence but had slight differences in the acyl residues bound to ornithine at

position one. Ornibactins are the first peptide siderophores isolated from non-fluorescent pseudomonads (Stephan *et al* 1993). Subsequently, Meyer *et al* (1995) showed that ornibactin-mediated iron uptake was independent of acyl chain length and was a common iron transport system in *Burkholderia* species but not in other *Pseudomonas* species . More recently, production of the four siderophores pyochelin, cepabactin, azurechelin and ornibactins by CF *B. cepacia* isolates has been correlated with RAPD type; ornibactins being the most common (Darling *et al* 1998).

Antibiotic resistance

B. cepacia is intrinsically resistant to most antibiotics due to a highly impermeable and selective outer membrane. It is resistant to β-lactam antibiotics and can even utilise penicillin G as a carbon source. It is also constitutively resistant to aminoglycosides and other agents commonly used in the treatment of gram-negative infections such as polymyxin B. Susceptibility to carbapenems is variable (Lu *et al* 1997). Another problem is the difficulty in estimating the level of antibiotic that will be attained in bronchial secretions (Pitt *et al* 1996). Desai *et al* (1998) showed that resistance of *B. cepacia* to antibiotics such as ceftazidime and ciprofloxacin increased just before the cells entered the stationary phase of growth. They also showed that biofilm or biomass grown *B. cepacia* (as may occur in the CF lung), are less susceptible to antibiotics than planktonic cells. The susceptibility to an antibiotic may also decrease during therapy and this is shown by the appearance of different antimicrobial sensitivity patterns in isolates of the same strain from the same patient (Pitt *et al* 1996). However, some success in treatment of CF *B. cepacia* infections has been demonstrated. The carbapenem meropenem, has been shown to be effective against many multiresistant *B. cepacia* isolates (Ciofu *et al* 1996). Synergistic effects

can be demonstrated using antibiotics such as ceftazidime with amikacin or ciprofloxacin (Lu *et al* 1997) and some non-antimicrobial agents e.g. chlorpromazine and prochlorperazine may enhance the action of antibiotics such as gentamicin and ceftazidime against the species (Rajyaguru and Muszynski 1998).

1.4. Aim of study

The first part of this study will concentrate on comparing traditional phenotypic methods of identifying *B. cepacia* with present day genotypic methods. Acid production in ammonium salt sugars, plate enzyme assays, rapid ID kits and PCR for target genes within the 16S and 23S rRNA operon of *B. cepacia,* will all be evaluated for specificity and sensitivity. Furthermore, the efficiency of PCR assays in detecting *B. cepacia* in primary sputum specimens will be assessed and compared with conventional culture methods on selective and non-selective media.

The presence of *B. cepacia* in the natural environment will be determined by culturing environmental samples on selective media and confirming the identity of any isolates by the above methods.

The distribution of putative epidemic markers in *B. cepacia* isolates and their correlation with genomovar group (determined by the above plate and tube assays) and RAPD fingerprint will be investigated.

Finally, the distribution of various virulence factors in *B. cepacia* will be monitored. These will include LPS constitution and serum resistance, and production of various enzymes specifically protease, lipase, lecithinase, gelatinase, haemolysin and onion-macerating enzymes. The presence or absence of these will also be correlated with genomovar group.

2. Methods

2.1. Bacterial isolates

The type strains of *B. cepacia, B. gladioli* and *P. aeruginosa* were obtained from the National Collection of Type Cultures (NCTC) and the American Type Culture Collection (ATCC). Other *Burkholderia* and *Ralstonia* species were provided by the LMG Culture Collection, Belgium and the National Collection of Plant Pathogenic Bacteria (NCPPB) UK. Isolates of other gram-negative species were selected from collections kept in the Laboratory of Hospital Infection (LHI). Clinical isolates of *B. cepacia* from CF patients included those sent to LHI for identification or typing. Some isolates from the ribotyping study of Pitt *et al* (1996) were also included. Environmental isolates of *B. cepacia* and some representatives of the UK epidemic strain including CF 5610 from the index case, were provided by Prof JRW Govan, University of Edinburgh Medical School, Scotland. An isolate of *Clostridium perfringens* was provided by Dr. Moira Brett of The Food Safety Microbiology Laboratory (FSML) at CPHL.

2.2. Sputum samples

Sputum samples from *B. cepacia*- positive CF patients were kindly provided by the Royal Brompton Hospital, Sydney Street, London, UK.

2.3. Identification

2.3.1. Biochemical identification and enzyme production

All media were prepared in the Media Department at CPHL except where stated. Isolates were tested in the API 20NE gallery (Biomerieux, Basingstoke, UK) and

examined for gram-stain reaction, motility, production of catalase, oxidase, nitrate reductase and growth on MacConkey agar, *B. cepacia* selective media (Mast, Bootle, UK), cetrimide and poly-hydroxybutyrate agars.

Overnight cultures of *B. cepacia* on nutrient agar (NA) 30 ^{0}C were suspended in sterile distilled water to give a light suspension of 0.5 McFarland equivalents and 20 µl drops were spotted onto substrate plates or agar slopes in tubes. These were incubated at 30 ^{0}C and the results (as determined by the changes described below) were read after 5 days. API 20NE strips were inoculated as per manufacturer's instructions and read after incubation at 30 ^{0}C for 48 h.

Casein agar plates were prepared at a concentration of 2.5% skim milk (w/v). A positive reaction was indicated by a zone of clearing in the opaque medium around the bacterial growth.

Tyrosine plates were prepared at a concentration of 0.5% (w/v). Tyrosine hydrolysis was indicated by clearing in the agar around the colonies. Some *B. cepacia* isolates also produced a brown melanin-like pigment on this medium which was recorded.

Gelatin plates were prepared at a concentration of 0.4% (w/v). Gelatin hydrolysis was detected by clearing around the growth after flooding the agar surface with 30% trichloracetic acid.

Egg yolk emulsion plates were prepared at a concentration of 10% (v/v). Lecithinase production was indicated by a zone of turbidity developing in the medium around and under the bacterial growth.

Tween 20 and 80 were added to molten nutrient agar at a concentration of 1% (v/v). Lipase production was indicated by a precipitate around the bacterial colonies.

Blood plates were prepared in LHI as described in section 2.7.2.

Enzyme production detected on the above media contributed to identifying and genomovar grouping *B.cepacia* and for a comparison with the production of extracellular virulence factors in section 3.5.1.

Ammonium salt sugars were prepared as agar slopes with the appropriate carbohydrate added at a concentration of 1% (v/v). A positive reaction due to acid production was indicated by a colour change from green to yellow.

Hugh and Leifson's agar media in tubes were prepared with glucose at a concentration of 1% (v/v). Isolates of *B. cepacia* were inoculated by stabbing to the bottom of the tube using a thin loop. Typical, oxidative *B. cepacia* turn the upper part of the medium yellow, while assacharolytic variants form a blue colour.

2.3.2. PCR identification

Primer sets

Oligonucleotide primer pairs for the amplification of specific regions of *B. cepacia* 16S and 23S rRNA and the internal transcribed spacer ITS region of *B. gladioli*, were synthesised by Cruachem Ltd, Glasgow, UK. The sequences are shown in Table 4.

Table 4
Primer sets used for confirmation of *B. cepacia* and *B. gladioli*

Primer	Ref	Sequence	Target	Amplicon size (bp)	Reference
P1	PC480 PC1250	5'GGTACCGGAAGAATAAGC3' 5'CTGTTCCGACCATTGTAT3'	16S rRNA *B. cepacia*	770	O'Callaghan *et al* (1994)
P2	PC1 PC2	5'GCTGC GGATG CGTGC TTTGC 3' 5'GCCTT CTCCA ATGCA GCGAC 3'	23S rRNA *B. cepacia*	323	Tyler *et al* (1995)
P3	PSR1 PSL1	5' TTTCG AGCAC TCCCG CCTCT CAG 3' 5'AACTA GTTGT TGGGG ATTCA TTTC 3'	16S rRNA *B. cepacia*	209	Campbell *et al* (1995)
P4	PG1 PG2	5' TTCAA TGACA AACGT TCGGG 3' 5' GCTTT CGCTT GACAG GCC 3'	ITS rRNA *B. gladioli*	274	Tyler *et al* (1995)

DNA extraction

Crude bacterial DNA was prepared by emulsifying five colonies of 48 h growth from NA in 100 µl of sterile tissue culture water (Sigma, Dorset, UK). Cell walls were disrupted in a Dri-block (Techne, UK) at 100 ^{0}C for 5 min., vortexed and centrifuged (IEC Micromax) at 13,000 *g* for 5 min. For the PCR, 3 µl of the supernatant was added to 12 µl of water; a water blank (15 µl) was also included.

PCR

PCR reagents were added to the samples prepared above to give a final volume of 25 µl. The cocktail included 100 pmol of each of a primer pair, 50 pmol of $MgCl_2$, 2.5 pmol of each of the deoxynucleotide triphosphates, 1.25 U of *Taq* DNA Polymerase and 2.5 µl of 10x PCR buffer (Life Technologies, Paisley, UK). For a multiplex PCR, each extra primer added replaced 1 µl of the water.

Amplification was carried out in a Thermal cycler (Techne) for 25 cycles. DNA was denatured at 96 ^{0}C for 5 min for 1 cycle, and at 96 ^{0}C for 15 s for cycles 2 to 25. The optimum annealing conditions were determined between temperatures of 58 ^{0}C and

63 ^{0}C for 30 s and primer extension was at 72 ^{0}C for 90 s. An additional primer extension was carried out at 70 ^{0}C for 5 min.

Detection of products

A 1.5% (w/v) Nusieve agarose gel (Flowgen, Sittingbourne, UK) was prepared in 60 ml of 0.5% 10 x TBE buffer (Life Technologies). The PCR products were separated at 100 V for 1.5 h and molecular weights were determined by comparison with a 123 bp ladder (Life Technologies) and the positive control NCTC 10661.

2.4. Detection of B. cepacia in sputum

2.4.1. Sputum preparation

Sputum was treated according to the method of Ochert *et al* (1994). Sputum samples were treated with 2% N-acetyl-L-cysteine (w/v) (BDH, Poole, UK) in N-acetyl-L-cysteine buffer (Woodhams and Mead 1965) in a ratio of 1:1.and incubated at room temperature until the viscosity was reduced. The samples were then inoculated onto *B. cepacia* selective media and NA and incubated at 30 ^{0}C. Each sample was centrifuged at 13000 *g* for 20 min in a microfuge and the deposit resuspended in cold phosphate buffered saline (PBS). This was repeated twice and the deposit resuspended in five times its volume of sterile tissue culture water. After mixing thoroughly, an equal volume of 25% chelex (Bio-Rad Laboratories Ltd, UK) (w/v) in sterile tissue culture water was added. This was vortexed for 10 s and incubated in a 56 ^{0}C waterbath for 30 min, vortexed again and heated in a 95 ^{0}C waterbath for 8 min. Finally, the sample was cooled on ice, vortexed and centrifuged at 13000 *g* for 10 min.

2.4.2. Detection of *B. cepacia*

For PCR detection of *B. cepacia* in sputum, 3 µl of the supernatant was added to 12 µl of sterile tissue culture water with a water blank of similar volume. The procedures were then followed as in section 2.3.2.

2.5. Isolation of B. cepacia from natural environments

2.5.1. Sampling

Two environmental complexes were chosen. One was the botanical gardens at Kew, and the other was a reservoir in North London. Various sites in these two locations were sampled. Sterile swabs were used to collect samples and these were immediately placed into 10 ml aliquots of Malka minimal broth (Appendix 1) supplemented with 300 U of colistin per ml.

2.5.2. Isolation and identification

The Malka broths were incubated for 5 days at 30 ^{0}C and a loopful plated onto *B. cepacia* selective media. The plates were incubated for two days at 30 ^{0}C. Any colonies growing on the selective medium were tested for oxidase production and for oxidation in O/F medium. Any isolates which were positive for one or both tests were confirmed as *B. cepacia* by PCR as in section 2.3.2.

2.6. *Epidemicity factors*

2.6.1. PCR for the detection of epidemic markers

Control isolates

Representative isolates from outbreaks in the UK, including the UK epidemic strain, were selected. NCTC 10661 was included as a negative control as were the *B. gladioli* strains ATCC 25417 and NCTC 12378.

Primers

Oligonucleotide primer pairs (Cruachem Ltd) for the detection of the genes encoding cable pili, the insertion sequence hybrid and the *B. cepacia* epidemic strain marker are shown in Table 5.

Table 5
Primer sets for the detection of epidemic markers in *B. cepacia*

Ref	Sequence	Target	Amplicon (bp)	Reference
CBL1 CBL2	5' CCAAAGGACTAACCCA 3' 5' ACGCGATGTCCATCACA 3'	*CblA*	664	Sajjan *et al* (1995)
IS402A IS1356B	5'CAACCGAGACTGAGGAGATG 3' 5' TCCGGCGACACCTCGATGCC 3'	IS hybrid	592	Tyler *et al* (1996)
BCESM1 BCESM2	5' CCACGGACGTGACTAACA 3' 5' CGTCCATCCGAACACGAT 3'	Epidemic marker	1.400	Mahenthir-alingham *et al* (1996)

PCR

DNA extraction, PCR reagents and detection of PCR products were as described in preceding sections.

2.6.2. Random Amplified Polymorphic DNA typing

Preparation of DNA

Bacterial DNA for RAPD typing was prepared by the guanidium thiocyanate-EDTA-sarkosyl (GES) extraction method of Pitcher *et al* (1989). DNA was diluted 1 in 100 in distilled water and quantified in a Ultrospec III Spectrophotometer (Pharmacia) using the formula below.

$Absorbance_{260}$ x 5.0 = μg/μl DNA

Samples were diluted to give DNA concentrations of 50 ng in 15 μl of sterile tissue culture water for use in the PCR.

RAPD PCR

The sequence of the RAPD primer P272 was 5' AGCGGGCCAA 3' (Mahenthiralingam *et al* 1997). PCR was carried out in a 25 μl volume. This contained 25 pmol of primer 272, 50 pmol of $MgCl_2$, 2.5 pmol of each of the deoxynucleotide triphosphates, 1.25 U of Taq DNA Polymerase and 2.5 ul of 10 x PCR buffer (Life Technologies). Amplification was carried out in a TouchDown Thermal cycler (Techne) for 45 cycles. DNA was denatured at 95 ^{0}C for 5 min for 1 cycle, and 95 ^{0}C for 1 min, 36 ^{0}C for 5 min, and 72 ^{0}C for 2 min for cycles 2 to 45. PCR products were detected as in preceding sections.

2.7. *Putative virulence factors of B. cepacia*

2.7.1. Extracellular virulence factors

Lecithinase production in broth culture

Broth cultures of *B. cepacia* and *Clostridium perfringens* were grown in tryptone soy broth (TSB) and peptone water broth (PWB) at 30 ^{0}C for 48 h and Robertson's cooked meat (RCM) broth at 37 ^{0}C for 48 h respectively. The bacteria were deposited by centrifugation and doubling dilutions of 0.5 ml of the supernatant were prepared in 0.05 M Hepes buffer (Sigma). Egg yolk substrate was prepared by adding egg yolk emulsion (Oxoid) to 0.05 M Hepes buffer in a ratio of 1:5. This was clarified with 20% NaCl (w/v) (Sigma) and filtered through a 0.45 μm membrane filter (Sartorius) under vacuum; 0.5 ml of substrate was added to each dilution of supernatant and incubated at 37 ^{0}C for 2 h. Lecithinase production was evident by the appearance of turbidity in the clarified egg yolk. The highest dilution of supernatant showing turbidity was taken as the titre of lecithinase activity.

Phospholipase C production

This was based on a modification of the method of Berka *et al* (1981) where phospholipase C hydrolyses *p*-nitrophenylphosphorylcholine (NPPC) with the production of the yellow chromogen *p*-nitrophenol. Agar grown isolates of *B. cepacia* were incubated for 48 h at 30 ^{0}C in TSB. The tubes were vortexed briefly (Vibro Gene, Stuart Scientific, UK) and the absorbances of 1 ml samples read at 540 nm in a Pharmacia LKB Ultrospec III spectrophotometer. The cells were deposited by centrifugation and 100 μl of supernatant was added to 900 μl of NPPC reagent

(Sigma) and incubated at 37 ^{0}C for 1 h. The absorbance at 405 nm was recorded and phospholipase C activity was calculated as follows.

Phospholipase C = Absorbance at 405 nm ÷ Absorbance at 540 nm

Protease production in broth culture

Protease production was measured by a modification of the method of Kreger and Gray (1978).

Preparation of standard curve

Trypsin (Sigma) concentrations of 5,10,15, 20 and 25 μg/ml were prepared in duplicate in 0.5 ml aliquots of protease assay buffer (0.2 M Tris (hydroxymethyl) aminomethane). To each of these was added 0.5 ml of 5 mg/ml azocasein suspension (Sigma). After incubation at 37 ^{0}C for 45 min, the enzyme action was stopped by the addition of 3.0 ml of cold 5% (w/v) trichloroacetic acid (TCA). Undigested azocasein was removed by centrifugation at 3000 *g* for 10 min and 2 ml of the supernatant was neutralised with an equal volume of 0.5 M NaOH and the absorbancy read at 420 nm. A standard curve was plotted as trypsin concentration against increasing absorbancy due to release of azo dye from the casein.

Protease production assay

B. cepacia were grown for 48 h at 30 ^{0}C and 37 ^{0}C in TSB and PWB. The cells were deposited by centrifugation at 3000 *g* for 20 min and 0.5 ml of the supernatant was mixed with 0.5 ml of azocasein (5 mg/ml). The same procedure as above was

followed and the absorbance at 420 nm was used to estimate the concentration of protease in the supernatant.

2.7.2. Haemolytic activity and serum sensitivity

Haemolysin production

Plate assay. Isolates were tested for their ability to lyse red blood cells from a variety of species. Venous blood samples were obtained from sheep, horse, rabbit, fowl and human blood group "O". Two ml volumes in 10 mm diameter tubes, were centrifuged at 3000 *g* for 20 min and the packed cell volume was estimated against volumes of water. The remainder of the blood was centrifuged as above and the cell deposit was washed thrice in phosphate buffered saline (PBS). Packed cells were added to peptone water agar at 56 ^{0}C to a concentration of 5% (v/v) except for the horse red cells which were used at 7% (v/v). 10 ml volumes of this agar were dispensed into 9 cm petri dishes and set plates were dried for 15 min at 37 ^{0}C. Plates were inoculated with a suspension of bacteria at a concentration of 0.5 McFarland units in sterile distilled water and incubated in a moist atmosphere in a plastic box at 30 ^{0}C and examined daily for 5 days for zones of haemolysis.

Broth culture. Red blood cells were prepared as above and resuspended to a concentration of 1%. Isolates were grown for 48 h at 30 ^{0}C and 37 ^{0}C in TSB as a rich growth medium and PWB as a poor growth medium. The broth cultures were centrifuged at 3000 *g* for 20 min and doubling dilutions of the supernatants were made in PBS. 50 µl aliquots of these dilutions were mixed with an equal volume of 1% red cell suspension in a microtitre plate. A control well of PBS and red cells served as a negative control and distilled water and red cells as a positive control. Two sets of microtitre plates were prepared and incubated at 37 ^{0}C and 4 ^{0}C for 2 h and examined

for haemolysis in comparison with controls. The titre was taken as the highest dilution giving haemolysis equivalent to the positive control.

Serum sensitivity

Blood samples were collected from three healthy volunteers. The red cells were centrifuged (Denley BS 400) at 3000 *g* for 20 min and the serum pooled, aliquotted, stored at –20 ^{0}C and used within a month.

Isolates of *B. cepacia* were grown on NA plates for 48 h at 30 ^{0}C and 2-5 colonies were inoculated into TSB and incubated at 30 ^{0}C for a further 48 h. The broth culture was diluted 1 in 10 (10 µl and 90 µl) with Hank's buffered salt solution (HBSS) (Life Technologies). This was further diluted in logarithmic steps to give 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions and 20 µl of each were spotted onto NA plates.
50 µl of each dilution was mixed with 50 µl of pooled normal human serum (NHS) in the wells of a microtitre plate. Control wells of 50 µl of culture dilution and 50 µl of HBSS were also prepared. The wells were sealed with a plastic cover and the plate was shaken briefly on an IKA Schuttler MTS 4 and incubated at 37 ^{0}C. After 3 h, 20 µl samples from each well were spotted onto NA plates and the microtitre plate reincubated. This was repeated after 6 h and the plate discarded. All NA plates were incubated at 30 ^{0}C for 48 h and the growth was recorded as confluent (CL), semi-confluent (SCL), ++, + or +/-. Where there were less than 10 colonies, these were counted.

Preparation of LPS extracts

A modification of the method of Hitchcock and Brown (1983) was used to prepare LPS extracts. Isolates of *B. cepacia* were grown in 3 ml of TSB and incubated at 30 ^{0}C for 48 h. The cells were deposited by centrifugation (MSE Coolspin 2) at 3000 *g* for 15 min. The supernatant was discarded and the cells were resuspended in 100 µl of 2 x sample buffer by vortexing. (Appendix 2). The suspension was boiled in a water bath for 10 min and 100 µl of distilled water was added to each tube and the entire contents transferred to a 1.5 ml eppendorf tube containing 40 µl of (0.25% w/v) protease K (Sigma) stock solution (Appendix 2) and incubated in a waterbath at 60 ^{0}C for 2 h. The extracts were then stored at –20 ^{0}C.

Blocking of serum killing by incubation with LPS extracts

Serum samples (from serum sensitive isolates) and broth cultures were prepared as above. The broth cultures were diluted to 10^{-2} and 50 µl of this dilution was mixed with 50 µl of NHS in microtitre plate wells and control wells of broth culture and HBSS were also prepared. A further 50 µl of NHS was mixed with an equal volume of LPS extract and incubated at 37 ^{0}C. The order and contents of the wells were as follows.

A: HBSS + broth culture
B: NHS + broth culture
C: NHS + LPS extract
D: HBSS + NHS
E HBSS + LPS extract

After incubation at 37 ^{0}C for 3 h, 20 µl samples from rows A and B were spotted onto NA and incubated at 30 ^{0}C for 48 h. 50 µl of a 10^{-2} dilution of the isolate under test

was added to rows C, D and E and re-incubated for a further 3 h. After incubation, wells C to E were spotted onto NA and incubated at 30 ^{0}C for 48 h.

SDS-PAGE of LPS extracts

SDS-PAGE was carried out according to Laemmli's method (1970). Two mini-gels (Appendix 3) were prepared in side-arm flasks. The resolving gel was prepared first and degassed for 15 min. The gel plates were cleaned and assembled with a gasket between them as a sealant and Temed (Sigma) was added with gentle mixing. The solution was pipetted into the gel mould to a level 1 cm below the comb edge and covered with water to exclude air. The stacking gel was prepared similarly and pipetted into the gel mould after the resolving gel had set and the water had been removed. The comb was immediately put in place taking care to exclude air bubbles.

The gel was left to set at room temperature for 1 h, the comb and sealant was removed and the gel was placed in an electrophoresis tank (Bio-Rad) containing the reservoir buffer. Care was taken to remove bubbles trapped under the gel. Proteinase K treated LPS extracts stored at –20 ^{0}C were defrosted and samples were loaded into the wells with a Hamilton syringe (Sigma) using a range of volumes to determine the optimum amount of LPS. The gels were run at 50 mA (109 V) for approximately 2 h.

Detection of bands

After electrophoresis, the gels were placed in a fixing solution (Appendix 3) with 0.7% periodic acid and shaken at room temperature for 5 min. They were rinsed twice with distilled water followed by washing 6 times in distilled water with 5 min shaking between each wash. The silver stain (Appendix 3) was prepared by adding ammonium

hydroxide to sodium hydroxide and then gradually adding silver nitrate solution with gentle mixing followed by the water. After the final wash, the gel was placed in the stain and shaken gently for 10 min. The gels were rinsed twice with distilled water and washed 6 times as above and the developing solution (Appendix 3) was added. The gels were gently shaken until bands appeared when the developing solution was replaced with distilled water. The gels were rinsed thoroughly before photographing.

2.7.3. Onion maceration assay

Representatives of clinical and environmental isolates of *B. cepacia* were compared for their ability to digest onion tissue (Wigley and Burton 1999). Large onions were cut horizontally into slices of approximately 5 mm in thickness and placed into 9 cm plastic petri dishes. Nicks of approximately 2 mm in depth were made in the inner region with a sterile scalpel blade and 100 µl of 48 h TSB cultures of *B. cepacia* were applied. Controls of *P. aeruginosa* and sterile saline were also included. The tests were incubated in a sealed plastic container with moistened tissue paper at 30 ^{0}C for 48 h and onion slices were examined for colour and texture changes compared with the saline control. The surface of the onion was also swabbed and inoculated onto selective medium to determine the survival of the isolate.

3. Results

3.1. Identification of Burkholderia cepacia

3.1.1. Biochemical identification of *B. cepacia* and enzyme production

All isolates were confirmed as *B. cepacia* by their reactions in API 20NE, gram-stain, production of catalase and oxidase and growth on *B. cepacia* selective medium, cetrimide and polyhydroxybutyrate agars, acid production in ammonium salt sugars. growth at 42^0C the Hugh and Leifson's O/F test, hydrolysis of casein, tyrosine, gelatin, tween 20 and 80 and egg yolk agar substrate. The results for the reference strains and the percentage positives for 122 CF and three environmental isolates are shown in Table 6 and illustrated in Figures 1 to 4.

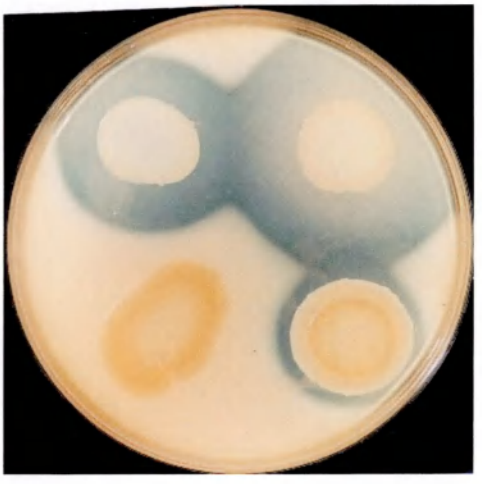


Figure 1 Casein medium showing strong, medium, weak and no proteolysis.

Figure 2 Tyrosine medium showing lysis by 3 of 4 isolates and production of a melanin-type pigment by one isolate.

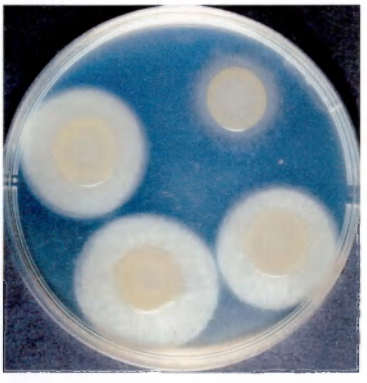


Figure 3 Tween 80 medium showing strong hydrolysis in three isolates and weak hydrolysis in one isolate.

Figure 4 Lecithin medium showing strong production of phospholipase in 3 isolates and weak production in one isolate.

Table 6
Reactions of reference strains of *B. cepacia* in biochemical tests and percentage positives of test isolates for each character.

Test	Reference strains					% positive of test isolates
	ATCC 17765	ATCC 17759	ATCC 25416	ATCC 17616	NCTC 10661	
Growth at 42^{0}C	+	-	+/-	+	+	88
Hugh & Leifson O/F	O	O	O	O	O	74
Casein	+	+	+	-	+	24
Gelatin	-	-	-	-	+	4
Egg yolk	+	+	+	+	+	54
Tween 20 hydrolysis	+	+	+	+	+	97
Tween 80 hydrolysis	+	+	+	+	+	94
Tyrosine hydrolysis	+	+	+	+	-	98
Tyrosine pigment	-	-	-	-	-	12
Adonitol	+	+	+	+	+	72
Acid production in:						
Arabinose	+	+	+	+	+	78
Cellobiose	+	+	+	+	+	74
Dulcitol	+	+	+	+	+	75
Fructose	+	-	+	+	+	77
Glucose	+	+	+	+	+	78
Inositol	+	+	+	+	+	74
Lactose	+	+	+	+	+	77
Maltose	+	+	+	+	+	77
Mannitol	+	+	+	+	+	69
Salicin	+	+	-/+	-/+	+	38
Sorbitol	+	+	+	+	+	76
Sucrose	+	+	+	+	+	27

3.1.2. PCR Identification

The primer pair Pc 480 and Pc 1250 was found to be sensitive for the detection of *B. cepacia* over a range of annealing temperatures but also produced bands with *B. gladioli* and *Achromobacter* species. This was not affected by raising the annealing temperature and at temperatures above 60 ^{0}C, the clarity of all bands was reduced. This primer pair was therefore considered to be unsuitable for further evaluation.

The reactions of *Burkholderia, Ralstonia* and *P. aeruginosa* strains with the three sets of primers are shown in Table 7 and illustrated in Figure 5 as a multiplex PCR.

Table 7
Reactions of reference strains of *Burkholderia* and *Ralstonia* with primer sets

Species	Strain no.	P2	P3	P4
B. cepacia	NCTC 10661	+	+	-
B. cepacia	NCTC 10744	+	+	-
B. cepacia	ATCC 29424	+	+	-
B. cepacia	ATCC 25608	+	+	-
B. cepacia	ATCC 27515	+	+	-
B. cepacia	ATCC 17460	+	+	-
B. cepacia	ATCC 25610	+	+	-
B. cepacia	ATCC 17765	+	+	-
B. gladioli	NCTC 12378	-	-	+
B. gladioli	ATCC 25417	-	-	+
B. gladioli	ATCC 10247	-	-	-
B. gladioli	ATCC 10248	-	-	+
B. glumae	LMG 1277	-	+	-
R. plantarii	LMG 10908	-	-	-
B. vietnamiensis	LMG 6998	+	-	-
B. vandii	LMG 16020	+	+	-
R. solanacearum	LMG 2295	+	-	-
B. andropogonis	LMG 2126	-	-	-
B. caryophylli	LMG 2155	-	+	-
B. cocovenenans	LMG 11626	+	-	+
R pickettii	NCTC 11149	-	-	-
Ps .aeruginosa	NCTC 10332	-	-	-

P2: 23S rRNA for *B. cepacia*

P3: 16S rRNA for *B. cepacia*

P4: ITS for *B. gladioli*

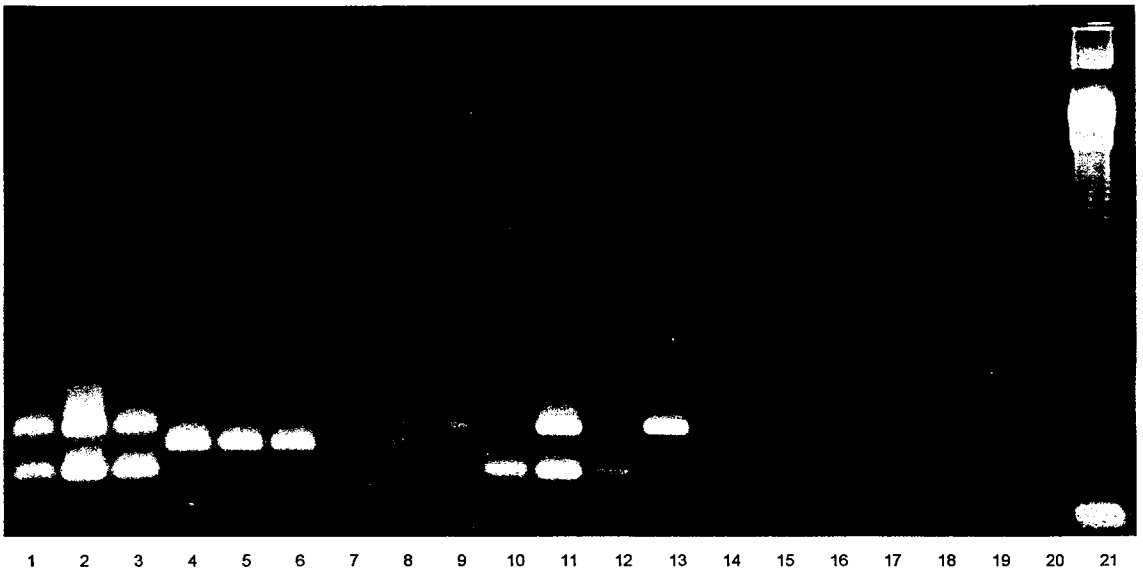


Figure 5 Multiplex PCR with three primers (see Table 7) and reference strains of *Burkholderia* spp and other species. Lane 1: *B. cepacia* NCTC 10661; lane 2: *B.cepacia* NCTC 10774; lane 3: *B. cepacia* ATCC 25417; lane 4: *B. gladioli* NCTC 12378; lane 5: *B. gladioli* ATCC 10248; lane 6: *B. gladioli* ATCC 25417; lane 7: *R. pickettii* NCTC 11149; lane 8: *B. cocovenenans* LMG 11626; lane 9: *R. solanacearum* LMG 2295; lane 10: *B. caryophylli* LMG 2155; lane 11: *B. vandii* LMG 16020; lane 12: *B. glumae* LMG 1277; lane 13: *B. vietnamiensis* LMG 6998; lane 14: *B. plantarii* LMG 10908; lane 15: *B. andropogonis* LMG 2126; lane 16: *Ps aeruginosa* NCTC 10332; lane 17: *Ps aeruginosa* NCTC 10662; lane 18: *A. baumannii*; lane 19: *S. maltophilia*; lane 20: water blank; lane 21: 123 bp size markers.

The optimal annealing temperature for each of the primers was 63 ^{0}C and all *B. cepacia* reference strains produced amplicons with both sets of homologous primers but not with the *B. gladioli* primers and vice versa. However, one reference strain of *B. gladioli* failed to react with primer P4. Four other *Burkholderia* spp. and the *R. solanacearum* reference strain were positive in the PCR with either primer P2 or P3 but only *B. vandii* reacted with both primers. *B. andropogonis, R. plantarii* and *R. pickettii* were negative with each of the three primer sets.

Over 18 months, 177 isolates were submitted for species confirmation by clinical diagnostic laboratories as presumed *B. cepacia* from CF patients and most isolates grew on the selective medium. Table 8 summarises the results of the PCR with the three primer pairs.

Table 8
Reactions of presumptively identified '*B. cepacia*' with primer sets

Biochemical Identification	Number	P2	P3	P4	No reaction
Typical *B. cepacia*	78	75	75	3	0
Asaccharolytic *B. cepacia*	15	15	15	0	0
"*B. cepacia*"	5	0	0	0	5
B. gladioli	11	10	10	0	1
P. aeruginosa	28	0	0	0	28
Other glucose nonfermenters	24	0	0	0	24
Glucose fermenters	3	0	0	0	3
Not identified	13	0	0	0	13
Total	**177**	**100**	**100**	**3**	**74**

100 isolates reacted with primers P2 and P3. Of these, 75 were typical of *B. cepacia* in biochemical tests, 15 were asaccharolytic and 10 gave biochemical reactions most consistent with an identification of *B. gladioli*. None of the latter isolates formed products with primer P4. However, P4 reacted with three biochemically typical *B. cepacia* isolates. Five clinical isolates which were negative in the PCR, grew on the selective medium and were classified by colonial morphology, pigment and other phenotypic tests as closest to *'B. cepacia'* although the API 20NE classified them as 'doubtful profile' for the species. The 24 other glucose non-fermenters included alkaline forming pseudomonads and *S. maltophilia*; 28 strains proved to be *P. aeruginosa* and three others fermented glucose. None of these reacted in the PCR. Ten

environmental isolates of *B. cepacia* from the Edinburgh Botanical Garden were confirmed by PCR as was the UK epidemic strain.

3.2. Detection of B. cepacia in sputum

The results of PCR detection of *B. cepacia* in sputum and the correlation with isolation of the organism, are shown in Table 9. Figure 6 illustrates the PCR products after gel electrophoresis.

Table 9
Detection of *B. cepacia* in sputum and its isolation and identification

	Growth from sputa on medium		No. sputa PCR positive with primers		No. PCR positive isolates with primers		No. isolates biochemically identified		
No. sputa	PC	NA	P2	P3	P2	P3	B. cepacia	Others on PC	Others on NA
35	16	29	20	32	15	15	15	1	11

1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 6 PCR on CF sputum isolates using *B. cepacia* specific 16S and 23S rRNA primers. Lane 1: 123 bp ladder; lanes 2 to 10: sputum samples from presumptive *B. cepacia* positive CF patients; lane 11: *B. cepacia* NCTC 10661; lane 12 *B. gladioli* NCTC 12378; lane 13 water blank.

The 16S rRNA primer pair P3 generally gave stronger reactions than the 23S rRNA primer pair P2 which in some cases failed to produce a band. This is also illustrated in Figure 6. Thirty two out of the 35 sputum samples were positive with the 16S rRNA primer pair. Of the three negative samples, one failed to yield bacterial growth on either medium, the second grew gram-negative rods on NA and PC agar which were

identified by API 20NE as *Bordetella bronchiseptica* and the third grew *P. aeruginosa* on NA.

B. cepacia could not be isolated from 16 PCR-positive sputa. This may have been due to the organism being non-viable or that the cell numbers were too low to be detectable by culture. However, *B. cepacia* was cultured from the 16 remaining sputum samples on NA and PC agar. Other organisms especially staphylococci were also grown from some of the sputum samples.

3.3. Isolation of B. cepacia from natural environments

Tables 10 and 11 show the results of growth following enrichment on PC agar from samples taken from two environmental sites.

Table 10
Isolation of organisms from botanical garden

Area	Growth on PC agar	H&L	Oxidase
Temperate soil	-		
Temperate soil	+	Alkaline	+
Humid soil	+	Alkaline	+
Tropical orchids	-		
Tropical moss	-		
Temperate orchids	-		
Temperate moss	-		
Soil in moist tropics	-		
Soil in moist tropics	+	Alkaline	+
Pond in moist tropics	+	Alkaline	+
Soil in dry tropics	-		
Soil in dry tropics	-		
Soil in dry tropics	+	Alkaline	+
Soil in wet tropics	-		
Pond in wet tropics	+	No reaction	-
Carniverous plants	-		
Humidfier spray in wet tropics	-		
Soil in wet tropics	-		
Tropical ferns	-		
Soil in temperate zone	+	No reaction	-

Table 11
Isolation of organisms from reservoir

Area	Growth on PC agar	H&L	Oxidase
Soft mud	-		
Water next to boating club	-		
Sediment from above	-		
Water under bridge	-		
Sediment from above	-		
Tree bark scrapings	-		
Playing field soil	-		
Water next to playing field	-		
Sediment from above	-		
Water next to jetty	-		
Sediment from above	-		

No *B. cepacia* was isolated from either environmental complex. The alkaline producers from the botanical garden were probably *Alcaligenes* species.

3.4. PCR for the detection of epidemic markers and RAPD typing

Control isolates

Results of the reactions of the control isolates with the primers for the detection of epidemic markers and their correlation with RAPD profile, are shown in Table 12 and illustrated in Figure 7.

Table 12
Comparison of RAPD profiles of *B. cepacia* and related species and their reactions with 3 primers for the detection of epidemic markers

		PCR reactions		
Strain number	RAPD	*CblA*	IS Hybrid	BCESM
*CF 5610	Epidemic	+	+	+
C 1394	Nonepidemic	-	-	+
ID 1091 [a]	Epidemic	+	+	+
ID 1092 [a]	Epidemic	+	+	+
ID 1118	Nonepidemic	-	-	+
ID 5318	Epidemic	+	+	+
ID 5566	Epidemic	+	+	+
ID 1120	Nonepidemic	-	-	+
ID 1100	Epidemic	+	+	+
ID 5314	Nonepidemic	-	-	-
CF 1382	Epidemic	+	+	+
ID 5306	Epidemic	+	+	+
ID 1122	Nonepidemic	-	-	+
ID 5308	Epidemic	+	+	+
ID 5309	Epidemic	+	+	+
NCTC 10661[b]	Nonepidemic	-	-	-
ATCC 25417[c]	Nonepidemic	-	-	-
NCTC 12378[c]	Nonepidemic	-	-	-

* Control UK *B. cepacia* epidemic strain

[a] = Asaccharolytic strains

[b] = *B. cepacia*

[c] = *B. gladioli*

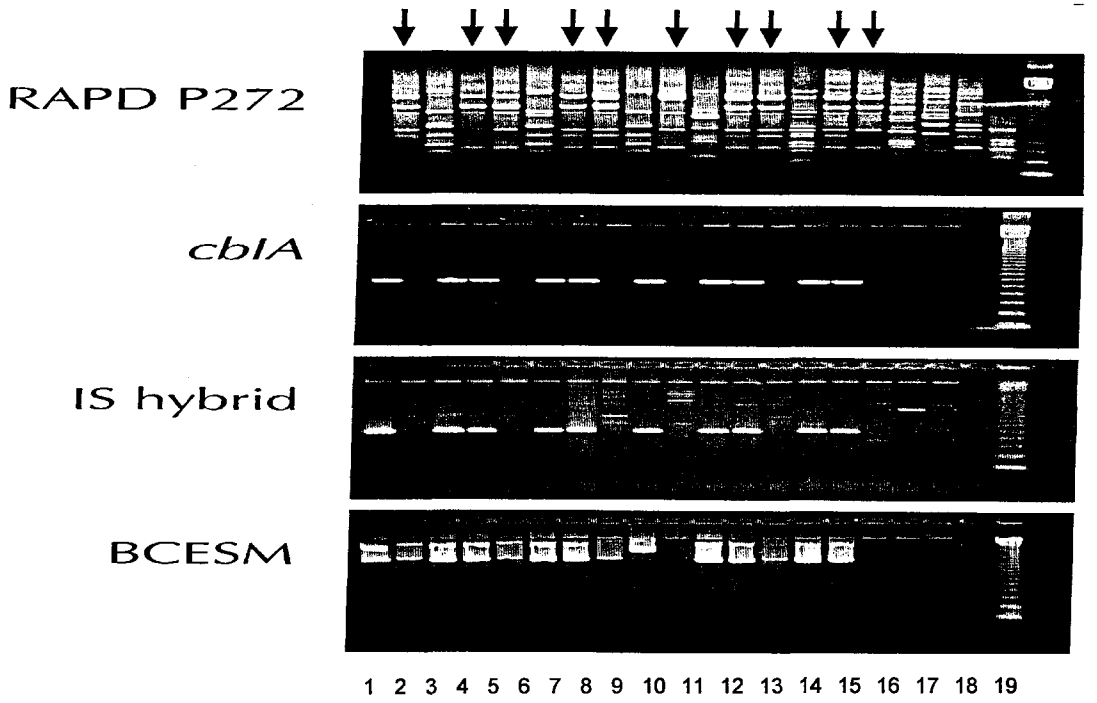


Figure 7 CF isolates of *B.cepacia* showing RAPD fingerprinting and corresponding PCR detection of the *cblA* gene and IS hybrid and BCESM genes. Arrows indicate isolates giving RAPD profiles indistinguishable from the UK epidemic strain. Lane 19 is a waterblank

The optimum annealing temperature for primer pair CBLI and CBL2 was 58 ^{0}C and for primer pairs IS402A and IS1356B and BCESM1 and BCESM2 it was 63 ^{0}C. The UK epidemic strain from the index case CF 5610 and all the isolates which were indistinguishable from this strain, were positive for all three epidemic markers but NCTC 10661 and the two *B. gladioli* reference strains were negative for all three. The other isolates from outbreaks in the UK were negative for *cblA* and the IS hybrid but all except for one were positive for BCESM.

The results of RAPD fingerprinting and presence of epidemic markers for 116 CF isolates are shown in Table 13.

Table 13 Numbers of epidemic and non-epidemic isolates of *B. cepacia* and presence of epidemic markers

Isolates	RAPD	CBLA present	IS hybrid present	BCESM present
UK epidemic	39	39	37	39
Non-epidemic	77*	0	0	11

* Includes four small clusters of cross-infection involving two to three patients.

The results in Table 13 show that one third of the 116 isolates are the UK epidemic strain and of the epidemic markers, only *cblA* was exclusively present in all the epidemic isolates.

3.5. Putative virulence factors of B. cepacia

3.5.1. Extracellular virulence factors

The results of enzyme production in plate assays in section 3.1.1, show that CF isolates of *B. cepacia* varied in their ability to produce proteolytic and phospholytic enzymes. However, a positive reaction in a plate assay does not necessarily mean that the isolate produces the enzyme extracellulary. To test whether *B. cepacia* from CF patients produced lecithinase, phospholipase C and proteolytic activity in broth culture, representative isolates were selected for testing including those giving strong, weak and no reaction in the corresponding plate assay.

Lecithinase production

Table 14 shows the results of lecithinase activity in doubling dilutions of six isolates of *B. cepacia* grown in TSB and PWB and a *C. perfringens* control grown in RCM.

Table 14

Lecithinase production by *B. cepacia* in broth culture

			Dilutions						
Isolate	Plate assay	Broth	Neat	10^1	10^2	10^3	10^4	10^5	Titre of lecithinase activity
1	-	TSB	-	-	-	-	-	-	-
2	-	TSB	-	-	-	-	-	-	-
3	+	TSB	-	-	-	-	-	-	-
4	++	TSB	-	-	-	-	-	-	-
5	+	TSB	-	-	-	-	-	-	-
6	+	TSB	++	+	+	+	+/-	-	10^4
1	-	PWB	-	-	-	-	-	-	-
2	-	PWB	-	-	-	-	-	-	-
3	+	PWB	-	-	-	-	-	-	-
4	++	PWB	-	-	-	-	-	-	-
5	+	PWB	-	-	-	-	-	-	-
6	+	PWB	+	-	-	-	-	-	1
C. perfringens	++	RCM	++	+	+	+	+	+/-	10^5

Only one *B. cepacia* isolate gave a turbid reaction in egg yolk emulsion and this did not give a strong reaction in the plate assay. This isolate appeared to give a better production of lecithinase in TSB than PWB. Overall, the results suggest that most *B. cepacia* isolates do not produce lecithinase in broth culture.

Phospholipase C production

Sixteen isolates of *B. cepacia* were tested for phospholipase C activity in TSB and the results are shown in Table 15.

Table 15
Phospholipase C activity in broth culture and in comparison with plate assay

Isolate	Absorbance at 540nm	Absorbance at 405nm	Phospholipase C activity 405 nm/540 nm	Lecithinase production in plate assay
1	0.749	0.061	0.080	+
2	0.932	0.101	0.110	+
3	0.540	0.071	0.132	+
4	0.740	0.072	0.100	+
5	0.485	0.078	0.200	+
6	0.337	0.069	0.210	+
7	0.205	0.065	0.320	+
8	0.575	0.070	0.122	+
9	0.359	0.071	0.200	+
10	0.658	0.090	0.140	++
11	0.686	0.075	0.110	-
12	0.150	0.068	0.500	-
13	0.443	0.063	0.140	-
14	0.672	0.055	0.080	-
15	0.235	0.069	0.200	-
16	0.174	0.069	0.400	-

There was little correlation between phospholipase C production in broth culture and lecithinase production in the plate assay. The plate positive isolates gave readings for phospholipase C activity which were just as low as the plate negative isolates including one which gave a strong (+ +) reaction. The highest reading for phospholipase C activity was found with a plate negative isolate but this may have been due to pigment production which can effect the results of this assay.

Protease production

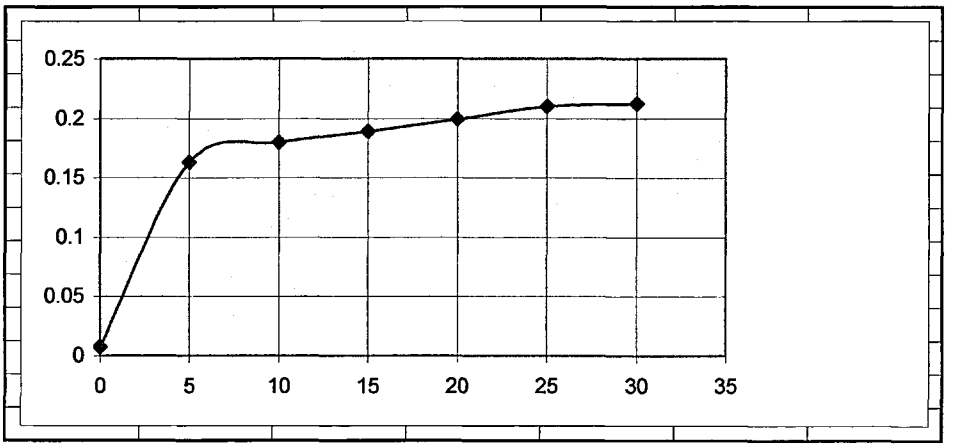
Preparation of standard curve

Table 16 shows the increase in absorbancy due to the release of azo dye from azocasein with increasing trypsin concentration. The standard curve is illustrated in Figure 8.

Table 16
Protease activity of trypsin

Concentration of trypsin µg/ml	Absorbance reading at 420nm	Average
0	0.120 0.003	0.008
5	0.164 0.162	0.163
10	0.179 0.181	0.180
15	0.193 0.185	0.189
20	0.196 0.201	0.199
25	0.218 0.201	0.210
30	0.211 0.212	0.212

Figure 8
Standard curve of trypsin concentrations



Protease production in broth cuture

The protease activities of three clinical isolates of *B. cepacia* in TSB and PWB and in comparison with plate assay results are shown in Table 17.

Table 17
Protease activity of three isolates of *B. cepacia* in broth culture

Isolate	Plate assay	Conditions	Absorbance 420 nm
842	++	PW 30^0C	0.051
		TSB 30^0C	0.039
		PW 30^0C	0.016
		TSB 30^0C	0.029
1270	+	PW 30^0C	0.018
		TSB 30^0C	0.022
		PW 30^0C	0.012
		TSB 30^0C	0.054
1182	-	PW 30^0C	0.013
		TSB 30^0C	0.023
		PW 30^0C	0.010
		TSB 30^0C	0.023

The absorbance readings of the clinical isolates were too low to be read off the standard curve. The absorbances for the clinical isolates were generally nearer to that of the protease assay buffer blank which suggests that there may be no proteolytic activity in *B. cepacia* isolates grown in broth culture.

In summary, no extracellular enzymes which may be associated with virulence, were detected in CF isolates of *B .cepacia.*

3.5.2. Haemolytic activity and serum sensitivity

A panel of 116 isolates of *B. cepacia* including the controls NCTC 10661, the UK epidemic strain CF 5610 and two environmental isolates were tested for haemolytic activity and serum sensitivity. Some representatives of serum sensitive and resistant isolates were also examined for presence of LPS.

Haemolysin production

The volume of packed red blood cells required in peptone water agar was determined in Table 18

Table 18
Estimation of packed cell volume from various species after centrifugation

Source of blood	Volume of whole blood in ampoule (ml)	Volume of packed cells in ampoule (ml)	(%) Concentration of blood in plates	Total of whole blood (ml) required.
Rabbit	2.0	1.0	5.0	10
Fowl	2.0	0.8	5.0	12.5
Sheep	2.0	1.4	5.0	7.14
Horse	2.0	0.8	7.0	17.5
Human "O"	2.0	0.4	5.0	25.0

pw = peptone water

Plate assay

Before testing the panel of 116 isolates, 12 representative isolates were tested on all five media to evaluate use of the different blood types and incubation times. These results are shown in Table 19 and illustrated in Figure 9.

Table 19
Haemolytic activity of 12 representative isolates of *B. cepacia* on blood from different species

Isolate	Days	Fowl	Rabbit	Sheep	Horse	Human
1	1	-	-	-	-	-
	2	-	-	-	+	-
	3	-	-	-	+	-
	4	-	-	+	+++	-
	5	-	-	++	++++	-
2	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-
3	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	+/-	-	-
4	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	+/-	-
	4	-	-	+	+++	-
	5	-	-	++	++++	-
5	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-
6	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	+/-	-
	5	-	-	-	+/-	-
7	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	+/-	-
	5	-	-	-	+/-	-
8	1	-	-	-	-	-
	2	-	-	-	+	+/-
	3	-	-	-	++	++
	4	-	-	-	+++	+++
	5	-	-	-	++++	+++
9	1	-	-	-	-	-
	2	-	-	-	+	+/-
	3	-	-	-	++	++
	4	-	-	-	+++	+++
	5	-	-	-	++++	+++
10	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-
11	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	+/-
	4	-	-	-	+/-	+
	5	-	-	+	+	++
12	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	+/-	+/-
	5	-	-	+	+	++

These results highlight the importance of a five-day incubation period in order to determine haemolytic activity on blood agar plates. They also show that only horse and human blood cells were suitable for determining haemolysin production in *B. cepacia.* There were however some discrepancies between the results for horse and human cells and as a consequence the panel was tested on both horse and human blood agar plates.

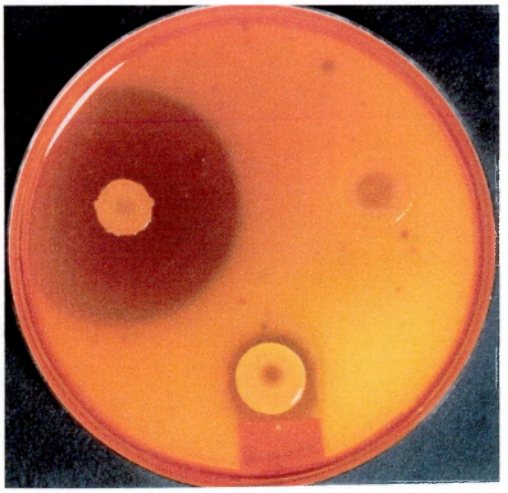


Figure 9 Isolates of *B cepacia* showing strong, weak and no haemolysis on human blood agar.

Haemolysin production in broth culture

Six isolates which had given strong, medium and no haemolysis on the blood plates, were tested for haemolysin production in broth culture. None of them gave a reaction comparable to the positive control with any of the dilutions used. Where slight precipitation of the red blood cells appeared, this could not be correlated with the plate haemolysis result.

Serum sensitivity

Before examining the panel of *B. cepacia* isolates for their sensitivity to NHS, five isolates were selected to determine the optimum dilution of broth culture and incubation time. Bacterial growth was graded as confluent (CF), semi-confluent (SCF), ++ (>50 colonies), + (between 20 and 50 colonies) and +/- (<20 colonies). The results of the bactericidal action of normal human serum (NHS) are shown in Table 20 and illustrated in Figure 10. Control wells of culture and HBSS were also included.

Table 20
Growth of *B. cepacia* after incubation with NHS and HBSS

Isolate	Time (h)		Dilutions				
			10^1	10^2	10^3	10^4	
1 CF	0		CF	CF	CF	SCF	R
	3	NHS	CF	CF	SCF	++	
		HBSS	CF	CF	SCF	++	
	6	NHS	CF	CF	SCF	++	
		HBSS	CF	CF	SCF	++	
2 CF	0		CF	CF	SCF	SCF	R
	3	NHS	SCF	SCF	++	++	
		HBSS	SCF	SCF	++	++	
	6	NHS	SCF	SCF	++	++	
		HBSS	SCF	SCF	++	++	
3 CF	0		CF	CF	SCF	SCF	
	3	NHS	CF	CF	SCF	SCF	R
		HBSS	CF	CF	SCF	SCF	
	6	NHS	SCF	SCF	++	++	
		HBSS	SCF	SCF	++	++	
4 CF	0		CF	CF	SCF	SCF	
	3	NHS	+/-	+/-[2]	-	-	S
		HBSS	SCF	SCF	SCF	++	
	6	NHS	+/-	-	-	-	
		HBSS	SCF	SCF	++	++	
5 Epidemic CF	0		CF	CF	SCF	SCF	S
	3	NHS	+/-[7]	+/-[1]	-	-	
		HBSS	CF	SCF	++	+	
	6	NHS	-	-	-	-	
		HBSS	SCF	SCF	++	++	

SS: Serum sensitivity
R: Resistant
S: Sensitive

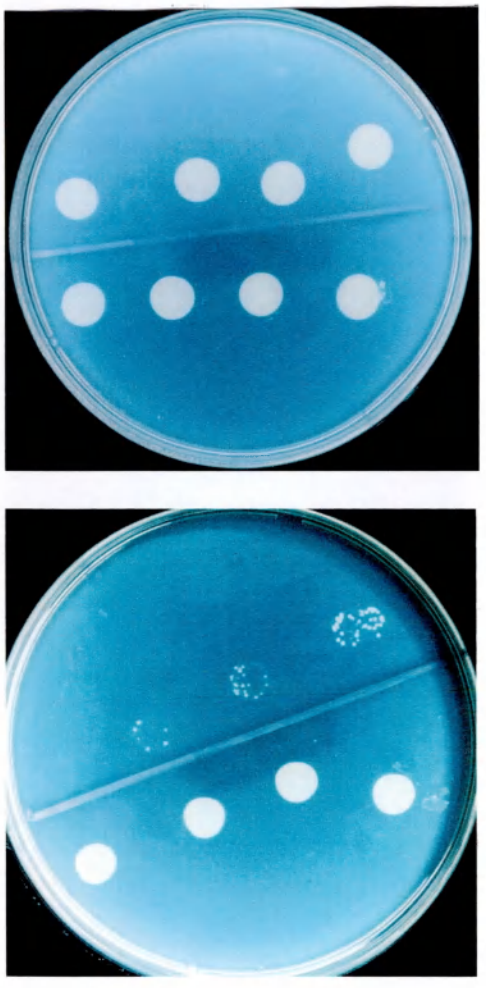


Figure 10 Growth of CF *B. cepacia* isolates on NA plates after incubation with NHS (above) and HBSS (below). All samples diluted 10-1 to 10-4.Upper left; resistant isolate, upper right; intermediate sensitivity, below left; sensitive isolate.

Table 20 shows that the first three isolates were serum resistant and the last two were sensitive. There appeared to be little difference between initial bacterial counts and after incubation with HBSS for 3 h. There was also little advantage in incubating for 6 h. The panel of 116 CF isolates and four controls were therefore diluted 1 in 100 which seemed to be the optimum dilution and incubated with NHS and HBSS for 3 h. Isolates varied from completely resistant where there was little or no difference between growth after incubation with serum and HBSS, to serum sensitive where the decrease in growth was by more than three grades for example from CL to + or +/-. There were also some isolates that demonstrated intermediate sensitivity where the

decrease in growth was only by one or two grades for example from CL to SCL or ++.

Serum sensitivity after serum incubation with LPS

To determine whether LPS from a serum sensitive isolate blocked the bactericidal action of serum, isolates of serum sensitive *B. cepacia* were incubated with NHS as in the previous section and the results compared with the same isolates pre-incubated with homologous LPS. A representative panel of 12 isolates was first tested with and without homologous LPS to evaluate the experiment and these results are shown in Table 21.

Table 21

Serum sensitivity of *B. cepacia* isolates with and without LPS

	1	2	3	4	5	6	7	8	9	10	11	12
A	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF	SCF
B	-	+/-^{3}	-	+/-	+	+/-	+	-	+/-^{7}	+	-	+
C	-	SCF	CF	++	+/-^{9}	CF	++	+/-^{6}	+/-^{4}	+	+	+/-^{1}
D	-	-	-	-	+	-	+/-	-	+/-^{4}	+/-^{4}	-	+
E	-	-	-	-	-	-	-	-	-	-	-	-

A: HBSS and culture (Control)
B: NHS and culture
C: NHS and LPS (culture added after 3h)
D: HBSS and NHS (culture added after 3h) (Control)
E: HBSS and LPS (culture added after 3h) (Control)

The columns highlighted are the serum sensitive isolates which became resistant or intermediate in susceptibility to serum killing after incubation with LPS, suggesting that LPS is the target for the serum killing reaction. The non-highlighted isolates in Table 21 presumably have a different serum target site. Why the HBSS and LPS in control row E should have a bactericidal effect is not clear.

A summary of the results of haemolytic activity, serum sensitivity and blocking of serum sensitivity by LPS for 116 CF isolates and controls, is shown in Table 22.

Table 22 Comparison of haemolytic activity and serum resistance in 116 CF isolates of *B. cepacia* and controls

	Control isolates				CF isolates	
Test	NCTC 10661	Environ. 1	Environ. 2	CF 5610	77 non-epidemic B. cepacia	39 epidemic B. cepacia
HA	-	-	-	+	16	16
SR	+	+	+	-	54	4
SI	-	-	-	-	4	26
SS	-	-	-	+	19	9
SSbl	NA	NA	NA	-	17	0

HA: Haemolysin production

SR: Serum resistant

SI: Intermediate sensitivity

SS: Serum sensitivity

SSbl: Serum sensitivity blocked by LPS

Statistical analysis indicated a significant relationship between isolates of the UK epidemic strain and both haemolytic activity ($\chi^2 = 33.18$; $P = 0.1\%$) and serum sensitivity ($\chi^2 = 9.57$; $P = 1.0\%$).

Detection of lipopolysaccharide by SDS-PAGE

The results in Table 22 show that both the UK epidemic and other CF *B. cepacia* isolates may be serum resistant or sensitive. Furthermore, the sensitive non-epidemic isolates can be grouped into those which become resistant after blocking by LPS and those which remain sensitive. It was interesting to find four resistant UK epidemic isolates as this strain has been described as constitutively lacking the O-side chain (Butler, unpublished).

To analyse further the role of LPS in serum sensitivity, LPS extracts prepared in section 2.7.2 from 14 isolates were selected and included serum sensitive and resistant isolates from UK epidemic and other CF isolates. Volumes of 10, 15 and 20 μl were run on SDS-PAGE gels and the results are shown in Table 23 and the banding patterns of 12 of the isolates are illustrated in Figure 11.

Table 23
Presence of O-side chain in serum sensitive and resistant *B. cepacia* isolates

Isolate	Serum sensitivity	Resistant after blocking by LPS	Presence of O-side chain
UK epidemic strain (CF 5610)	S	-	-
UK epidemic	R	na	+
UK epidemic	R	na	+
UK epidemic	R	na	+
UK epidemic	R	na	+
UK epidemic	S	-	-
(NCTC 10661)	R	na	+
CF	S	+	+
CF	R	na	+
CF	R	na	+
CF	S	+	+
CF	S	-	+
CF	S	-	+
CF	S	-	-

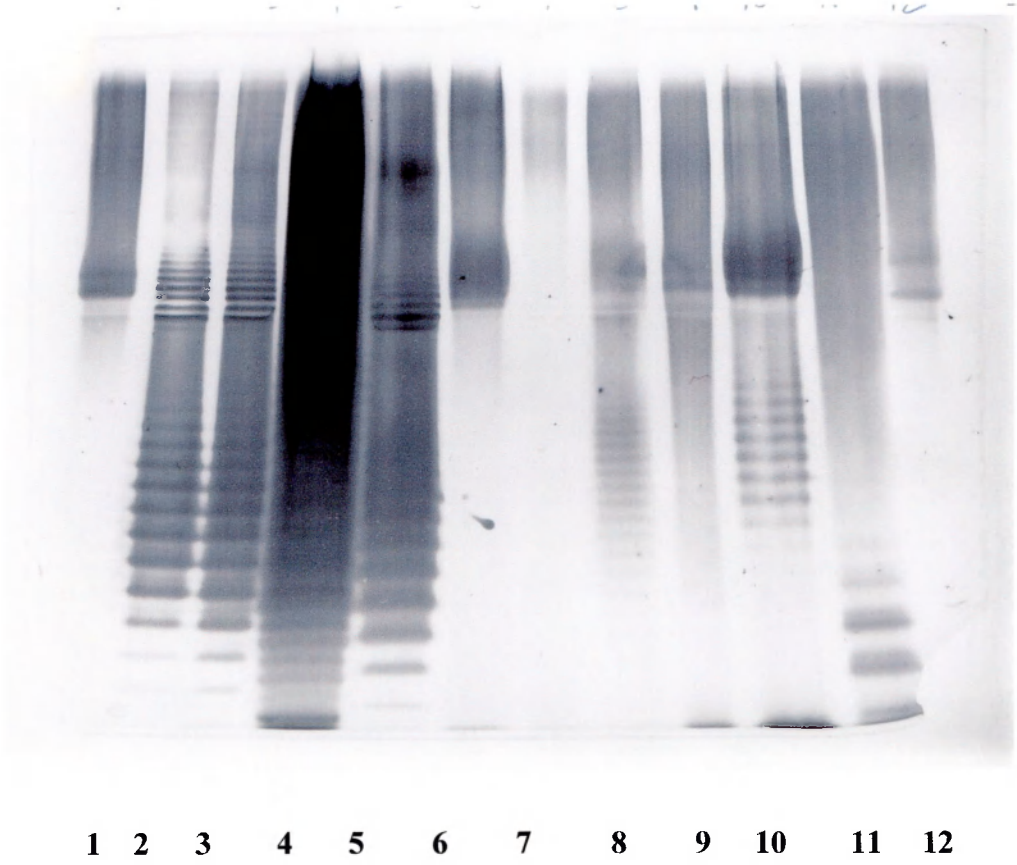


Figure 11 SDS-PAGE of LPS extracts of *B. cepacia* isolates Lane 1 epidemic rough, 2 to 5 epidemic smooth, 6 epidemic rough, 7 to 11 non-epidemic smooth, 12 non-epidemic rough

The volume of LPS extract found to give the clearest bands was 20 µl. The results in Table 23 and Figure 11 suggest that in terms of presence or absence of O-side chain and sensitivity or resistance to NHS, four categories of *B. cepacia* could be identified: 1) isolates with an O-side chain which were serum resistant, 2) serum sensitive isolates with O-side chain, which became resistant after blocking with homologous LPS, or 3) which remained sensitive and 4) serum sensitive isolates lacking an O-side chain. Figure 11 shows the presence of O-side-chains in LPS extracts of four serum resistant UK epidemic isolates. Lanes 7 and 9 do not show the LPS bands clearly but these were found to be present on repeat testing.

3.5.3. Onion maceration assay

Onion maceration and discolouration after inoculation with *B. cepacia* is shown in Table 24.

Table 24
Onion appearance and isolation of *B. cepacia*

Genomovar	Onion appearance after 48h	Growth on selective media
Saline	No change-no discolouration or softness.	-
I/IV	Slightly brown but not soft	+
II	No change	+
IIIa	Slightly yellow and soft	+
IIIa	Slightly yellow and soft	+
IIIa epidemic	No change	+/-
IIIb	Slight discolouration	+
IIIb	No change	+
IIIb epidemic	Slightly brown but not soft	-
IIIc	Slightly yellow and soft	+
IIIc	No change	+
IIIc epidemic	Slightly yellow and soft	-
I/IV	Brown discolouration but only slightly soft.	+
I/V	Slightly yellow, not soft	+
Environmental I	No change	+
Environmental II	Very brown and soft	+
NCTC 10661 I	Yellow and soft	+
Epi 5610 III	Slightly soft but no discolouration	+
P. aeruginosa	Brown and soft	+ *

* Kings agar

Only one of the two environmental isolates tested caused significant maceration of the onion tissue, the other isolate appeared to have no effect.

The clinical isolates showed varying degrees of discolouration and maceration but none of them were comparable with the environmental isolate. Interestingly, the UK epidemic strain isolates had little effect on the onion tissue and were the only isolates

which could not be grown from the onion tissue after incubation. Figure 12 illustrates the difference between onion maceration due to environmental isolate II and a clinical isolate.

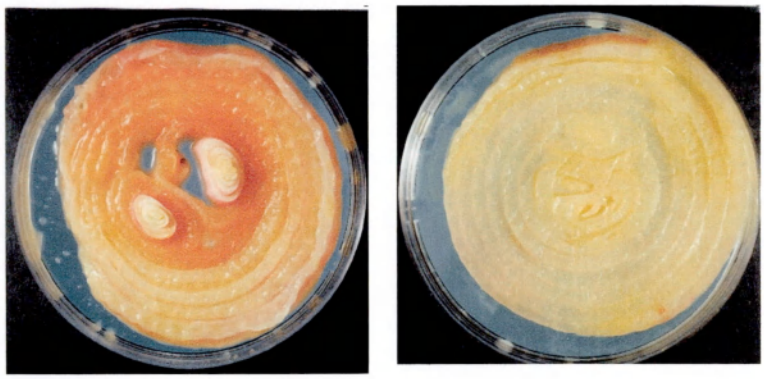


Figure 12 Maceration of onion tissue after inoculation of an environmental *B. cepacia* isolate (left). No effect after inoculation with a CF isolate of *B. cepacia* (right).

Summary of results

Appendix 4 shows a summary table of the biochemical and epidemiological properties of CF isolates identified as *B. cepacia*.

4. Discussion

B. cepacia is not as common a cause of lung infections in CF patients as *P. aeruginosa* but it is associated with a more serious disease and in some cases a rapidly fatal outcome. Consequently, investigations into the isolation and accurate identfication of the organism, its source and epidemiology, and putative virulence factors have become very important areas of investigation.

Culturing sputum from the CF lung on to the colistin-containing selective media Pseudomonas cepacia (PC) agar (Gilligan *et al* 1985) and Oxidation-fermentation-lactose-polymyxin B (OFPBL) agar (Welch *et al* 1987), has greatly improved the isolation of the slowly growing *B. cepacia* in mixed flora specimens. However, Hutchinson *et al* (1996) found that although 24 of 35 nebulizer circuits from CF patients were contaminated with colistin- resistant gram-negative rods, only three of the isolates growing on the PC selective agar were subsequently identified as *B. cepacia.* At least 15 other species including *S. maltophilia* and *Pseudomonas, Acinetobacter* and *Alcaligenes* spp. grew on the selective medium. In this study, assacharolytic organisms isolated on PC agar from an environmental source were probably *Alcaligenes* species. Of the test isolates received in LHI, 68 of 177 were not *B. cepacia* or closely related species, but had either grown on selective media or were colony picks from non-selective media. As many of these were colonisers of the CF lung, this highlights the importance of not only using selective media but also of employing accurate identification methods.

However, difficulties may be encountered in distinguishing between *B. cepacia* and other *Burkholderia* species and gram-negative rods that grow on the selective media (Burdge *et al* 1995) and in identifying assacharolytic variants. Between the years 1965 to 1984, 4840 isolates of gram-negative non-fermentative bacteria were submitted to the National Collection of Type Cultures (NCTC) for identification (Holmes 1986). Of these, 195 isolates (4%) were identified as *B. cepacia* which suggests that the organism is not uncommon in clinical material but that some laboratories have difficulty in identifying it. Kiska *et al* (1996) found that three out of four commercial kits incorrectly identified *B. gladioli, R. pickettii* and some other gram-negative rods as *B. cepacia.* The kits also failed to identify *B. cepacia* in some instances. In this study, API 20NE kits were employed in the first instance but failed to identify asaccharolytic isolates or distinguish between *B. cepacia* and *B. gladioli* which agrees with the findings of Simpson *et al* (1994). However van Pelt *et al* (1999), found that API 20NE gave the most accurate identification of *B. cepacia* although it was not apparent whether they used *B. gladioli* as a negative control. Recently developed genotypic methods of identification have the advantage of more rapid turnaround times and greater sensitivity and specificity. O'Callaghan *et al* (1994) exploited highly variable regions within the 16S rRNA for the identification of various gram-negative rods including *B. cepacia.* Although the PCR identification of *B. cepacia* was sensitive and specific in so far as the species tested, *B. gladioli* was not used as a negative control. This study revealed that a product band was formed with *B. gladioli.* Similar findings have since been reported by van Pelt *et al* (1999). Campbell *et al* (1995) also designed primers directed at variable 16S rRNA sequences and Tyler *et al* (1995) examined *B. cepacia* specific 23S rRNA sequences and *B. gladioli* specific ITS sequences. This study found both the 16S rRNA and 23S rRNA *B. cepacia*

primers to be sensitive in identifying all isolates including asaccharolytic variants and a batch of isolates biochemically identified as *B. gladioli.* In contrast, van Pelt, *et al* (1999) found that the 23S rRNA primers lacked sensitivity and only identified 22 of 25 *B. cepacia* isolates. In this study *B. gladioli* ITS primers failed to produce a band with one of the reference strains but gave positive reactions for three isolates identified as *B. cepacia* by API 20NE. With regards to specificity, all three primer pairs gave negative results for other gram-negative rods of clinical significance including the closely related *R. pickettii.* However, other *Burkholderia* species tested produced a band with one or more of the three primer pairs, although only *B. vietnamiensis* (out of the species tested here) has been linked to human disease. Vandamme *et al* (1997) concluded that *B. cepacia* comprises at least five different species or genomovars including *B. vietnamiensis* and named this the *B. cepacia* complex. Their results showed that the majority of CF isolates could be placed in genomovar II or III but few in genomovar I, IV or *B. vietnamiensis.* This agrees with the results of the present study where out of 116 isolates, 48 and 58 were biochemically classified in genomovars II and III respectively while only nine could be classified in genomovars I/IV. However, in the biochemical schemes of both Vandamme *et al* (1997) and Holmes, *et al* (1986), there is a problem of long incubation times and mis-identification may occur. This may have been the reason for the PCR positive *B. cepacia* isolate which was biochemically '*B. gladioli*' (Appendix 4) and an example of the hybrid described by Simpson *et al* (1994). Also, a negative reaction in ammonium salt sugars is inadequate for the identification of asaccharolytic *B. cepacia* without further tests. Biochemical tests therefore do not offer an advantage over PCR methods for identifying *B. cepacia* but the former can be used to classify the complex into genomovar groups and to identify *B. vietnamiensis.* A further

advantage of PCR techniques is that the differentiation of genomovar groups including *B. vietnamiensis* by PCR (Bauernfeind *et al* 1999), (Mahenthiralingam *et al.* 2000), (LiPuma *et al.* 1999) and RFLP analysis of amplified 16S rDNA (Segonds *et al* 1999) has recently been described.

PCR identification of *B. cepacia* and other CF pathogens may be applied directly to clinical specimens, hence minimising the need for growth and isolation. Karpati and Jonasson (1996), found by using one species specific and one universal primer that PCR detection of *P. aeruginosa* in sputum was 93% sensitive but detection of *B. cepacia* was only 78% sensitive. In contrast, O'Callaghan *et al* (1994) found that non-specific PCR products were produced with their 16S rRNA primers on sputum specimens which had been solubilised by dithiothreitol. They concluded that further purification of specimens may be necessary. In this study, 32 of 35 sputum specimens from *B. cepacia* positive CF patients were positive in the PCR using the 16S rRNA primers described by Campbell *et al* (1995). Two of the three which were PCR negative were culture-positive for other gram-negative rods and the third was culture-negative. Furthermore, exactly one half of the PCR positive sputa were culture-negative suggesting a high degree of sensitivity as well as specificity which agrees with the findings of Whitby *et al* (1998).

B.cepacia is both an environmental and a clinical pathogen and consequently determining the source of the organism in human infections is a cause for concern. This has recently been highlighted by the proposed use of *B. cepacia* as a biopesticide (Govan and Vandamme 1998). There are four possible routes of colonisation: i) directly from environmental isolates in the natural environment, ii) indirectly via

contaminated equipment or solutions, iii) directly from another patient, iv) indirectly from an environment contaminated with patient isolates.

Contrary to popular belief, *B. cepacia* is sparse in the natural environment with few reported isolations (Butler, *et al* 1995), (Honicky, *et al* 1993). In this study, *B. cepacia* could not be isolated from samples taken from environmental sites which included soil, water and vegetation. It has been suggested that environmental and clinical isolates of *B. cepacia* differ in their plant pathogenic capacity (Tabacchioni *et al* 1995), (Bevivino, *et al* 1994), including their ability to macerate onion tissue (Gonzalez and Vidaver 1979). This study found only one isolate capable of causing significant maceration of onion tissue and this was from an environmental source. In contrast, Butler *et al* (1995), despite concluding that none of their environmental isolates had phenotypic properties equivalent to the UK CF epidemic strain, found that this strain could nevertheless cause soft rot in onions. There is however evidence that environmental isolates can cause human disease and have been associated with a type of inflammation and maceration of the toe-webs of troops training in swamps (Taplin, *et al* 1971). Gonzalez and Vidaver (1979) found that both plant and clinical isolates were toxic for mice and had similar antibiotic sensitivities. Furthermore, LPS from both clinical and environmental isolates has been reported to have greater endotoxic activity than LPS from *P. aeruginosa* (Hughes *et al* 1997), (Shaw, Poxton *et al* 1995).

The presence of *B. cepacia* in the immediate environment of colonised CF patients would suggest that these patients were the source of the organism and that this posed a risk to others. However *B. cepacia* does not appear to be more abundant in the homes of colonised patients than in control homes (Mortensen *et al* 1995) and only sparsely contaminated a CF ward with no carriage by staff or patients (Doring *et al* 1997).

Nelson *et al* (1991) found that colonised CF patients in hospital did contaminate their environment but that this was minimal and the risk of indirect carriage from the environment subsequently low. In contrast, medical equipment and contaminated antiseptic solutions do appear to be potential sources of *B. cepacia* infection in CF and non-CF patients (Ederer and Matsen 1972), (Berkelman *et al* 1982), (Gravel-Tropper *et al* 1996), (Hutchinson *et al* 1996), (Reboli *et al* 1996), (Noriega *et al* 1975), (Smith *et al* 1985), (Geftic *et al* 1979), (Speller *et al* 1971), (Bassett *et al* 1973) (Hobson *et al* 1995).

The possibility of patient-to-patient transmission of *B. cepacia* is an emotive issue as colonised CF patients are often treated on separate wards and advised to avoid social contact with non-colonised patients. Some would argue that cross-infection is not a problem as many patients have sporadic isolates (Steinbach *et al* 1994) and even if a particular strain is common in a hospital, this may not be due to direct patient to patient spread as some patients do not acquire the strain despite close contact with carriers (Segonds *et al* 1997), (LiPuma *et al* 1988), (Taylor *et al* 1992). However, a strain common to more that one centre is strongly indicative of patient-to-patient transmission by hospital transfer or social contact (Govan *et al* 1993), (Pitt *et al* 1996), (Segonds *et al* 1997) (Smith *et al* 1993), (Smyth *et al* 1993). Furthermore, a reduction in the incidence of *B. cepacia* colonisation and the absence of endemic strains in hospitals where carriers are segregated has been supported (Paul *et al* 1998), (Thomassen *et al* 1986). There is now strong evidence for the presence of a strain of *B. cepacia* capable of causing epidemic spread in the UK CF population within and between hospitals and in the social context (Govan *et al* 1993), (Pitt *et al* 1996), (Simpson *et al* 1994). This has been variously described as ribotype 1 (Pitt *et al* 1996)

and ET12 (Johnson *et al* 1994) depending on the fingerprinting method employed and was assigned to genomovar III by Vandamme *et al* (1997). It is not known why particular strains have the potential to cause epidemic spread whilst others are confined to single cases or a few patients in close contact, although, in recent years various "epidemic" markers have been described which are associated with these strains. The most well known of these is the *cblA* gene encoding long intertwined cable-like pili which, unlike the other pilin structures in *B. cepacia,* appear to be associated with optimal colonisation of the CF lung enhancing epidemic spread (Sajjan *et al* 1995), Goldstein *et al* (1995). Sun *et al* (1995) found that the PFGE and ribotype profiles of *cblA*-positve isolates involved in an outbreak in Toronto, were not only indistinguishable from one another but also from the UK epidemic strain. Tyler, *et al* (1996) looked for an association between the presence of insertion sequence (IS) elements, *cblA*, electrophoretic type (ET) and ribotype (RT) in isolates of *B. cepacia.* An IS hybrid of IS*1356* inserted within IS*402* was found exclusively in the epidemic strain from Ontario and the UK, being detected in approximately 95% of isolates. It is not known whether the hybrid is linked to the apparent increase in transmissibility and virulence of this strain or whether it is simply a marker for the lineage. Mahenthiralingam *et al* (1996), identified a conserved region in seven epidemic *B. cepacia* strains (BCESM) including one *cblA*-positive isolate. They suggested that the presence of this marker was related to a strain's ability to infect multiple patients and become epidemic. In this study, all the control isolates which were indistinguishable from the UK epidemic strain by RAPD, were positive for all three epidemic markers and those from smaller clusters and sporadic isolates were negative for *cblA* and the IS hybrid but four of five were positive for BCESM. However looking at the results of the CF test isolates, it appears that only the UK epidemic strain is widespread in the

UK and was found in 13 hospitals. BCESM was present in all these isolates but was absent in four smaller clusters although present in a minority of sporadic isolates. The IS hybrid and *cblA* were exclusive to the UK epidemic strain but whereas *cblA* was present in all isolates, the IS hybrid was missing in a minority. All isolates of the epidemic strain apart from one were biochemically classifed as genomovar III.

Despite much research, little is known about the virulence factors of *B. cepacia* and why the outcome in CF patients colonised with the same strain can vary from asymptomatic carriage to a rapidly fatal necrotising pneumonia. For most pathogenic bacteria apart from some exotoxin producers, colonisation and avoidance of host defences is of paramount importance in the disease process. Adherence to host tissues is often the the first step and is particularly important in the lung to avoid the innate immune response of muco-ciliary clearance (albeit impaired in CF patients). Many bacterial species produce polar or peritrichous fimbriae which attach to specific receptors on host surfaces. Fimbriated *B. cepacia* display a much higher level of adherence to mucosal surfaces than non-fimbriated and this has been linked to a poorer prognosis (Sajjan *et al* 1992). *B. cepacia* has also been shown to invade and sequest in epithelial cells where it may be protected from host immune responses and antibiotics (Burns *et al* 1996), (Tipper *et al* 1998).

Many bacteria also avoid active immune responses by protecting themselves with an outer capsule or slime layer such as the alginate polysacchharide of *P. aeruginosa.* Gram-negative bacteria may also be protected by the long oligosaccharide side-chains of LPS which hinder the insertion of the complement mediated membrane attack complex. *P. aeruginosa* isolated from the CF lung are most often deficient in O-side-

chains and are serum sensitive. However, Butler *et al* (1994) found that even though all rough LPS isolates of *B. cepacia* were serum sensitive or intermediate, smooth LPS isolates may also be sensitive suggesting that factors other than the presence of an O side-chain may be important. This study also found that all rough isolates of *B. cepacia* were serum sensitive or intermediate but four isolates of the UK epidemic strain were shown to possess O-side chains and were serum resistant. This contrasts with the statement of Butler *et al* (1994) that this strain is always rough. However the relationship between serum sensitivity and epidemic or non-epidemic isolates was shown to be significant. Smooth LPS isolates in this study were either serum sensitive or resistant and furthermore, sensitive smooth LPS isolates either remained sensitive or became resistant after serum blocking with homologous LPS. This supports the theory that factors other than LPS may be involved in serum resistance. The only significance of genomovar grouping with regards to serum sensitivity is that genomovar III has all the UK epidemic isolates so that the majority of this group are sensitive or intermediate.

Enzymes produced by bacterial species are theoretically toxins in that their substrate is invariably host tissue. However, a link with disease or even minor tissue damage cannot always be demonstrated. In this study, some *B. cepacia* were shown to be strong producers of enzymes such as haemolysin, protease and lecithinase and these were mainly non-epidemic genomovar IIIa isolates. In contrast, genomovar II and IIIc isolates (including the UK epidemic strain) were mainly poor enzyme producers and this characteristic varied in genomovars I/IV. With few exceptions, none of the enzymes detected in plate assays were produced extracellularly in broth culture. Approximately 30% of isolates in this study gave zones of lysis on human blood agar

after five days incubation. Haemolysis was comparable on horse blood agar but there was little activity on fowl, rabbit or sheep blood cells in agar. These results are contrary to those of Nakazawa *et al* (1987), Gessner and Mortensen (1990) and McKevitt and Woods (1984), who all found that only about 4% of *B. cepacia* isolates produced lysis on any blood agar. However, each of the groups incubated the blood agar plates for approximately 48 h which highlights the importance of a five day incubation period as employed here. As with serum sensitivity, the relationship between epidemic isolates and haemolysis was statistically significant. One problem which this study encountered was diffusion of the melanin-like pigment of the UK epidemic strain into the blood agar making interpretation difficult in some cases.

Approximately 50% of isolates studied here produced lecithinase (phospholipase C) on egg yolk agar plates and these were randomly distributed throughout the genomovars, although less in IIIc. There was more activity on tween 20 and 80 plates than on egg yolk agar although Gessner and Mortensen (1990) found that most isolates produced both enzymes. Lonon *et al* (1988), also found that phospholipase C was not produced in broth culture and that lipase activity on tween plates was unrelated to phospholipase C activity. The role of *B. cepacia* lipase in pathogenicity is unclear although Straus *et al* (1992) showed that phagocytosis of the organism was reduced in the presence of lipase.

In this study 31% of isolates, mainly from genomovars IIIa and I/IV produced a proteolytic reaction on casein agar. *B. cepacia* does not produce the potent exotoxins and elastase enzymes equivalent to those of *P. aeruginosa* although McKevitt *et al* (1989) described an extracellular proteinase which was antigenically similar to *P.*

aeruginosa elastase in culture supernatants of *B. cepacia*. This could explain the systemic spread of low doses of *P. aeruginosa* in the burned mouse model in comparison with localised higher inocula of other *Pseudomonas* species including *B. cepacia* (Stover *et al* 1983). In the CF lung, however, the opposite situation is seen whereby *P. aeruginosa* remains localised whilst *B. cepacia* is capable of systemic spread. The factors involved in this seemingly paradoxical situation are not known and it is important to note that isolates of genomovar IIIc, which are devoid of detectable enzyme activity, have nevertheless been associated with fatal disease in CF patients (Wilsher *et al* 1997), (Clode *et al* 1999). Other possible pathogenic mechanisms not investigated in this study are the ability to invade cells, avoid host defence mechanisms, the tissue damaging action of endotoxin, siderophore production and antibiotic resistance.

In conclusion, this study has shown that PCR methods for the identification of *B. cepacia* are more sensitive, specific and time saving than traditional biochemical and kit methods. They also allow for the detection of the organism directly in clinical specimens hence eliminating the need for selective media. The classification of *B. cepacia* and related species into genomovar groups has caused much speculation about the association of epidemic and virulent strains with particular genomovars. This is largely unsupported by published data other than the association of the UK epidemic strain and some smaller clusters of multiply antibiotic resistant strains (Simpson *et al* 1994) with genomovar III (Vandamme *et al* 1997). This study found that only the UK epidemic strain is widespread in the UK and that it is most reliably identified by the detection of the *cblA* gene. The presence of intracellular enzymes in *B. cepacia* may have some correlation with genomovar group but the possible role of

these enzymes in pathogenicity is uncertain and these and other virulence factors would have to be correlated with clinical condition in a future study.

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6. Appendicies

6.1. Appendix 1

6.1.1. Malka minimal medium (Butler *et al* 1995)

Solution A

Reagent	**gm per litre**	**Concentration**	**Total volume in ml**
Na_2HPO_4	73.4	X 50	20
KH_2PO_4	32.4		

5-10 ml chloroform to 100 ml to sterilise

Solution B

Reagent	**gm per litre**	**Concentration**	**Total volume in ml**
$MgSO_4$ H_2O	20.5	X 50	20

5-10 ml chloroform to 100 ml to sterilise

Solution C

Reagent	**Molarity**	**Concentration**	**Total volume in ml**
Mono-sodium L glutamate	1 M	X 50	20

Sterilise by filtration

Solution D

Reagent	**gm per litre**	**Concentration**	**Total volume in ml**
$FeSO_4$ $7H_2O$	1.83	X 1000	1

One drop of H_2SO_4 added to sterilise

Solution E

Reagent	**gm per litre**	**Concentration**	**Total volume in ml**
$(NH_4)_2SO_4$	50	X 50	20

Make up to 200 ml with sterile distilled water

Mix sterile solutions

20A + 20B + 20C + 1D + 20E + 919 distilled water

6.2. *Appendix 2*

6.2.1. Preparation of SDS-PAGE buffers and enzyme solutions (Laemmli 1970)

Resolving gel buffer

Reagent	Weight /volume	Concentration
Tris	45.4 gm	0.75 M
Sterile distilled water	500 ml	
pH 8.8		

Stacking gel buffer

Reagent	Weight /volume	Concentration
Tris	15.12 gm	0.75 M
Sterile distilled water	250 ml	
pH 6.8		

Reservoir buffer

Reagent	Weight /volume	Concentration
Tris	1.2 gm	0.025 M
Glycine	5.76 gm	0.192 M
10 % SDS	4 ml	0.1 %

Make up to 400 ml with sterile distilled water

x2 Sample buffer

Reagent	Volume (ml)	Concentration
Glycerol	10	20 %
Stacking gel buffer	12.5	0.125 M
10 % SDS	20	4 %
2-mercaptoethanol	1	2 %
Sterile distilled water	6.5	
Total Volume	**50**	

0.25 % Protease K stock solution

Reagent	Volume (ml)
Glycerol	2
Stacking gel buffer	2.5
10 % SDS	4
Sterile distilled water	11.5

0.05 gm Protease K

6.3. *Appendix 3*

6.3.1. SDS-PAGE gels and staining solutions (Laemmli 1970)

Preparation of 10 % acrylamide resolving gel for two mini-gels

Reagent	Volume (ml)
30 % acrylamide solution	6.7
0.75 M resolving gel buffer	10
10 % SDS	0.2
Sterile distilled water	2.1
1.5 % ammonium persulphate	1
TEMED 10 µl	

Preparation of 5 % acrylamide stacking gel for two mini-gels

Reagent	Volume (ml)
30 % acrylamide solution	1.7
0.5M stacking buffer	10
10 % SDS	0.1
Sterile distilled water	5.2
1.5 % ammonium persulphate	0.5
TEMED 10 µl	

Silver stain fixing solution

Chemical	Weight/volume	Concentration
Methanol	900 ml	
Glacial acetic acid	300 ml	
Periodic acid	100 ml	0.7 %

Silver stain

Chemical	Weight/volume	Concentration
Sodium hydroxide	14 ml	0.1 M
Ammonium hydroxide	1.0 ml	
Silver nitrate	2.5 ml	20 %

Silver stain developing solution

Chemical	**Weight/volume**	**Concentration**
Sodium carbonate	50 ml	0.28 M
Formalin	1.0 ml	40 %
Sodium thiosulphate	4.0 ml	4.3 %

6.4. *Appendix 4*

6.4.1. Table 25 Summary of biochemical assays, plate assays, presence of epidemiological markers and epidemiological typing of *B.cepacia* isolates

Hosp	Genom	CA	IH	ESM	RAPD	SS	BI	Cas	Lec	Gel	Hyn
1	B.gladioli	+	+	+	E	R		-	+	-	++
	1										
2	I/IV	-	-	-	NE	S	N	-	+	-	-
2	I/IV	-	-	-	NE	R		++	+	-	+++
3	I/IV	-	-	-	NE	R		-	-	-	++
4	I/IV	-	-	+	NE	S	N	+	-	-	+
5	I/IV	-	-	-	NE	S	N	+	+	-	++
6	I/IV	-	-	-	NE	S	N	-/+	+	-	+++
7	I/IV	-	-	+	NE	S	Y	++	++	+	+
8	I/IV	-	-	-	NE	S	N	+	+	-	++
9	I/IV	-	-	-	NE	R		-	+	-	-
	9										
10	II	-	-	-	NE	R		-	+	-	-
2	II	-	-	-	NE	S	Y	-	-	-	-
2	II	-	-	+	NE	R		-	-	-	-
6	II	-	-	-	NE	S	Y	-	-	-	-
6	II	-	-	-	NE	S	Y	-	-	-	-
11	II	-	-	-	NE	R		-	+	-	-
12	II	-	-	-	NE	R		-	-	-	-
13	II	-	-	-	NE	R		-	-	-	-
13	II	-	-	-	NE	R		-	+	-	-
13	II	-	-	-	NE	R		-	+	-	-
14	II	-	-	-	NE	R		-	-	-	-
7	II	-	-	-	NE	R		-	+	-	-
15	II	-	-	-	NE	R		-	+	-	-
15	II	-	-	-	NE	S	Y	-	-	-	-
15	II	-	-	+	NE	R		-	+	-	-
15	II	-	-	-	NE	R		-	-	-	-
16	II	-	-	-	NE	R		-	-	-	-
16	II	-	-	-	NE	R		-	-	-	-
17	II	-	-	-	NE	R		-	+	-	-
18	II	-	-	-	NE	R		-	+	-	-
19	II	-	-	-	NE	R		-	-	-	-
20	II	-	-	+	NE	R		-	+	-	-
20	II	-	-	-	NE	R		-	+/-	-	-
1	II	-	-	-	NE	R		-	+	-	-
1	II	-	-	-	NE	R		-	+	-	-
1	II	-	-	-	NE	I	Y	-	+	-	-

1	II	-	-	-	NE	R		-	-	-	-
1	II	-	-	-	NE	S	Y	-	+	-	-
1	II	-	-	-	NE	R		-	+	+	-
1	II	-	-	-	NE	R		-	-	-	-
1	II	-	-	-	NE	R		-	-	-	-
1	II	-	-	-	NE	R		+/-	-	-	-
1	II	-	-	-	NE	R		-	+	-	-
1	II	-	-	-	NE	R		-	+	-	-
21	II	-	-	-	NE	R		-	+	-	-
22	II	-	-	-	NE	S	Y	-	-	-	-
23	II	-	-	-	NE	S	Y	-	+	-	-
24	II	-	-	-	NE	S	Y	-	+	-	-
25	II	-	-	-	NE	R		-	+	-	-
26	II	-	-	-	NE	R		-	-	-	-
27	II	-	-	-	NE	R		-	-	-	-
28	II	-	-	+	NE	S	Y	+	+	+	-
29	II	-	-	+	NE	R		-	-	-	-
29	II	-	-	+	NE	R		-	+	-	-
5	II	-	-	-	NE	R		-	-	-	-
5	II	-	-	-	NE	R		-	+	-	-
5	II	-	-	-	NE	R		-	+	-	-
30	II	-	-	-	NE	R		-	+	-	-
		48									
2	IIIa	-	-	-	NE	R		++	++	-	+++
											+
6	IIIa	-	-	-	NE	I	Y	+	+	-	+++
											+
6	IIIa	-	-	-	NE	R		+	+	-	-
31	IIIa	-	-	-	NE	R		+	+	-	+++
											+
15	IIIa	-	-	+	NE	I	Y	++	+	-	+/-
8	IIIa	+	+	+	E	I	N	-	-	-	+
32	IIIa	-	-	-	NE	R		++	+	-	+++
33	IIIa	+	+	+	E	I	N	+	-	-	+
3	IIIa	-	-	+	NE	R		+	+	-	+
34	IIIa	-	-	-	NE	R		+	+	-	+
22	IIIa	-	-	-	NE	R		+	+	-	+++
9	IIIa	-	-	-	NE	R		++	+	-	-
5	IIIa	+	+	+	E	I	N	+	-/+	-	+
5	IIIa	+	+	+	E	I	N	+	-	-	+
5	IIIa	+	+	+	E	I	N	+	-	-	+
5	IIIa	+	+	+	E	I	N	+	-	-	+
5	IIIa	+	+	+	E	I	N	+	-	-	+
5	IIIa	+	+	+	E	I	N	+	-	-	+
5	IIIa	+	+	+	E	I	N	+	-	-	+
5	IIIa	-	-	-	NE	S	Y	+	+	+	+
5	IIIa	+	+	+	E	I	N	+	-	-	+
5	IIIa	+	+	+	E	R		+	-	-	+
		22									
6	IIIb	+	+	+	E	S	N	-	-	-	-

35	IIIb		-	-	-	NE	S	Y	-	+	-	-
32	IIIb		-	-	-	NE	S	Y	-	+	+	+++
												+
36	IIIb		-	-	+	NE	R		+	+	-	-
		4										
37	IIIc		-	-	-	NE	R		-	-	-	-
3	IIIc		-	-	-	NE	S		-	-	-	-
38	IIIc		+	+	+	E	S	N	-	-	-	-
8	IIIc		+	-	+	E	S		-	+/-	-	-
8	IIIc		+	+	+	E	I	N	-	-	-	-
8	IIIc		+	+	+	E	S		-	-	-	-
8	IIIc		-	-	+	NE	R		-	-	-	-
8	IIIc		+	-	+	E	S	N	+/-	-	-	-
8	IIIc		+	+	+	E	S		+/-	-	-	-
33	IIIc		+	+	+	E	I	N	-/+	-	-	+
1	IIIc		+	+	+	NE	I		-	-	-	-
1	IIIc		+	+	+	E	S	N	-	-	-	-
1	IIIc		+	+	+	E	I	N	-	-	-	-
1	IIIc		-	-	-	NE	R		-	-	-	-
1	IIIc		-	-	-	NE	R		-	-	-	-
39	IIIc		+	+	+	E	S	N	-	-	-	-
40	IIIc		+	+	+	E	R		-	-	-	-
40	IIIc		+	+	+	E	I	N	+/-	+/-	-	-
40	IIIc		+	+	+	E	I	N	+	+	-	-
40	IIIc		+	+	+	E	I		+	-	-	-
40	IIIc		+	+	+	E	I	N	-	-	-	-
40	IIIc		+	+	+	E	S	N	-	+/-	-	+++
41	IIIc		+	+	+	E	I	N	-	-	-	-
3	IIIc		+	+	+	E	R		-	-	-	-
22	IIIc		+	+	+	E	I	N	-	-	-	+
22	IIIc		+	+	+	E	I	N	-	-	-	-
22	IIIc		+	+	+	E	I	N	-	-	-	++
22	IIIc		+	+	+	E	I	N	-	-	-	-
40	IIIc		+	+	+	E	I	N	-	-	-	-
40	IIIc		+	+	+	E	I	N	-	-	-	-
21	IIIc		+	+	+	E	I	N	-	-	-	-
41	IIIc		+	+	+	E	I	N	-	-	-	++
		32										
		116										

Genom: genomovar

CA: *CblA*

IH: Insertion Sequence hybrid

ESM: Epidemic strain marker

SS: Serum sensitive

Bl. Sensitivity blocked

Cas: Casein hydrolysis

Lec: Lecithinase production

Gel: Gelatin hydrolysis

Hyn: Haemolysin production

R: Resistant

S: Sensitive

N: No

Y; Yes

E: Epidemic

NE: Non-epidemic