LABORATORY STANDARDS FOR PROCESSING MICROBIOLOGICAL SAMPLES FROM PEOPLE WITH CYSTIC FIBROSIS

Report of the UK Cystic Fibrosis Trust Microbiology Laboratory Standards Working Group

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Preface

To deliver optimum care to those with Cystic Fibrosis (CF) it is absolutely essential that the microbiology of each individual is fully understood. Therapeutic decision making and Infection Prevention and control practices depend on it. The role of the microbiology laboratory has never been more important in CF care.

Although life expectancy for children born with CF is better today than ever before, all patients still face the challenge of respiratory infection. Whilst many respiratory pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* are seen in other patient groups, some of the most significant, such as *Burkholderia cenocepacia* are rarely encountered outside of CF. The microbiology of CF is further complicated because many common pathogens do not conform with the normal laboratory appearance. Examples include auxotrophic *P. aeruginosa* and small colony variants of *S. aureus*. As patients live longer, the range of micro-organisms causing infection broadens and antibiotic resistance develops. Many infections, such as those caused by non-tuberculous mycobacteria, can be a difficult diagnostic as well as therapeutic challenge. Finally, the introduction of molecular techniques has resulted in wide-ranging revisions to bacterial taxonomy and contributed to a greater understanding of pathogenicity and cross-infection.

In order to support the drive to achieve the highest standards in the microbiology laboratory, the UK CF Trust has commissioned a best practice guideline. The UK Cystic Fibrosis Trust Microbiology Laboratory Standards Working Group includes medical microbiologists, clinical scientists, biomedical scientists and CF clinicians. This document has been produced following their review of the available evidence and current practice. We have also shared this document with the Standards Methods Working Group of the Health Protection Agency, and this will underpin the development of a new National Standard Operating Procedure for the processing of respiratory samples submitted from people with CF.

We hope that this guideline will contribute to delivering the highest standards of care for people with Cystic Fibrosis.

Professor Stuart Elborn
Professor of Respiratory Medicine,
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**GRADING SCHEME FOR RECOMMENDATIONS USED**

The criteria for the grading of recommendations in this document are based upon a paper by Petrie et al published on behalf of the Scottish Intercollegiate Guidelines Network.

Much of the data in the document are derived from observational studies where randomisation is not appropriate or possible but many are from peer reviewed scientific studies therefore this grading is not always appropriate.

### Levels of evidence

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<thead>
<tr>
<th>Level</th>
<th>Type of evidence (based on AHCPR, 1992)</th>
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<tr>
<td>Ia</td>
<td>Evidence obtained from meta-analysis of randomised controlled trials.</td>
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<td>Ib</td>
<td>Evidence obtained from at least one randomised controlled trial.</td>
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<tr>
<td>IIA</td>
<td>Evidence obtained from at least one well designed controlled study without randomisation.</td>
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<td>IIB</td>
<td>Evidence for at least one other type of quasi-experimental study.</td>
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<tr>
<td>III</td>
<td>Evidence obtained from well-designed non-experimental descriptive studies, such as comparative studies, correlation studies and case control studies.</td>
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<tr>
<td>IV</td>
<td>Evidence obtained from expert committee reports or opinions and/or clinical experience of respected authorities.</td>
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### Grades of recommendations

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<tr>
<td>A (levels Ia, Ib)</td>
<td>Requires at least one randomised controlled trial as part of the body of literature of overall good quality and consistency addressing the specific recommendation.</td>
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<tr>
<td>B (levels IIA, IIB, III)</td>
<td>Requires availability of well conducted clinical studies but no randomised clinical trials on the topic of the recommendation.</td>
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<tr>
<td>C (level IV)</td>
<td>Requires evidence from expert committee reports or opinions and/or clinical experience of respected authorities. Indicates absence of directly applicable studies of good quality.</td>
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SUMMARY OF KEY RECOMMENDATIONS

SAMPLING ISSUES (CHAPTER 2)

- Respiratory sampling should be undertaken at each hospital visit and at times of respiratory exacerbation [C].
- Sputum is the recommended specimen for routine sampling [B].
- A cough swab should only be used if a patient cannot expectorate [C].

SAMPLE PROCESSING (CHAPTER 3)

- The presence of *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex (Bcc) and *Staphylococcus aureus* should always be reported, irrespective of quantity [C].
- Laboratories should use a selective medium to enhance the detection of Bcc [B].
- Selective media should be used to enhance the detection of *S. aureus* and *Haemophilus influenzae* from respiratory samples of people with CF [B].
- Sabouraud medium should be used to enhance the recovery of fungi from respiratory samples of people with CF [B].
- Culture of respiratory samples from people with CF for mycobacteria should be performed using both an automated liquid culture system and a solid medium (e.g., LJ medium) to maximise sensitivity [B].
- There is currently insufficient data to recommend the routine application of molecular detection methods directly to samples in addition to routine culture methods [C].

IDENTIFICATION (CHAPTER 4)

- All non-fermenting Gram-negative bacilli should be identified to species level [C].
- *P. aeruginosa* isolates with typical characteristics (e.g., green pigmentation, positive oxidase test, growth at 42°C) can be reliably identified with these tests alone [B].
- An excellent identification from a commercial kit such as API 20NE, in conjunction with colonial appearance, characteristic oxidase test and antibiogram, can be used to confirm the identity of *Stenotrophomonas maltophilia*, *Achromobacter* spp. and non-pigmented *P. aeruginosa* [B].
- Commercial kits/systems should NOT be used to identify members of the *Burkholderia cepacia* complex, *Pandoraea* spp., *Ralstonia* spp., and *Burkholderia gladioli* [B].
- The identity of members of the *Burkholderia cepacia* complex, atypical isolates of *S. maltophilia*, *Achromobacter* spp. and *P. aeruginosa*, and any colomycin resistant non-fermenting Gram-negative bacilli must be confirmed using molecular identification methods [B].
- recA PCR should be used to confirm any suspicious isolates as a member of the *Burkholderia cepacia* complex [B].
- All recA PCR-positive isolates should be further characterised to species level by recA sequencing. RecA-based PCR tests are no longer specific enough for this purpose with the exception of *Burkholderia multivorans* [B].
The identification and susceptibility testing of NTM is a highly complex area and should only be carried out in laboratories with sufficient knowledge and expertise e.g. mycobacteriology reference laboratory [B].

Molecular typing of P. aeruginosa should be performed using a standard method as determined by the Health Protection Agency’s CF Microbiology Reference Laboratory, in the Centre for Infection, Colindale, London [C].

All confirmed B. cenocepacia isolates should undergo molecular typing to establish whether they are members of the ET-12 and other epidemic lineages [C].

SUSCEPTIBILITY TESTING (CHAPTER 5)

- Susceptibility testing should be performed on isolates of P. aeruginosa associated with early and intermittent colonisation [C].
- Susceptibility testing should be performed using a standardised and validated method (e.g. BSAC disc diffusion) [B].
- There is no evidence to support the routine use of multiple combination bactericidal antibiotic testing [A], or biofilm testing methods [B].
- Conventional susceptibility tests on P. aeruginosa isolates associated with chronic infection in CF are poorly reproducible and may not predict clinical response. Their omission does not adversely affect short-term clinical outcomes [B].
- Susceptibility testing of P. aeruginosa using automated devices cannot be recommended at this time [B].
- Repeated isolation of Aspergillus spp., in spite of long-term treatment with anti-fungal drugs may indicate the need for referral of isolates to a reference laboratory for susceptibility testing [C].

POST ANALYTICAL PROCESSES (CHAPTER 6)

- CF clinicians and microbiologists should agree the structure, content and communication of laboratory reports [C].
- All new or suspect isolates of Bec and MRSA should be communicated urgently to the CF clinical team [C].
- A reliable system of identifying those with initial infection with P. aeruginosa needs to be in place in order to commence timely eradication therapy [C].
- Regular surveillance of P. aeruginosa, including molecular typing, should be carried out as recommended by the CF Trust Infection Control Group [C].
- In addition to providing a laboratory service and advice on Infection Prevention, microbiologists should participate in regular multi-disciplinary meetings to discuss the management of individuals with CF [C].
1. MICROBIOLOGY AND CYSTIC FIBROSIS: AN OVERVIEW

1.1 Introduction

The quality of clinical care for patients with Cystic Fibrosis (CF) provided by a Specialist CF Centre is dependent on good microbiological support. The organisms infecting a patient will determine the treatment, quality of life, prospects for transplantation and overall survival. The accurate and prompt identification of respiratory pathogens is essential for ensuring timely commencement of eradication treatment for early infection with bacterial pathogens, the use of appropriate long term and rescue antibiotics for those with chronic bacterial infection and the application of appropriate infection control measures.

1.2 Pathogens

The microbiology of the CF lung is complex and challenging. It had been thought that a limited spectrum of respiratory pathogens was seen in CF, but increasing numbers of other species are being recognized as potential pathogens.

1.2.1 Staphylococcus aureus

*Staphylococcus aureus* is a frequent isolate and may be cultured early in infancy. In the UK, continuous anti-staphylococcal antibiotic prophylaxis is recommended for all children with CF under three years of age (CF Trust Antibiotic Working Group, 2009) [IV]. The overall prevalence of lower respiratory tract infection with meticillin-resistant *S. aureus* (MRSA) among people with CF seems to be increasing. The prevalence of lower respiratory tract infection with MRSA differs between Specialist CF Centres, possibly reflecting regional differences in prevalence of MRSA strains in local healthcare and community settings and differences in screening policies, infection control practices and use of eradication treatment (CF Trust Infection Control Working Group, 2008) [IV]. At the time of writing the clinical consequence of lower respiratory tract infection with MRSA for people with CF, in comparison with meticillin-sensitive *S. aureus*, remains unclear.

1.2.2 Haemophilus influenzae

*Haemophilus influenzae* is associated with childhood and has been reported to be the most common CF pathogen at age one year (Rosenfeld et al, 2001 [III]). *H. influenzae* is a natural commensal of the upper respiratory tract and therefore there is the need to distinguish infection from contamination when *H. influenzae* is cultured from respiratory samples. It is important to note that *H. influenzae* responsible for CF and non-CF lung infections are mostly non-capsulate and non-typeable, and are not prevented by vaccines for capsule type b *H. influenzae*.

1.2.3 Streptococcus pneumoniae

*Streptococcus pneumoniae* is occasionally isolated from young CF patients but is unusual.

1.2.4 Pseudomonas aeruginosa

*Pseudomonas aeruginosa* is the most common pathogen in CF and the prevalence increases with age. Early infection can often be cleared with aggressive antibiotic treatment, such as with an oral...
quionolone plus an inhaled antibiotic. After the initial isolation, *P. aeruginosa* may be found intermittently in respiratory secretions. Specialist CF Centres that have instituted a practice of screening for and prompt treatment of initial *P. aeruginosa* infection, together with infection control measures to prevent cross-infection, have reported significantly later onset of chronic infection (Lee et al 2004 [III]; Lebecque et al 2006 [III]). Chronic infection is associated with a faster deterioration in lung function, worsening of chest X-ray scores, increased hospitalisation and a reduction in overall survival. Use of long-term suppressant therapy with inhaled antibiotics is recommended for patients with chronic *P. aeruginosa* infection to help preserve lung function and decrease the need for additional intravenous antibiotics. Acute respiratory exacerbations are usually treated with two intravenous antibiotics that have different mechanisms of action, to reduce the potential for encouraging bacterial resistance from frequent therapy and to benefit from any potential antibiotic synergy. The standard treatment course is for two weeks. Some patients receive regular courses of intravenous antibiotics as maintenance therapy.

Transmission of some strains of *P. aeruginosa* can occur between people with CF and most national infection control guidelines recommend regular surveillance and the use of molecular fingerprinting, such as pulsed-field gel electrophoresis, multi-locus sequencing, or variable number tandem repeat to identify cross-infection at CF Centres (CF Trust Infection Control Working Group, November 2004) [IV].

Atypical colonial forms of *P. aeruginosa* may be found in chronic infection and can be difficult to identify unless molecular methods are used [III] (Wellinghausen 2005).

### 1.2.5 *Burkholderia cepacia* complex

Other Gram-negative bacteria can also infect the lung, usually later in the progression of Cystic Fibrosis. The most clinically significant have been the species of the *Burkholderia cepacia* complex (Bcc), which are associated with a significant increase in morbidity and mortality. There is also the potential for some strains to cause epidemics through patient-to-patient spread. Bcc consists of a number of closely related species, or genomovars, of which there are 17 currently recognised (Table 2, 4.2). Bcc had a major impact in the 1980s and 90s with outbreaks leading to many deaths. Most of the previous Bcc outbreaks in the UK and Canada were associated with *Burkholderia cenocepacia*, and the ET12 strain in particular (Govan et al, 2007 [IV]; Mahenthiralingam et al, 2001 [III]). The number of patients with *B. cenocepacia* has declined rapidly following measures to prevent person-to-person spread. However, cross-infection involving *Burkholderia multivorans* and other genomovars, in particular *Burkholderia dolosa*, has also been reported (Mahenthiralingam et al, 2002 [IV], Agodi et al 2001 [III], Kalish et al 2006 [III]).

Infection with members of the Bcc may be regarded as a contraindication to lung transplantation due to its association with poor post-transplant survival (Murray et al, 2008 [III]; Kreider and Kotloff, 2009 [IV]). Accurate identification and speciation of Bcc from specimens of sputum from people with CF is thus of the utmost importance as false-positive identification may have serious psychological, social and organisational consequences, such as exclusion from social events and scientific and other conferences and rejection as a potential lung transplant recipient. False-negative identification could result in transmission of Bcc to others with CF if appropriate Infection Prevention measures are not in place (Miller & Gilligan, 2003 [IV]). In the clinical microbiology laboratory, the use of selective media and appropriate identification procedures is vital for optimum culture and reliable diagnosis of Bcc in sputum and other clinical specimens (van Pelt et al, 1999 [III]; Miller & Gilligan, 2003 [IV]). Identification of Bcc by common commercial systems is unreliable. Unless state-of-the-art facilities are available on site, all new isolates suspected as Bcc should be sent to a laboratory experienced in the phenotypic and DNA-based identification of the group and of
individual Bcc species.

Recently, cases of *Burkholderia pseudomallei*, a species closely related to the Bcc and the cause of the subtropical infection melioidosis, have been described in patients with CF returning from travel to areas where *B. pseudomallei* is endemic (Visca et al 2001 [IV], Holland et al 2002 [IV]). The Cystic Fibrosis Trust has published a factsheet providing advice on *B. pseudomallei* for CF individuals planning travel to South-East Asia and subtropical areas of Australia.

1.2.6 Other bacterial species

The impact of other *Burkholderia* spp., *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Ralstonia* (formerly *Pseudomonas*) spp. and *Pandoraea* spp. on individuals with CF and their propensity for cross-infection is unresolved and warrants further study. Recent reports from reference laboratories indicate that many Gram-negative bacteria in CF are incorrectly identified using standard laboratory tests. Some are colistin resistant and may be mis-identified as *Burkholderia* spp. (Saiman et al 2001 [III], Wellinghausen et al 2005 [III]). It is important that bacteria are carefully identified, as the range of antibiotics that may have activity are species specific as are the growth conditions required for testing antibiotic susceptibility in the laboratory.

More recently there has been a recognition that other bacterial species – usually considered part of the normal oral flora including anaerobes – are found in significant numbers in the sputum of patients with CF (Tunney et al 2008 [III], Rogers et al 2004 [III]). The presence of bacteria in the lung does not necessarily imply a direct pathogenic effect. These bacteria may be harmless commensals or interact with other bacteria influencing their growth or behavior. For example, a viridans streptococcus and a coagulase-negative staphylococcus from CF sputum have been suggested to be involved in the up-regulation of genes involved in pathogenicity in *P. aeruginosa* (Duan et al 2003 [IIb]).

1.2.7 Non-tuberculous mycobacteria

The significance of the isolation of non-tuberculous mycobacteria (NTM) in the respiratory secretions from a patient with CF presents a difficult diagnostic dilemma. Infections with rapidly-growing NTM, in particular *Mycobacterium abscessus*, can cause severe disease in CF and are a major therapeutic challenge. Infections with the *Mycobacterium avium* complex are also frequently seen (Roux et al, 2009 [III]). It is often unclear whether the presence of these mycobacteria represents transient contamination of the airways, colonisation, or true infection and therefore repeated sampling is required. The American Thoracic Society has recently published its revised guidance for the diagnosis of NTM disease (Griffith et al 2007) [IV]. The use of liquid culture for mycobacteria has reduced the time needed for culture and improved sensitivity. However the large numbers of *P. aeruginosa* in CF sputum can make decontamination difficult and methods still need optimisation to realise the potential advantages of liquid culture systems.

1.2.8 Fungi

Fungal infections have become more prevalent in people with CF in recent years. Infection with *Aspergillus* spp. has long been recognised as a problem in CF, usually presenting as allergic bronchopulmonary aspergillosis. Recently it has been suggested that *Aspergillus* infection can cause respiratory exacerbations by stimulating a fungal-associated bronchitis that responds to specific antifungal therapies (Shoseyov et al, 2008 [IV]). Other fungi are increasingly recognised as complications of CF care e.g. *Scedosporium apiospermum* and *Wangiella (Exophiala) dermatitidis*. 
1.2.9 Respiratory viruses

It has been recognised for many years that respiratory viruses can have a significant clinical impact on wellbeing and respiratory function in people with CF (Wang et al, 1984 [III]; Hiatt et al, 1999 [III]; van Ewijk et al, 2008 [IIb]). The introduction of molecular detection techniques has allowed a faster and more accurate identification of the causative agents of exacerbations (Wat et al, 2008 [III]). These techniques have been used to demonstrate that people with CF have been infected with the H1N1 influenza virus during the 2009 pandemic (Whitaker et al, 2009 [IV]). Admission to hospital because of respiratory syncytial virus (RSV) infection has also been associated with an increased risk of subsequent colonisation by *P. aeruginosa* (Armstrong et al, 1998 [IIb]). There is also evidence that RSV mediates binding of *P. aeruginosa* to respiratory epithelial cells (van Ewijk et al, 2007 [IIb]).
2. SAMPLING ISSUES

2.1 Sampling methods

There are a number of methods that can be used to obtain respiratory samples for culture or non-culture-based diagnosis from people with CF who do not expectorate sputum: cough swab, cough plate, oropharyngeal culture, laryngeal or naso-pharyngeal aspirate, induced sputum following hypertonic saline, bronchoalveolar lavage and bronchoscopy brush specimens. Several studies have compared yields of pathogens using these different methods:

Thomassen et al, (1984) compared bacteriological cultures of sputum and specimens obtained at thoracotomy from 17 patients with CF. All organisms found in the surgical specimens were also detected in the sputum. The authors concluded that the observed correlation of sputum and lung specimen culture results supports the value of sputum culture in the management of lung disease in CF (Thomassen et al, 1984 [III]).

A comparative study of oropharyngeal cultures and bronchial sampling demonstrated a sensitivity and specificity of 70% and 83% for isolation of Pseudomonas aeruginosa and 80% and 91% for isolation of Staphylococcus aureus respectively, in 26 non-expectorating people with CF. OPS cultures yielding P. aeruginosa or S. aureus were highly predictive, but the absence of these organisms in such cultures did not rule out their presence in the lower airways (Ramsay et al, 1991 [III]).

Pathogens obtained by oropharyngeal suction (OPS) and bronchoalveolar lavage (BAL) were compared in 44 children with chronic lung infection (five had CF). Twenty-seven of 44 (61%) BAL samples yielded a positive bacterial culture. The sensitivity of OPS in detecting the same pathogen as found in BAL was 89% (24/27 samples), the specificity was 94% (16/17 samples) and the predictive value was 91% (40/44 samples). The authors concluded that OPS is a simple non-invasive method, which may be helpful in the diagnosis of chronic pulmonary infection (Avital et al, 1995 [III]).

The yield of potential pathogens isolated from throat swabs by one of three different methods was studied using samples taken from 50 children with CF (Hoppe et al, 1995 [III]). Swabs were cultured directly or placed into either saline or Ringer’s lactate, left for 30 minutes, and the liquid was then inoculated onto solid media for culture. A total of 124 isolates not considered part of normal throat flora were obtained (Candida albicans (39); P. aeruginosa (36); S. aureus (22); coliforms (12); other pseudomonads (5); Aspergillus fumigatus (5); Haemophilus influenzae (2); other Candida spp. (2)). No method cultured all 124 isolates. The three methods gave sensitivities of 91.9% (Ringer’s lactate), 82.3% (saline), and 59.7% (direct culture), respectively.

Seventy-five of 90 (83%) infants with CF diagnosed by neonatal screening had 150 simultaneous broncho-alveolar lavage and oropharyngeal culture samples collected for quantitative bacterial culture at an age of 17 months. Ten children undergoing bronchoscopy for stridor served as controls. S. aureus (19%), P. aeruginosa (11%), and H. influenzae (8%) were the most frequent isolates from BAL samples; the corresponding frequency from oropharyngeal samples were S. aureus (47%), Escherichia coli (23%), H. influenzae (15%), and P. aeruginosa (13%). The sensitivity, specificity, and positive and negative predictive values of oropharyngeal cultures for pathogens causing lower respiratory infections was 82%, 83%, 41%, and 97%, respectively. When the same species was isolated from paired oropharyngeal cultures and BAL cultures, PFGE showed some strains were unrelated. The authors concluded that oropharyngeal cultures do not reliably predict the presence of bacterial pathogens in the lower airways of young CF children (Armstrong et al, 1996 [III]).

One study enrolled ten adults with CF and the recovery of bacteriological pathogens was 79% for induced sputum, 76% for spontaneously expectorated sputum, and 73% for BAL (Henig et al, 2001...
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H. influenzae (13%). The sensitivity, specificity, and positive and negative predictive values for isolation of S. aureus were 35.7%, 73.5%, 83.3% and 96.2% for oropharyngeal cultures in non-expectorating patients and 91.7%, 94.1%, 100% and 100% for sputum cultures from expectorating patients, respectively. Molecular typing showed that genotypes of P. aeruginosa isolates recovered from oropharyngeal swabs and sputum differed from the strains recovered by bronchoscopy in 55% and 40%, respectively. Longitudinal variations in the genotype occurred in 62% of samples and were mostly evident in bronchoscopy samples only. The study concluded that sputum was of equal value as BAL for detection of P. aeruginosa, but cultures from the oropharynx are not suitable for characterising bacterial conditions in the CF lung. Different genotypes within the same host and longitudinal genetic alterations are common and may only be detectable by culturing BAL.

Equi et al (2001 [III]) compared cough swabs and spontaneously expectorated sputum samples from 30 children with CF. Cough swabs yielded 12 isolates and the corresponding sputum samples 36 isolates, giving a specificity of 100% and sensitivity of 34% for cough swabs compared to sputum. No details of the identity of these isolates were provided. Positive and negative predictive values for cough swab were 100% and 21% respectively.

The study of Kabra et al (2004, [III]) compared cough swabs and throat swabs taken before and after physiotherapy with sputum samples; 387 samples were collected from 48 people with CF. Sensitivity for isolation of P. aeruginosa was 40%, 42% and 82% for cough swabs, throat swabs taken before physiotherapy and throat swabs taken after physiotherapy, respectively, and for S. aureus 57%, 50% and 100%, respectively. Specificity was high (99-100%) for all three sampling methods for P. aeruginosa and S. aureus.

In a study of 43 children with CF, results of culture of sputum or cough swab samples in those unable to expectorate sputum were compared with the results from induced samples taken following administration of 6% hypertonic saline solution (HTS). The procedure of sputum induction was tolerated in 41/43 patients. Four patients were able to expectorate sputum before and 19 after HTS induction. Four pre-HTS samples were positive when post-HTS samples were negative. Thirteen post-HTS samples were positive when pre-HTS samples were negative. The authors concluded that cultures on HTS-induced samples can provide additional microbiological information (Ho et al, 2004 [III]).

Maiya et al (2004, [III]) carried out a randomised prospective study of respiratory sample collection involving 31 patients with CF aged between eight and 16 years old and found that of 20 who had a positive sputum culture, 16 were also positive by cough plate (sensitivity 80%); this compared with seven positive cultures each for moistened swab and dry swab (sensitivity 35%). P. aeruginosa was isolated from cough plates as effectively as from sputum but not from cough swabs. Conversely
Aspergillus spp. were isolated from sputum samples but not from either cough plates or cough swabs.

The performance of BAL, protected brush specimens and spontaneously expectorated sputum from 12 CF adults as specimens for bacteriological culture of the lung was evaluated by Aaron et al (2004 [III]). P. aeruginosa was isolated from each of the samples from all 12 subjects. Genotyping of isolates showed full correspondence between the specimens, indicating that bronchoscopy did not offer any advantage in sensitivity.

**Recommendations**

- **Respiratory sampling should be undertaken at each hospital visit and at times of respiratory exacerbation [C].**
- **Sputum is the recommended specimen for routine sampling [B].**
- **A cough swab should only be used if a patient cannot expectorate [C].**
- **Other methods should be considered to identify lower airway infection in patients who are clinically deteriorating, particularly those with persistently negative cough swabs. These include induced sputum and BAL. Current evidence does not strongly support one particular method [C].**

**2.2 Transport and storage of samples**

Traditional teaching has been that processing the sample as soon as possible after collection will yield the most accurate microbiological results. It was postulated that any delay in processing, particularly with storage at room temperature, increased the overgrowth of more-rapidly multiplying bacteria, which could mask true pathogens; conversely, preventing overgrowth by refrigeration could result in the death of fragile pathogenic organisms. As both storage at room temperature and refrigeration could influence the eventual interpretation of cultures, the emphasis has been on prompt processing to optimize the quality of the analysis.

There are no studies evaluating the optimal environmental conditions for storing CF sputum specimens prior to microbiological analysis. However, the survival of organisms in non-CF sputa under different storage conditions has been evaluated.

**2.2.1 Refrigeration**

Some studies have reported negative effects of refrigeration on sputum cultures. Gould et al (1996, [III]) found that *H. influenzae, Moraxella catarrhalis* and *S. pneumoniae* were lost in 8.7% of samples kept at 4°C for 48h. Sputum samples from patients with bronchiectasis were studied using a quantitative method, comparing counts obtained from immediate processing with those following storage at 4°C for 24h or following storage at 20°C for 24h, including the survival when samples were posted back by first-class mail (Pye et al, 2008 [III]). Fifteen of the 38 samples studied had *P. aeruginosa* as the predominant organism. There was at least a 10-fold loss in viability in 24% of samples stored at 4°C compared with only 8% stored at 20°C. There is also evidence that freezing of sputum from patients with non-CF bronchiectasis reduces the recovery of *P. aeruginosa* (Murray et al, 2009 [III]) and Bcc isolates “die” (actually become dormant) when kept at 4°C (Pitt and Govan, 1993 [III]).

However, other studies have not demonstrated a negative impact of refrigeration on the quality of sputum samples. Quantities of *S. aureus, P. aeruginosa, H. influenzae* or *S. pneumoniae* were unaffected by storage at 4°C for up to two days (Wong et al, 1984 [III]). Williams et al (1978, [III]) also found that pneumococci survived refrigeration for several days. A prospective study on 50 sputum samples
(patient details were not provided) showed that, although refrigeration of sputum led to changes in the interpretation of the Gram stain, it did not lead to significant loss of bacterial growth after 20 hours of refrigeration (Penn and Silberman, 1984 [III]). Refrigeration at 2-4°C for up to 14 days was shown to minimise the reduction in viability of mycobacteria (Tраore and Slosareк, 1981 [III]). During the same study, samples kept at 37°C saw significant increases in numbers of non-specific microflora and almost complete loss of mycobacteria between the fourth and eighth day.

### 2.2.2 Storage at room temperature

A 50% decrease in the isolation rate for *H. influenzae* and *S. pneumoniae* was observed in 102 samples of sputum submitted by post from patients attending chest clinics. Samples were typically delayed by 24 to 30 hours prior to culture (May et al, 1964 [III]). *S. pneumoniae* was shown to survive at room temperature for more than two days but overgrowth occurred after 12 hours (Williams et al, 1978 [III]). A study of 34,314 sputum samples submitted to a reference laboratory showed that fungi (including *Aspergillus* species) could still be isolated even after delays of up to three months after collection (Hariri et al, 1982 [III]).

The ability to grow mycobacteria from sputum specimens was reduced by storage at room temperature in a tropical country although smear-positivity was unaffected for four weeks. Culture positivity declined from 88% at the time of storage to 83%, 68%, 22%, 13% and 0% after three, seven, 14, 21, and 28 days, respectively. It was therefore recommended that samples should not be stored for more than three days before culture (Paramasivan et al, 1983 [IV]).

### 2.2.3 National recommendations

The HPA National Standard Method (HPA BSOP 57, 2008 [IV]) advises that respiratory specimens should be transported and processed as soon as possible; it does not make specific recommendations regarding CF specimens. It advises that sputum may be refrigerated for up to 2-3 hours without an appreciable loss of pathogens and, if processing is delayed, refrigeration is preferable to storage at ambient temperature.

The American Society for Microbiology Cumulative Techniques and Procedures in Clinical Microbiology (Gilligan et al, 2000 [IV]) states that if transport is needed, the specimens should be transported at 4°C; “*P. aeruginosa* survives this temperature for 24 hours, and most of the other pathogens of CF patients will survive this temperature for a minimum of 1 to 2 hours”.

#### Recommendations

- **Samples should be sent promptly to the laboratory and processed as soon as possible; there is insufficient evidence to judge whether refrigeration of samples produces better microbiological results than keeping samples at room temperature. However, current consensus is that if samples are delayed for more than a few hours they should be refrigerated at 4°C** [C].

- **The results from the culture of respiratory samples from people with CF submitted to the laboratory by post should be interpreted with caution** [C].
3. SAMPLE PROCESSING

3.1 Homogenisation

The viscosity and mucopurulent nature of sputum presents challenges in obtaining appropriate samples for microbial culture, and particularly when quantitative culture is required. Methodologies to liquefy or reduce viscosity include mechanical disruption by glass beads or chemically by mucolytic agents.

Dithiothreitol (DTT) was evaluated as a mucolytic agent using sputum samples from 79 people with CF (Hammerschlag et al, 1980 [IIb]). Un-liquefied sputum cultures failed to yield three of 47 *Pseudomonas aeruginosa* isolates but liquefied sputum cultures missed five of 13 isolates of *Candida albicans*. Neither liquefied nor un-liquefied samples were completely robust for isolating *Staphylococcus aureus*. There were too few isolates of *Haemophilus influenzae* to evaluate the two methods but the final concentration of DTT (50 mg/l) used was potentially inhibitory for some isolates of *H. influenzae*. Although there was no evidence that liquefaction with DTT improved recovery of CF-associated pathogens, it did allow them to be quantified.

DTT was compared to homogenisation with saline or glass beads for recovery of bacteria from sputum samples obtained from 18 people with non-CF bronchiectasis (Pye et al, 1995 [IIb]). The quantity of pathogen recovered was significantly higher from samples homogenised using DTT compared to either saline or glass beads, particularly of *H. influenzae*. DTT had no inhibitory effect on the recovery of *P. aeruginosa*, *H. influenzae*, *Streptococcus pneumoniae*, or *Moraxella catarrhalis*.

Homogenisation using DTT and quantification of bacterial counts on selective media was studied using 60 sputum samples from children with CF (Wong et al, 1984 [IIb]). There was a poor correlation between the predominant organism identified after qualitative culture of non-homogenised sputum and the numbers of bacteria obtained using homogenisation with DTT and quantification. When *P. aeruginosa* was the predominant organism on routine qualitative culture it was also the most numerous on quantitative culture. The same was also true for *H. influenzae*. However, when *S. aureus* was the predominant organism on routine qualitative culture there were higher numbers of *P. aeruginosa* than *S. aureus* isolated using quantitative culture.

**Recommendation**

- **There is insufficient evidence to properly assess the value of homogenisation when processing sputum samples from people with CF. However, the use of mucolytic agents, such as dithiothreitol, allows dilution and facilitates retrieval of micro-organisms present in the sample [C].**

3.2 Microscopy

3.2.1 Gram stain

The utility of sputum Gram stain in assessing both salivary contamination and in predicting the presence of pathogens was investigated using 287 respiratory samples from 270 people with CF (Sadeghi et al, 1994 [IV]). Microscopy was performed by a single observer, assessing sample quality based on the ratio of leucocytes to squamous epithelial cells (a ratio of >5 indicated acceptable sample quality) and predicting culture results on the basis of observed bacterial morphotypes. Overall 220 (77%) of samples were of acceptable quality. The appearance of bacteria on direct microscopy...
of sputum had a positive predictive value for subsequent culture of 98% for *P. aeruginosa*, 84% for *Burkholderia cepacia* complex, 86% for *S. aureus*, and 100% for *H. influenzae*.

This was further evaluated using sputum samples from 101 people with CF (Nair et al, 2002 [IIB]). Microscopy was performed by two different observers in a blinded fashion. Subjectively all but one sample appeared purulent but only 59 samples would have been accepted for culture using a quality score based upon leucocytes and squamous epithelial cells. In addition, there was agreement for the quality score between both observers for only 21 samples, suggesting poor reproducibility. All but four of the 41 samples that would have been rejected using the quality score grew CF-associated Gram-negative bacilli. The positive predictive value of large quantities of Gram-negative bacilli and subsequent growth of *P. aeruginosa* was 85%. However, the negative predictive value for absence of Gram-negative bacilli and negative culture was very poor at just 10%.

**Recommendation**

- **There is insufficient evidence to support the routine use of Gram staining of sputum samples from people with CF, either as a marker of specimen quality or as a predictor of subsequent culture results [B].**

### 3.2.2 Acid-fast staining for mycobacteria

The 2007 American Thoracic Society (ATS) Guidelines recommend the use of a fluorochrome technique, such as auramine-phenol, in preference to other staining methods, such as Ziehl-Neelsen as the former is more sensitive for the detection of mycobacteria, including NTM (Griffith et al, 2007 [IV]). However, it was noted in the 2007 ATS Guidelines that some rapid-growing NTM such as *Mycobacterium abscessus* may over-decolourize and not stain using fluorochrome techniques (Griffith et al, 2007 [IV]).

**Recommendation**

- **Acid-fast staining for mycobacteria in CF respiratory samples should be performed using a fluorochrome method e.g. auramine-phenol [C].**

### 3.3 Decontamination methods – mycobacteria

To improve the recovery of mycobacteria from CF respiratory samples a decontamination step is used, primarily to prevent the overgrowth of mycobacterial cultures with *P. aeruginosa*. The efficacy of different methods has been studied.

Whittier et al (1993, [III]) compared the impact of adding oxalic acid to a standard decontamination procedure. The standard decontamination procedure consisted of 0.25% N-acetyl-L-cysteine and 1% sodium hydroxide (NALC-NaOH) and was applied to 121 respiratory samples from people with CF prior to mycobacterial culture. Subsequently, 74% of Lowenstein-Jensen (LJ) slopes and 36% of liquid culture vials were contaminated with *P. aeruginosa*. Fourteen of the samples were smear-positive for acid-fast bacilli (AFBs), nine of which yielded NTM on culture. The decontamination procedure was modified by adding 5% oxalic acid after the initial phase of NALC-NaOH, and tested using a further 441 respiratory samples from people with CF. Contamination with *P. aeruginosa* was significantly reduced, with only 5% of LJ slopes and 3% of liquid culture vials affected. Ninety of the samples were smear-positive, all of which yielded NTM on culture.
Another study compared contamination rates and yield of mycobacteria following two different
decontamination procedures using 406 respiratory samples from 148 people with CF (Bange et al,
1999 [III]). All samples were cultured using the BACTEC MGIT 960 liquid culture system.
Contamination rates were 58% with NALC-NaOH and 26% with NALC-NaOH-oxalic acid (NALC-
NaOH-Oxa). Eleven samples yielded mycobacteria. Five samples were positive after each method,
three only after NALC-NaOH and three only after NALC-NaOH-Oxa. The authors suggested
mycobacterial growth was inhibited either by bacterial overgrowth of NALC-NaOH-treated samples
or by the direct toxicity of oxalic acid.

The proficiency of 20 laboratories for detecting NTM was tested using five simulated sputum
samples (Whittier et al, 1997 [III]). All laboratories were expected to use the NALC-NaOH-Oxa
decontamination procedure. The decontamination procedure worked well, with only 6% of samples
contaminated with P. aeruginosa. Most laboratories gave correct smear and culture results on negative
samples or those with a heavy inoculum of an NTM. However, only 55% of laboratories were able
to isolate Mycobacterium chelonae when the inoculum was low. The authors concluded that the oxalic acid
might kill some mycobacteria present in samples. The culture methods used by the participating
laboratories were not stated.

A comparison between three decontamination methods was made using serial dilutions containing
Mycobacterium abscessus in mock samples contaminated with P. aeruginosa and Bcc. Methods utilised
either 4% NaOH, 5% oxalic acid, or NALC-NaOH-Oxa (Jordan et al, 2008 [III]). Cultures were
performed both on LJ slopes and in a liquid culture system (BacT/Alert, Biomerieux). Both oxalic
acid-containing methods were more sensitive in detecting M. abscessus than the use of NaOH alone.

The NALC-NaOH-Oxa method was compared with a chlorhexidine-based method using 827
sputum samples obtained from 289 people with CF (Ferroni et al, 2006 [III]). All cultures were
performed on LJ slopes. The initial phase of the study compared the two methods during recovery
of differing dilutions of M. abscessus and Mycobacterium avium. The chlorhexidine method was more
sensitive, yielding 10-100 times more CFU than the NALC-NaOH-Oxa method. During the field test
using 827 CF sputum samples, 20% of chlorhexidine-treated samples were contaminated in
comparison to only 14% of NALC-NaOH-Oxa samples (p = 0.0017). Sixty samples were culture-
positive for NTM. Of these 33 grew after decontamination with chlorhexidine, six after
decontamination with NALC-NaOH-Oxa and 21 after decontamination with both methods. In
summary, although contamination rates were higher with the chlorhexidine method, it was more
sensitive than NALC-NaOH-Oxa for detecting NTM.

A study compared the recovery rate of mycobacteria from LJ slopes and two automated liquid culture
systems, the BACTEC 460TB system (Becton Dickinson, Heidelberg, Germany) and the BACTEC
MGIT 960 system (Becton Dickinson). A total of 2,624 clinical samples were processed, of which 242
samples came from 106 people with CF (Leitritz et al, 2001 [III]). All samples were
decontaminated using NALC-NaOH. CF-associated samples were processed with the addition of
polymyxin B, amphotericin B, carbencillin and trimethoprim to LJ slopes and polymyxin B,
amphotericin B, nalidixic acid, trimethoprim and azlocillin to the two BACTEC systems.
Contamination rates for non-CF-associated samples were 5.3%, 3.3% and 6.9% for LJ slopes, the
460TB and MGIT 960 systems, respectively. For CF-associated samples, rates increased to 14.5%,
24.8%, and 16.9%, respectively for the same three culture methods.
Recommendations

- A decontamination procedure should be used to reduce P. aeruginosa contamination of CF-associated respiratory samples sent for mycobacterial analysis. Options include the use of NALC-NaOH-Oxa or chlorhexidine.

3.4 Conventional culture

3.4.1 Pseudomonas aeruginosa

There are few data on the utility of specific selective media for isolation of P. aeruginosa from the respiratory secretions of people with Cystic Fibrosis.

A comparison of different selective and non-selective media on 258 respiratory tract secretions from an unknown number of people with CF was conducted at a single North American CF centre (Doern et al, 1992 [III]). All plates were incubated at 35°C in 5-7% CO2 for 72 hours. Of the 137 isolates of P. aeruginosa obtained, all 137 (100%) grew on Maconkey agar, 136/137 (99.3%) grew on chocolate agar and 113 (82.5%) grew on cetrimide agar.

A novel chromogenic agar for isolating and identifying P. aeruginosa (PS-ID) was compared against 5% horse blood agar (BA) and Pseudomonas CN selective agar (PCN) using 100 sputa from distinct people with CF (Laine et al, 2009 [III]). Plates were incubated at 37°C in air for 72 hours. P. aeruginosa was isolated from 62 samples after 72 hours incubation, with 59 samples (95.2%) positive on both PS-ID and PCN and 56 samples (90.3%) positive on BA. Almost all isolates were grown within 48 hours incubation. Colonies producing a purple colour on PS-ID had a positive predictive value of 98.3% for being P. aeruginosa.

The expression of the mucoid phenotype on six different isolation media was examined using 15 P. aeruginosa isolates from people with CF (Pugashetti et al, 1982 [III]). All 15 expressed the mucoid phenotype on Pseudomonas Isolation Agar (PIA), and 14 of 15 expressed it on Maconkey agar. Expression on four other non-selective, basal media was much more variable. Govan and Deretic (1996, [IV]) observed that if an isolate was non-mucoid on PIA it was always non-mucoid on other isolation media.

Some constituents of selective media have been shown to be inhibitory for CF isolates of P. aeruginosa. Of 200 CF isolates tested, 22 were inhibited by 16mg/l Irgasan (the selective agent in PIA), and 45 and 15 were inhibited by 8mg/l nalidixic acid and 128mg/l cetrimide, respectively (the latter two being selective agents in pseudomonas selective agar) (Fonseca et al, 1986 [III]).

Recommendations

- There is insufficient evidence to suggest that use of a selective medium enhances the yield of P. aeruginosa from the respiratory secretions of people with CF. However, the use of some selective media may assist in identification, particularly the appearance of pigmentation and the mucoid phenotype.

- Plates should be incubated at 35-37°C in air and examined after overnight incubation and after at least another 24 hours.

- The presence of P. aeruginosa should always be reported, irrespective of quantity.
3.4.2 

**Burkholderia cepacia complex**

The use of a selective medium is essential for the isolation of *B. cepacia* complex (Bcc) and inhibition of other organisms commonly found in the respiratory secretions of people with Cystic Fibrosis.

*Pseudomonas cepacia* (PC) medium supported superior growth of 38 of 50 stock isolates of Bcc compared to non-selective Maconkey agar (0 of 50) after 48 hours incubation (Gilligan et al, 1985 [III]).

In a comparative clinical study respiratory secretions from 169 people with CF attending two CF Centres in the United States were cultured onto PC medium and non-selective Maconkey agar. Plates were incubated at 35°C in air for 48 hours. Bcc was isolated from 35 people using PC medium but only 21 people using Maconkey agar (Gilligan et al, 1985 [III]). In the same study PC medium only allowed six (2.7%) of 221 other CF-associated pathogens to grow through on the medium.

The use of a Bcc selective medium has been associated with significantly higher detection rates of Bcc. A study conducted in the USA found that 14 of 15 (95%) laboratories using a Bcc selective medium isolated Bcc from simulated CF sputum samples compared to 22 of 100 (22%) of laboratories that did not use such a medium (p < 0.0001) (Tablan et al, 1987 [III]). The selective media used were either PC medium or oxidation-fermentation polymyxin-bacitracin-lactose (OFPBL) medium.

The comparative performance of different selective media for Bcc has been assessed in trials. An evaluation of three selective media (PC medium, OFPBL, and trypan blue-tetracycline (TB-T) agar) for Bcc was conducted using 20 Bcc isolates from people with CF, 20 from people without CF and evaluation of three selective media (PC medium, OFPBL, and trypan blue-tetracycline (TB-T) agar) for Bcc. After 72 hours incubation, the sensitivities of BCSA, OFPBL, and PC medium were 100%, 96%, and 84% respectively. BCSA was also superior to OFPBL and PC medium for suppressing non-Bcc isolates; 40 non-Bcc organisms were isolated on BCSA, 263 from OFPBL and 116 from PC medium. No comment was made on recovery at different incubation temperatures.

A comparative evaluation of three selective media (PC medium, OFPBL, and *Burkholderia cepacia* selective agar (BCSA)) was conducted using 328 samples of respiratory secretions from 209 children with CF attending two paediatric CF Centres and 328 samples of respiratory secretions from 109 adults with CF attending a single adult CF Centre (Henry et al, 1999 [III]). All three Centres were in North America. Plates were either incubated at 35-37°C (two Centres) or 30°C (one Centre) in air for 72 hours. Five (1.5%) samples from four (1.9%) children and 75 (22.9%) samples from 16 (14.7%) adults grew Bcc. After 72 hours incubation, the sensitivities of BCSA, OFPBL, and PC medium for detecting Bcc were 100%, 96%, and 84% respectively. BCSA was also superior to OFPBL and PC medium for suppressing non-Bcc isolates; 40 non-Bcc organisms were isolated on BCSA, 263 from OFPBL and 116 from PC medium. No comment was made regarding the impact of different temperatures of incubation.

A comparative evaluation of two selective media (BCSA and MAST selective agar) was conducted using 149 expectorated sputum samples from 113 people with CF (32 Bcc-colonised, 81 non-Bcc-colonised) attending three CF Centres in Belfast and Manchester (Wright et al, 2001 [III]). Plates were incubated at 37°C in air for 48 hours. All 53 Bcc isolates (100%) were grown within 48 hours on BCSA, compared to 50 of 53 (94.3%) on MAST selective agar. The other three did grow on MAST selective agar after five days of incubation. Twenty-eight non-Bcc isolates grew on MAST selective agar, compared to 13 on BCSA (Wright et al, 2001 [III]).

**Recommendations**

- **Laboratories should use a selective medium to enhance the detection of Bcc** [B].
The Bcc selective medium should have a high sensitivity and specificity for Bcc e.g. BCSA, MAST selective agar [B].

Laboratories should incubate Bcc selective medium plates at 35-37°C in air for five days to maximize sensitivity; plates should be reviewed daily [C].

The presence of Bcc should always be reported, irrespective of quantity [C].

3.4.3 Staphylococcus aureus

The use of mannitol salt agar (MSA) has been associated with improved detection of S. aureus. A collection of 60 sputum samples from 14 people with CF yielded 50 samples positive for S. aureus from 11 people. Plates had been incubated at 37°C in air for 48 hours. The inclusion of MSA increased the number of samples from which S. aureus was isolated from 21 to 50 when compared to non-selective media (Sparham et al, 1978 [III]).

A retrospective review of all culture results of CF respiratory samples during a single calendar year in one US laboratory found 207 samples positive for S. aureus (Sharp & Searcy, 2006 [IV]). More than 98% of those samples with greater than 1+ of growth of yellow colonies on MSA were ultimately confirmed as S. aureus on further testing.

A comparative evaluation of MSA and CHROMagar Staph aureus selective and differential agar (CSA) was conducted using 220 respiratory samples from an unknown number of people with CF (Flayhart et al, 2004 [III]). Plates were incubated at 37°C in 5-10% CO2 for 72 hours. A total of 66 samples were positive for S. aureus, all of which were positive on CSA (100%) and 59 (89.4%) were positive on MSA. Incubation of plates for 48 hours resulted in an increase in S. aureus recovery of 8% for MSA and 11% for CSA compared to 24 hours incubation. No non-S. aureus isolates were misidentified on CSA, giving 100% specificity. The cost of CSA ($3.07 per plate) was higher than the cost of MSA ($1.31) in this study but could be offset by reducing the cost of additional subcultures ($1.03) and additional biochemical confirmatory tests e.g. slide coagulase (Flayhart et al, 2004 [III]).

A comparative evaluation of CSA, MSA, and 5% sheep’s blood agar (BA) was conducted using 200 sputum samples from an unknown number of people with CF (D’Souza & Barron, 2005 [III]). Plates were incubated at 37°C in air for 72 hours. S. aureus was isolated from 50 samples. After 16-20 hours incubation 39 samples (78%) were positive on CSA compared to 30 samples (60%) and 29 samples (58%) for BA and MSA, respectively. Two (4%) S. aureus isolates did not grow after 48 hours incubation. After 72 hours incubation the sensitivities of CSA, MSA, and BA were 98%, 96%, and 96% respectively (p = NS). The specificity of CSA was 99% (D’Souza & Barron, 2005 [III]).

The ability of two different chromogenic agars and four conventional media for isolating small-colony variants (SCV) of S. aureus was assessed using 53 well characterised strains, some of which had been obtained from the sputa of people with CF (Fipp et al, 2005 [III]). The media used were S. aureus ID (SAID), CSA, MSA, Baird-Parker agar, tryptic soy agar and Columbia blood agar. After 72 hours of incubation all SCV isolates were recovered on Columbia blood agar. However, one isolate failed to grow on either SAID or Baird-Parker agar, three failed to grow on either CSA or MSA and five failed to grow on tryptic soy agar.

Recommendations

A selective medium should be used to enhance the detection of S. aureus from respiratory samples of people with CF. Options include mannitol salt agar and CHROMagar Staph aureus selective and differential agar [B].
Select plates for *S. aureus* should be incubated at 35-37°C in air and examined after overnight incubation and after at least another 24 hours [C].

The presence of *S. aureus* should always be reported, irrespective of quantity [C].

### 3.4.4 Haemophilus influenzae

The recovery of *H. influenzae* from the respiratory secretions of people with CF has been enhanced by the use of a medium supplemented with N-acetyl-D-glucosamine (NAG), haemin (X factor), NAD (V factor) and cefsulodin disks (Moller et al, 1993 [III]). The NAG medium was compared with chocolate agar and chocolate agar with an added bacitracin disk for isolating *H. influenzae* from 203 sputum samples obtained from 30 people with CF. Plates were incubated overnight at 37°C in 5% CO₂. *H. influenzae* was isolated from 86 samples (42%) from 24 people. The recovery rate on NAG was superior to chocolate with or without a bacitracin disk, with 84/86 (97.7%) isolates growing on NAG, compared to 46/86 (53.5%) and 62/86 (72.1%) growing on chocolate with or without a bacitracin disk, respectively.

A comparative evaluation of two selective media, cefsulodin chocolate blood agar (CCBA) and haemin-bacitracin blood agar (HBBA), and non-selective chocolate agar (CA) was conducted on 73 respiratory samples from an unknown number of people with CF (Smith & Baker, 1997 [III]). CCBA plates were incubated overnight at 37°C in 5% CO₂. *H. influenzae* was isolated from 13 samples. All 13 were recovered on CCBA, ten on HBBA, and only three on non-selective CA.

**Recommendations**

- A selective medium should be used to enhance the recovery of *H. influenzae*. Options include chocolate agar supplemented with either bacitracin or cefsulodin [B].
- Plates should be incubated at 35-37°C in 5% CO₂ and examined after overnight incubation and after at least another 24 hours [C].
- As isolation of *H. influenzae* could indicate contamination from the upper respiratory tract, it may be helpful to report the approximate amount present [C].

### 3.4.5 Other Gram-negative bacilli

A novel Gram-negative selective agar (GNSA) was evaluated using a three-stage process (Moore et al, 2003 [Hb]). Stage one tested its ability to grow a laboratory collection of organisms, comprised of 31 examples of CF-associated Gram-negative bacilli, 11 non-CF-associated Gram-negative bacilli, 13 Gram-positive organisms, and seven fungi. Stage two tested its ability to recover *P. aeruginosa*, *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia* from simulated sputum samples and stage three tested its ability to recover organisms from 12 sputum samples submitted from 12 people with CF. Plates were incubated at 37°C in air for 48 hours. GNSA supported the growth of all Gram-negative bacilli but not Gram-positive organisms or fungi. It was able to support the growth of organisms in simulated sputum samples at quantities in the range 1.5 x 102 to 6.7 x 103 CFU/ml. GNSA successfully recovered all Gram-negative bacilli from the 12 sputum samples from people with CF, including the isolation of *Achromobacter xylosoxidans* from two people that was not recovered using standard culture media (Maconkey agar, Pseudomonas Isolation Agar and blood agar).

A comparative evaluation of vancomycin-amphotericin B-imipenem (VIA) agar, a selective medium for *S. maltophilia*, against bacitracin-chocolate agar (BC) with an additional imipenem disk was
conducted using 814 sputum samples from 87 people with CF (Denton et al, 2000 [III]). Plates were incubated at 37°C in air for 48 hours. Two hundred and thirty-five samples from 34 people with CF were positive for *S. maltophilia*. All 235 (100%) were positive on VIA but only 106/235 (45.1%; p < 0.0001) were positive on BC. Other imipenem-resistant Gram-negative bacilli, including *P. aeruginosa* and Bcc, were isolated on VIA.

### Recommendations

- **Laboratories may wish to consider additional selective media for isolating *S. maltophilia* and other Gram-negative bacilli only if they have particular clinical needs in enhancing their recovery [C].**

### 3.4.6 Fungi

An investigation into the prevalence of *Aspergillus fumigatus* and other fungal species in adults with CF was conducted at a single CF Centre in Germany (Bakare et al, 2003 [III]). Three hundred and sixty-nine sputum samples were received from 94 subjects and *A. fumigatus* was isolated from 109 (29.5%) samples from 43 subjects (45.7%). Of the samples positive for *A. fumigatus*, 85 (78%) grew on Sabouraud glucose agar (SGA) with additional antibiotics (0.5 g/l ciprofloxacin and 0.5 g/l amikacin), 82 (75.2%) on SGA without additional antibiotics, 46 (42.2%) on chocolate agar and 25 (22.9%) on blood agar. All media had been incubated at 37°C in air for three days. The incorporation of antibiotics into the SGA medium significantly reduced contamination rates with *P. aeruginosa*.

A comparative evaluation of Sabouraud dextrose agar (SDA) and a novel fungal medium “Medium B” (MB), both with or without the addition of selective antibiotics (128 mg/l cotrimoxazole, 50 mg/l chloramphenicol, 32 mg/l ceftazidime, 24 mg/l colistin), was conducted using a representative collection of fungal and bacterial species commonly encountered in respiratory samples from people with CF (Nagano et al, 2008 [IIb]). All four media were also evaluated for their ability to isolate fungi from the sputa of 28 people with CF. Plates were incubated at 22°C in air for two weeks. SDA without additional antibiotics was the best medium for supporting the growth of the representative collection of fungi, but was also the one most likely to become contaminated with bacteria. When used to isolate fungi from CF sputum samples, MB with the addition of antibiotics was more sensitive than either SDA or SDA with additional antibiotics (92.3% versus 84.6% for both versions of SDA, respectively). However, SDA with additional antibiotics was more specific for fungi than MB with additional antibiotics (89.2% versus 85.7%, respectively) (Nagano et al, 2008 [IIb]).

### Recommendations

- **Sabouraud medium should be used to enhance the recovery of fungi from respiratory samples of people with CF [B].**

- **The addition of appropriate antibiotics reduces contamination rates with *P. aeruginosa* [B].**

- **Plates should be incubated at 35-37°C in air and examined after overnight incubation and after at least another 24 hours. Prolonging cultures up to seven days and at different temperatures (e.g. 22°C) may increase yield [C].**
3.4.7 Mycobacteria

A meta-analysis of ten studies (none specifically performed on samples from people with CF) compared the performance of Lowenstein-Jensen (LJ) medium with the BACTEC 460 TB radiometric system and BACTEC 960/MGIT non-radiometric system for recovering 1,381 isolates (571 NTM) of mycobacteria from 14,745 clinical samples (Cruciani et al, 2004 [IIa]). Relative performance of the three systems for recovery of NTM is shown in the table below.

The sensitivity of both the 960/MGIT and 460 TB systems was increased to 76% and 88% respectively by using them together with LJ medium.

<table>
<thead>
<tr>
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<th>960/MGIT</th>
<th>460 TB</th>
<th>LJ Medium</th>
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<td>Sensitivity – all NTM</td>
<td>66%</td>
<td>75%</td>
<td>51%</td>
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<td>Sensitivity – M. avium complex</td>
<td>80%</td>
<td>73%</td>
<td>57%</td>
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<td>Contamination rate</td>
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<td>Mean time to positivity</td>
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<td>20.3 days</td>
<td>24.3 days</td>
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</table>

**Recommendation**

- Culture of respiratory samples from people with CF for mycobacteria should be performed using both an automated liquid culture system and a solid medium (e.g. LJ medium) to maximise sensitivity [B].

3.5 Molecular detection direct from samples

A number of molecular assays for detecting CF-associated pathogens have now been reported but few studies have subjected these techniques to rigorous evaluations against conventional culture methods using respiratory samples taken from people with CF.

3.5.1 *Pseudomonas aeruginosa*

A multiplex PCR (polymerase chain reaction) based on simultaneous amplification of two lipoprotein genes of *P. aeruginosa, oprI* and *oprL*, was evaluated using 49 sputum samples from people with CF (De Vos et al, 1997; [III]). Conventional culture was conducted using blood agar, chocolate-bacitracin agar and Maconkey agar with plates incubated at 35°C in air (chocolate-bacitracin in 5% CO₂) for 48 hours. All 40 samples positive for *P. aeruginosa* on conventional culture were PCR-positive. Four samples negative on culture for *P. aeruginosa* were also PCR-positive.

A PCR method for detecting *P. aeruginosa* was compared with conventional culture on cetrimide agar using 15 respiratory samples (ten sputum, five throat swabs) from an unknown number of people with CF (da Silva Filho et al, 1999 [III]). The PCR targeted the *algD* GDP mannose gene. PCR was positive in seven samples, six of which were also positive on culture. One sample was culture-positive but PCR-negative.

Molecular detection of *P. aeruginosa* using PCR was compared with conventional culture detection using a combination of blood agar, Maconkey agar, and *Pseudomonas Isolation Agar* (Xu et al, 2004 [III]). Both methods were used on sputum samples submitted from 57 adults with CF. PCR was performed using *oprL* and *exoA* targets. Plates used during conventional detection were incubated at 37°C in air for 48 hours. Thirty sputum samples were positive for *P. aeruginosa* using conventional methods and 35 were positive by PCR. The *oprL* target was more sensitive than the *exoA* target. Five adults with initially negative sputum samples on culture were PCR-positive and then subsequently became culture-positive a mean duration of 4.5 months later (range 4 – 17 months).
PCR amplifying the \( \text{algD} \) GDP mannose gene was compared with conventional culture and detection of serum antibodies against three \( P. \text{aeruginosa} \) antigens (elastase, alkaline protease, exotoxin A) for the early diagnosis of \( P. \text{aeruginosa} \) colonisation/infection in 87 people with CF with a mean age of 9.7 years (da Silva Filho et al, 2007 [III]). Culture was positive in 42/87 (48.2%) whilst PCR was positive in 53/87 (60.9%; \( p = \text{NS} \)). Serology was positive in 38/87 (43.6%). The combination of PCR plus serology was superior to each single method, to PCR plus culture, and to culture plus serology.

### 3.5.2 Burkholderia cepacia complex

A PCR method to detect Bcc was compared to conventional culture using Macconkey agar supplemented with an unspecified concentration of polymyxin (Whitby et al, 1998 [III]). Plates were incubated for five days. Both methods were applied to 219 sputum samples from adult (109 samples) and paediatric (110 samples) CF Centres. In total 63/219 (28.8%) samples were culture-positive for Bcc, of which 60 (95.2%) were PCR-positive. Conversely there were seven culture-negative samples that were PCR-positive for Bcc.

PCR amplification of the \( \text{recA} \) gene followed by restriction fragment length polymorphism (RFLP) analysis was compared with conventional culture on MAST selective agar incubated at 37\(^\circ\)C in air for 48 hours using 100 sputum samples from people with CF (McDowell et al, 2001 [IIb]). Seventeen samples were positive for Bcc on culture, all of which were PCR-positive. RFLP revealed that the Bcc species isolated were \( B. \text{cepacia} \) (11 were IIIA, four were IIIB) and \( B. \text{multivorans} \) (two). There were no positive PCR results with culture-negative samples.

A novel rRNA gene-based PCR assay successfully and rapidly identified 69 Bcc-positive sputa, irrespective of the Bcc species. The technique exhibited 100% sensitivity and specificity for all 17 Bcc species and detected 104 CFU/ml when applied to sputum (Brown and Govan 2007 [III]). More recently, a monoclonal antibody, mAb 5D8, was produced against Bcc LPS. mAb 5D8 reacted with a proteinase-K-sensitive 22 kDa antigen in all nine Bcc species tested, and also in \( B. \text{cepacia} \), suggesting that mAb 5D8 might be helpful in rapid diagnosis of \( B. \text{cepacia} \) spp. in people with CF (AuCoin et al 2010 [III]).

### 3.5.3 Other CF-associated pathogens/multiplex PCR

Routine microbiological culture was compared with molecular detection of three CF-associated pathogens using sputum samples from six people with CF (van Belkum et al, 2000 [III]). Conventional cultures were performed on Columbia agar, Macconkey agar, chocolate agar and mannitol salt agar. Bacterial genes for the small subunit ribosomal RNA (ssu rDNA) were specifically amplified from DNA extracted from the sputum samples and hybridised with probes for \( P. \text{aeruginosa} \), \( S. \text{aureus} \), and \( H. \text{influenzae} \). DNA from \( S. \text{aureus} \) and \( H. \text{influenzae} \) was identified from three and four samples, respectively, which had not been detected by conventional culture. No samples were positive for \( P. \text{aeruginosa} \).

Routine microbiological culture was compared with molecular detection of \( S. \text{maltophilia} \) using species-specific PCR on 13 sputum samples submitted from an unknown number of people with CF (Whitby et al, 2000 [III]). The conventional culture method used was unspecified. \( S. \text{maltophilia} \) was isolated from three sputum samples obtained from two people with CF. There was concordance between the results of conventional culture and PCR.

A multiplex PCR method targeting \( P. \text{aeruginosa} \), Bcc and \( S. \text{maltophilia} \) was compared with conventional culture on 257 respiratory samples obtained from 106 people with CF (da Silva Filho et al, 2004 [III]). \( P. \text{aeruginosa} \) was isolated by culture in 56% of samples, Bcc in 4.3%, and \( S. \text{maltophilia} \) in 2.7%. By comparison multiplex PCR identified \( P. \text{aeruginosa} \) in 78.7% of samples, Bcc in 3.9%, and
4. IDENTIFICATION

4.1 Phenotypic identification of non-fermentative Gram-negative bacilli

Accurate identification of non-fermentative Gram-negative bacilli (NFGNB) from CF-associated respiratory samples raises a number of challenges. Isolates of Pseudomonas aeruginosa may display atypical colonial morphology, lack of pigmentation, a mucoid phenotype, and slow or auxotrophic growth. Laboratories relying solely on phenotypic identification methods may therefore misidentify P. aeruginosa or fail to differentiate between Burkholderia spp. and other NFGNB which can sometimes be isolated from people with CF (Table 1). Commercial platforms e.g. API 20NE, VITEK 2 (Biomerieux), BDPhoenix (Becton Dickenson) and MicroScan Walkaway (DadeBehring), have also been shown to be unreliable (Joyanes et al, 2001 [III]; Wellinghausen et al, 2005 [III]; Snyder et al, 2008 [III]). Misidentification of P. aeruginosa or Bcc as another species or falsely identifying other organisms as P. aeruginosa or Bcc has important clinical consequences leading to unnecessary or inappropriate antimicrobial therapy or segregation of patients.

Molecular methods [see Section 4.2] are able to improve the identification of NFGNB in comparison to phenotypic methods (Wellinghausen et al, 2005 [III]; Bosshard et al, 2006 [III]; Kidd et al, 2009 [III]), although laboratories will differ in their ability to provide these in-house. A review of the performance of phenotypic and molecular tests is provided along with recommendations that can be adapted locally by laboratories processing CF samples.

Table 1: Examples of non-fermenting Gram-negative bacilli isolated from CF patients that can cause identification problems

A collection of 150 isolates of non-fermenting Gram-negative bacilli (NFGNBs) obtained from respiratory samples of people with CF was subjected to identification using four different commercial phenotypic identification kits (Kiska et al, 1996 [III]). Fifty-eight of the isolates were members of the Bcc. The accuracies of the four kits for identifying all of the NFGNBs ranged from 57 – 80%. Accuracies for identifying isolates as Bcc ranged from 43 – 86%. All the kits also misidentified some non-Bcc isolates as Bcc.

The accuracy of nine different commercial kits for identifying Bcc obtained from CF sputum cultures was assessed by comparing the identification results of 1,051 isolates sent by 108 referring centres (Devine et al, 2004 [IV]. Three samples were positive for mycobacteria. Mycobacterium xenopi was identified in one adult and one child and Mycobacterium chelonae in another adult. Eleven false-positives were documented as a result of cross-reactivity with Corynebacterium spp.

Recommendations

- There is currently insufficient data to recommend the routine application of molecular detection methods directly to samples in addition to routine culture methods [C].
- Direct PCR for M. tuberculosis in respiratory samples should be considered in patients in at risk groups who are smear positive for acid fast bacilli [C].
4. IDENTIFICATION

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Table 1: Examples of non-fermenting Gram-negative bacilli isolated from CF patients that can cause identification problems

| Achromobacter xylosidans |
| Burkhodleria cepacia complex |
| Burkhodleria gladioli |
| Cupriavidus respiraciuli |
| Inquilinus limosus |
| Pandoreoa apista |
| Pandoraea promenusa |
| Pandoraea pulmonicola |
| Pandoraea sputorum |
| Pseudomonas aeruginosa |
| Ralstonia insidiosa |
| Ralstonia mannitollytica |
| Ralstonia pickettii |
| Stenotrophomonas maltophilia |

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The accuracy of nine different commercial kits for identifying Bcc obtained from CF sputum cultures was assessed by comparing the identification results of 1,051 isolates sent by 108 referring...
laboratories with those of a reference laboratory (Shelly et al, 2000 [III]). Of the 770 isolates provisionally identified as Bcc by the referring laboratory, 682 (89%) were confirmed as Bcc by the reference laboratory. Of the 88 isolates not confirmed as Bcc, 28 (32%) were identified as *Burkholderia cepacia* complex (1). API 20NE reliably identified *Burkholderia cepacia* complex (1) and *Stenotrophomonas maltophilia* isolates within three hours.

The reliability of the automated VITEK 2 system (BioMerieux, Marcy l'Etoile, France) for identifying *P. aeruginosa* and *Stenotrophomonas maltophilia* was evaluated using 146 and 27 isolates of the two organisms, respectively (Joyanes et al, 2001 [IV]). It was not clear if any isolates were obtained from people with CF. The system correctly identified 91.6% of *P. aeruginosa* isolates and 100% of *S. maltophilia* isolates.

A study using 106 isolates of *S. maltophilia* from respiratory samples compared API 20NE, Vitek 2 and a 23S rRNA-based species specific PCR for their reliability in identification (Giordano et al, 2006 [III]). Most were reliably identified by API 20NE and Vitek 2. Only one isolate gave a low discrimination result with API 20NE, compared to 12 isolates with Vitek 2.

A comparison of the automated Phoenix system (BD diagnostics, Sparks, Maryland, USA) and the MicroScan WalkAway system (Dade Behring, West Sacramento, California, USA) was conducted using 45 isolates of NFGNBs, including eight obtained from CF sputum samples (Snyder et al, 2008 [III]). The Phoenix system misidentified one of 26 *P. aeruginosa* isolates. Both systems were unreliable in identifying isolates from CF sputum samples, such as *Burkholderia cenocepacia*, *Pandoraea pnomenusa*, and *Ralstonia mannitolilytica*. The authors advised caution in using either system for identifying less commonly encountered isolates from people with CF.

A study comparing the results of the API 20NE (BioMerieux, Marcy l’Etoile, France) with 16S rRNA molecular identification was conducted using 88 isolates of NFGNBs cultured from the respiratory secretions of people with CF that did not conform to typical phenotypic appearances for *P. aeruginosa* (Wellinghausen et al, 2005 [III]). API 20NE correctly identified only 15 (17%) of these 88 isolates. There was agreement between API 20NE and 16S rRNA identification for five of seven (71%) where API rated the identification as ‘Excellent’. However, agreement was poor when API rated the identification as ‘Very Good’ (six of 15 – 40%), ‘Good’ (three of 19 – 16%) or ‘Acceptable’ (one of 11 – 9%).

A study by an Australian reference laboratory determined if 2,267 isolates obtained from 561 people with CF were correctly identified as *P. aeruginosa* by 17 different referring laboratories (Kidd et al, 2009 [III]). The reference laboratory used a number of phenotypic and genotypic methods, including a *P. aeruginosa* species-specific PCR to confirm their identity. Overall, 2,214 (97.9%) of isolates from 531
(94.7%) people with CF had been correctly identified as *P. aeruginosa* by the referring laboratory. These laboratories had used a combination of phenotypic methods (colonial appearance, pigmentation, oxidase reaction, growth at 42°C, colistin susceptibility, commercial identification systems, such as API 20NE and Vitek, and growth on selective media) to confirm the identity of *P. aeruginosa* isolates. Only five of the 17 referring laboratories used molecular identification methods and only when they encountered an atypical isolate that could not be identified with first-line phenotypic tests. Of the 53 mis-identified isolates most were either *A. xylosoxidans* (40%) or *S. maltophilia* (28%). None were mis-identified isolates belonging to the *Bcc*.

Two Europe-wide quality assurance trials on CF microbiology conducted in 2007 and 2008 (Hogardt et al, 2009 [III]), despatched nine formulations containing CF-associated pathogens to 31 and 37 laboratories supporting large CF Centres in 18 and 21 European countries, respectively. Although laboratories rarely misidentified *P. aeruginosa* or *Staphylococcus aureus*, 4/31 (13%) laboratories failed to detect *B. cepacia* and 11/37 (30%) failed to detect *Burkholderia vietnamiensis*. Participants also had difficulty in isolating and correctly identifying rarer pathogens, such as *A. xylosoxidans*, *Inquilinus limosus*, and *Pandoraea pnomenusa*.

**Recommendations**

- **All non-fermenting Gram-negative bacilli should be identified to species level [C].**

- **P. aeruginosa** isolates with typical characteristics (e.g. green pigmentation, positive oxidase test, growth at 42°C) can be reliably identified with these tests alone [B].

- **An excellent identification from a commercial kit such as API 20NE, in conjunction with colonial appearance, characteristic oxidase test and antibiogram, can be used to confirm the identity of *Stenotrophomonas maltophilia*, *Achromobacter* spp. and non-pigmented *P. aeruginosa* [B].**

- **Commercial kits/systems should NOT be used to identify members of the *Burkholderia cepacia* complex, *Pandoraea* spp., *Ralstonia* spp., and *Burkholderia gladioli* [B].**

- **The identity of members of the *Burkholderia cepacia* complex, atypical isolates of *S. maltophilia*, *Achromobacter* spp. and *P. aeruginosa*, and any colomycin resistant non-fermenting Gram-negative bacilli must be confirmed using molecular identification methods [B].**

A suggested algorithm based on these recommendations is provided (see figure 1).
4.2 Molecular identification

4.2.1 Burkholderia cepacia complex

Taxonomic classification advanced sufficiently in the late 1990s so as to allow the separation of organisms originally described simply as "B. cepacia", into nine separate species or genomovars, known as the B. cepacia complex (Bcc) (Mahenthiralingham et al, 2000 [III]). Further recent studies have led to the addition of eight species so that currently the Bcc comprises seventeen species (Vanlaere et al, 2008 [III]; Vanlaere et al, 2009 [III]; Table 2). All species of the Bcc have been isolated from a wide variety of ecological niches, and with few exceptions all have been isolated from people with CF. Currently the most prevalent species in CF are Burkholderia multivorans and Burkholderia cenocepacia (Govan et al, 2007 [III]).

Table 2: Members of the Burkholderia cepacia complex and relevant references

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<tr>
<td>B. stabilis</td>
<td>Mahenthiralingham et al, 2000</td>
</tr>
<tr>
<td>B. vietnamiensis</td>
<td>Mahenthiralingham et al, 2000</td>
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<tr>
<td>B. dolosa</td>
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</tr>
<tr>
<td>B. ambifaria</td>
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<tr>
<td>B. ubonensis</td>
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<tr>
<td>B. latens</td>
<td>Vanlaere et al, 2008</td>
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<tr>
<td>B. diffusa</td>
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<tr>
<td>B. arboris</td>
<td>Vanlaere et al, 2008</td>
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<tr>
<td>B. seminalis</td>
<td>Vanlaere et al, 2008</td>
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<tr>
<td>B. metallica</td>
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<tr>
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Members of the Bcc are intrinsically multi-drug resistant, a characteristic which, when combined with the increased virulence and transmissibility associated with certain strains, can present significant problems for therapeutic management and infection control. Accurate and prompt identification of Bcc members is therefore crucial. Though growth on selective media may be suggestive of Bcc isolation, other CF-related organisms can also grow, making confirmation of identification by molecular means essential.

A number of approaches exist for the identification and speciation of the Bcc. 16S ribosomal RNA gene sequencing is useful for identification to genus level but has limited benefits for species.
4.2 Molecular identification

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Taxonomic classification advanced sufficiently in the late 1990s so as to allow the separation of organisms originally described simply as “B. cepacia”, into nine separate species or genomovars, known as the B. cepacia complex (Bcc) (Mahenthiralingham et al, 2000 [III]). Further recent studies have led to the addition of eight species so that currently the Bcc comprises seventeen species (Vanlaere et al, 2008 [III]; Vanlaere et al, 2009 [III]; Table 2). All species of the Bcc have been isolated from a wide variety of ecological niches, and with few exceptions all have been isolated from people with CF. Currently the most prevalent species in CF are Burkholderia multivorans and Burkholderia cenocepacia (Govan et al, 2007 [III]).

Table 2: Members of the Burkholderia cepacia complex and relevant references

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A number of approaches exist for the identification and speciation of the Bcc. 16S ribosomal RNA gene sequencing is useful for identification to genus level but has limited benefits for species
differentiation, since the species share a high degree of 16S rRNA sequence similarity (Vanlaere et al, 2008 [III]). Other options include multilocus sequence typing (Baldwin et al, 2005 [III]), as well as other single gene targets (Drevinek et al, 2008 [III]; Lynch & Dennis, 2008 [III]).

To date, the most successful method is based on species variation within the recA gene, a gene responsible for DNA repair and recombination. A simple PCR assay using primers BCR1 and BCR2, which span the entire recA gene, will identify an isolate as being a member of the Bcc (Mahenthiralingam et al, 2000 [III]).

In rare cases these primers will not successfully amplify in a true Bcc isolate. If other evidence suggests the possibility of Bcc (for example, previous Bcc isolation, growth on selective media, colistin resistance, an API profile suggesting the possibility of Bcc) the isolate can be identified to species level using one of the following methods:

### 4.2.1.1 Species-specific PCR for identification of Bcc species

Until recently, this method was routinely used to speciate the Bcc. However, current data suggest that, with the exception of B. multivorans, cross-reactions between some species can occur, leading to false positive results (Mahenthiralingam et al, 2000 [III]; Vermis et al, 2002 [III]; Turton et al, 2007 [III]). It is therefore advisable to confirm positive results by an alternative method such as recA sequencing.

### 4.2.1.2 recA PCR and restriction fragment length polymorphism (RFLP)

This method involves recA gene amplification using the BCR1 and BCR2 primers, followed by digestion of the PCR amplicon with restriction enzymes HaeIII and MnlI. Though this method works efficiently, the matching of RFLP pattern with species type requires some expertise.

#### Recommendations

- **recA PCR should be used to identify any suspicious isolates as a member of the Burkholderia cepacia complex [B].**

- **All recA PCR-positive isolates should be further characterised to species level by recA sequencing. RecA-based PCR tests are no longer specific enough for this purpose with the exception of B. multivorans [B].**

- **If the diagnostic laboratory does not have the facilities for molecular identification of the Bcc, the isolate should be sent to the Health Protection Agency CF Microbiology Reference Laboratory in the Centre for Infection, Colindale, London [C].**

### 4.2.2 Pseudomonas aeruginosa

There are several simple PCR assays that are both sensitive and specific for P. aeruginosa. PCR primers and the specific thermal cycler conditions can be found in the relevant references.

Single target PCR for the algD, 16S rDNA, gyrB, toxA, ecfX genes and the 16S -23S rDNA intergenic region have all been shown to identify P. aeruginosa with 100% specificity (Tyler et al, 1995 [III]; da Silvo Filho et al, 1999 [III]; Spilker et al, 2004; Lavenir et al, 2007 [III]). A number of other targets (16S rDNA, oprL, oprL, flc) have been evaluated in multiplex and real-time assays using P. aeruginosa and other NFGNB recovered from CF sputum (Mahenthiralingam et al, 2000 [III]; Lavenir et al, 2007 [III]). Laboratories with appropriate structures in place to carry out quality assured molecular assays can be confident in the ability of these PCRs to confirm the identification of P. aeruginosa.
Recommendations

- A validated species-specific PCR should be used when molecular identification of *P. aeruginosa* is required [C].

4.2.2.1 Serological diagnosis of *Pseudomonas aeruginosa* infection

Serological diagnosis of *P. aeruginosa* infection by detection of a range of species specific antibodies in serum or respiratory samples has been a subject of long interest in CF microbiology, particularly for the early detection of *P. aeruginosa* in non-sputum producing infants; but the use of serological testing with conventional antigens has proved controversial (Farrell & Govan, 2006 [IV]). Possible new targets have been developed that may be used in conjunction with molecular and culture methods, including A-band LPS (Weisner et al, 2007 [III]) and the outer membrane protein, OprL (Rao et al, 2009 [III]).

Recommendations

- Detection of *P. aeruginosa* antibodies is of little value in chronic infection. Although controversial, it is sometimes used in early detection of *P. aeruginosa* colonisation when used in conjunction with conventional culture and molecular detection methods [C].

4.2.3 Non-tuberculous mycobacteria (NTM)

A number of in-house molecular assays have been developed to facilitate the identification of NTM.

PCR and gene sequencing of the hypervariable areas of 16S rRNA was compared with conventional biochemical methods and nucleic acid hybridization (NAH) using a collection of 69 isolates of mycobacteria (Han et al, 2002 [III]). Overall concordance of gene sequencing with NAH and biochemical methods was 87% (59/68 isolates). Twenty-five isolates (36%) failed to identify using NAH. Most of the nine discordant identifications were novel species of mycobacteria that could not be reliably identified using biochemical methods.

PCR-restriction fragment length polymorphism analysis (PRA) of the *hsp65* gene was applied to 108 isolates (65 clinical, 43 reference) of 34 different species of mycobacteria (Devalois et al, 1997 [III]). A number of *Mycobacterium* species, including *Mycobacterium abscessus* and *Mycobacterium kansasii* yielded more than one specific PRA pattern.

PRA of the *hsp65* gene was compared to conventional biochemical methods for the identification of 541 clinical isolates of mycobacteria (Prammanan et al, 2005 [III]). Discordant results were obtained for 24 (4.4%) of isolates, including nine rapid growers (six *M. chelonae*, two *M. abscessus*, one *M. fortuitum*) and 15 slow growers (including one *M. avium*) isolate. The molecular assay had a turnaround time of 30 hours in comparison to conventional biochemical methods that had a turnaround time of two to four weeks.

A combination of in-house real-time PCR targeting the *rpoB* gene and 16S rRNA PCR followed by gene sequencing was used to identify 194 isolates of mycobacteria (Williams et al, 2007 [III]). Results were compared with those obtained by a reference laboratory. Using real-time PCR, 172 (89%) isolates gave identification concordant with those of the reference laboratory. The remaining 22 failed to amplify, giving a specificity of 100% and sensitivity of 83%. Sensitivity was increased to 96% when a bead-based DNA extraction method replaced the previous crude extraction method.

An in-house, multiplex real-time PCR assay was evaluated using 314 mycobacterial isolates obtained...
from 233 patients (Richardson et al, 2009 [III]). Primer targets included internal transcribed spacers, insertion sequence IS\textsubscript{1311}, and 16S rRNA. The assay was able to assign identity of isolates to the \textit{Mycobacterium chelonae / abscessus} group (68 isolates) and \textit{M. avium} complex (98 isolates) with a sensitivity of 100% and 99% and specificity of 100% for both, respectively. Further characterisation to species level was not obtained with this method.

A number of commercial molecular assays for identification of mycobacteria are also available.

The LiPA MYCOBACTERIA assay (Innogenetics NV, Ghent, Belgium), which combines PCR amplification of the 16 – 23S rRNA spacer region of mycobacteria followed by hybridization of the biotinylated PCR product with 14 specific oligonucleotide probes, was compared with PRA of the \textit{hsp65} gene, conventional biochemical testing and DNA probes for identifying 60 isolates of mycobacteria (Miller et al, 2000 [III]). Complete agreement between results of LiPA MYCOBACTERIA and the other methods occurred with 50/60 (83%) of isolates. The assay had problems differentiating \textit{M. avium} from \textit{M. intracellulare} and was unable to identify \textit{M. fortuitum} to species level. However, a further evaluation of the LiPA MYCOBACTERIA assay using 168 isolates of mycobacteria obtained from 2,532 respiratory and extrapulmonary sources showed its superior performance in comparison to a DNA probe system (AccuProbe, Gen-Probe, San Diego, USA) (Scaparo et al, 2001 [III]).

The utility of a commercial reverse line probe assay (GenoType Mycobacterium CM/AS, Hain Life Sciences, Nehren, Germany) was compared with HPLC and 16S rRNA gene sequencing for the identification of 131 isolates of NTM (Lee et al, 2009 [III]). Concordance between the commercial assay and the two reference methods was obtained for 119 (90.8%) of isolates. However, identification of \textit{M. abscessus} and \textit{Mycobacterium lentiflavum} was problematic using the assay. A much larger study of the GenoType Mycobacterium CM/AS assay was conducted on 1,181 isolates of mycobacteria collected from 12 different hospitals between 2005 and 2007 (Couto et al, 2009 [III]). The system correctly identified 144 of the 149 (96.6%) NTM isolates, including \textit{M. avium}, \textit{M. chelonae}, and \textit{M. abscessus}.

Commercial systems have been shown to mis-identify isolates of lesser known mycobacterial species. The AccuProbe, LiPA MYCOBACTERIA and GenoType Mycobacterium systems were all evaluated using a panel of 317 well characterised isolates of mycobacteria belonging to 136 taxa (species or complex), 61 of which had not been tested previously by any of the systems (Tortoli et al, 2009 [III]). The LiPA MYCOBACTERIA mis-identified 20 taxa, most of which cross-reacted with probes for \textit{M. fortuitum} or \textit{M. avium-intracellulare} complex and the GenoType Mycobacterium misidentified 28 taxa, most of which cross-reacted with \textit{M. intracellulare} or \textit{M. fortuitum} probes.

Recent taxonomic changes have separated \textit{M. abscessus} into three closely-related species, namely \textit{M. abscessus} sensu stricto, \textit{Mycobacterium massiliense}, and \textit{Mycobacterium bolletti}. This separation was based on the findings of nucleotide sequence analysis. However a recent study has suggested that the species assigned may vary depending on the molecular target used (Macheras et al, 2009 [III]). A collection of 59 isolates of \textit{M. abscessus} sensu stricto from 58 people with CF was subjected to species identification using nucleotide sequencing of three different target genes: \textit{rpoB}, \textit{hsp65}, and \textit{sodA}. Concordant results were obtained for 44 isolates but 15 isolates gave discordant results, with the identity varying with the target used. The authors suggested that the previous taxonomic separation of \textit{M. abscessus}, \textit{M. massiliense} and \textit{M. bolletti} should be reviewed.

A study of 40 clinical isolates belonging to the \textit{M. abscessus} group (27 \textit{M. abscessus} sensu stricto, 11 \textit{M. massiliense}, two \textit{M. bolletti}) was performed to correlate identification with clinical outcomes (Zelazny et al, 2009 [III]). Three isolates (11%) of \textit{M. abscessus} sensu stricto and three isolates (27%) of \textit{M. massiliense} were obtained from people with CF. There were too few isolates to infer any clear correlation between species identification and clinical impact.
Recommendation

- The identification of NTM is a highly complex area and should only be carried out in laboratories with sufficient knowledge and expertise e.g. mycobacteriology reference laboratory [B].

4.3 Molecular typing

4.3.1 Pseudomonas aeruginosa

Until recently, acquisition of P. aeruginosa was thought to involve unrelated organisms primarily acquired from the environment. The exceptions to this were CF siblings who frequently possessed the same strain. However, the introduction of microbiological surveillance and improved molecular techniques has implicated an increasing number of transmissible “epidemic” strains responsible for outbreaks in both paediatric and adult CF units in the UK and worldwide. These epidemic strains frequently exhibit phenotypic characteristics that are atypical of P. aeruginosa, making them difficult to identify without molecular analyses. Some are non-motile and non-pigmented, and in addition many are multi-resistant to the major anti-pseudomonal antibiotics (Chambers et al, 2005 [III]). Currently, the four most common transmissible strains in the UK are the Liverpool (LES), Manchester (MAN), Clone C and Midlands1 (Md11) epidemic strains. Their correct identification has important implications both for infection control and therapeutic management.

A multiplex PCR assay allows the identification of LES, Midlands1 and MAN (Fothergill et al, 2007 [III]). Primers for the specific identification of P. aeruginosa can also be incorporated in this multiplex PCR allowing both the identification of P. aeruginosa and the major epidemic strains (see Table 3). It should be noted, however, that the LES markers, PS21 and F9, are not unique to LES, nor are both present in all LES isolates (Fothergill et al, 2010 [III]).

Table 3: PCR primers for LES, Midlands1 and MAN multiplex PCR (Fothergill et al, 2007).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>For the identification of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS21F</td>
<td>5'-AAG CAG GCC AGC GTG TCT A-3'</td>
<td>Liverpool epidemic strain (LES)</td>
</tr>
<tr>
<td>PS21R</td>
<td>5'-AAA ACG TAG CAA GCA GTG-3'</td>
<td></td>
</tr>
<tr>
<td>LESF9 F</td>
<td>5'-AAC ACT TGC TCC ATC TGC-3'</td>
<td>Liverpool epidemic strain (LES)</td>
</tr>
<tr>
<td>LESF9 R</td>
<td>5'-CAC GAT ATC CAG CAA GAC-3'</td>
<td></td>
</tr>
<tr>
<td>MA 1SF</td>
<td>5'-GTC GGC AGA TAG CCT TTG TC-3'</td>
<td>Manchester epidemic strain (MAN)</td>
</tr>
<tr>
<td>MA 1SR</td>
<td>5'-CGA CTA ATA CCC GTC GCT TC-3'</td>
<td></td>
</tr>
<tr>
<td>MID 1F</td>
<td>5'-TTG GCC TCC ATC GTT TGA3'</td>
<td>Midlands 1 epidemic strain</td>
</tr>
<tr>
<td>MID 1R</td>
<td>5'-CTC CAG ATG CTT ACG AAA-3'</td>
<td></td>
</tr>
</tbody>
</table>

*For the positive identification of LES, a band of the correct size is required for both sets of LES-specific primers (PS21 and LESF9).

Molecular typing of P. aeruginosa in the last decade has been mainly performed using PFGE (Scott & Pitt, 2004 [III]). PFGE has proved a useful typing method but results are not easily comparable between laboratories, and it is time-consuming. A novel molecular typing technique for P. aeruginosa has recently undergone a successful trial alongside PFGE (Turton et al, 2009 [III]). Multiple-Locus Variable-Number Tandem-Repeat (VNTR) analysis is a PCR-based system that compares repetitive elements at multiple loci. It has been successfully used to type numerous pathogenic bacteria, including P. aeruginosa (Vu-Thien et al, 2007 [III]). Each strain is designated a VNTR code based on the number of repeats at selected loci. VNTR is a rapid technique that is both sensitive and specific.
Another advantage of VNTR is that the production of codes allows direct comparison of isolates between laboratories performing the test independently.

It is recommended that all Specialist CF Centres and CF Clinics undertake pro-active surveillance at local and national level to ensure that evidence of cross-infection with P. aeruginosa is rapidly detected to enable appropriate measures to be put in place to limit spread (CF Trust Infection Control Working Group, November 2004). Surveillance should be performed on all new isolates, and annually on a relevant proportion of positive patients.

**Recommendations**

- **Molecular typing of P. aeruginosa should be performed using a standard method as determined by the Health Protection Agency CF Microbiology Reference Laboratory, in the Centre for Infection, Colindale, London [C].**

**4.3.2 Burkholderia cepacia complex**

Epidemic strain markers are unreliable for accurate identification of Bcc with enhanced transmissibility. The *Burkholderia cepacia* Epidemic Strain Marker (BCESM) and the gene responsible for cable-like pili (*cblA*) have been used for the identification of the ET-12 strain of *Burkholderia cepacia* IIIA as it appeared this lineage was unique in possessing both markers. However, both these markers can be lost from ET-12 and recent data suggests that *cblA*-positive isolates exist that are distinct from the ET-12 lineage (Turton et al, 2009 [III]). Finally, there are numerous examples of Bcc strains responsible for epidemic spread that contain neither *cblA* nor BCESM.

**Recommendations**

- **All confirmed B. cepacia isolates should undergo molecular typing to establish whether they are members of the ET-12 or other epidemic lineages [C].**

- **Epidemic strain markers are not sufficiently reliable to determine the potential transmissibility of Bcc [B].**

- **If the diagnostic laboratory does not have the facilities for molecular typing of B. cepacia, the isolate should be sent to the Health Protection Agency CF Microbiology Reference Laboratory in the Centre for Infection, Colindale, London [C].**
5. SUSCEPTIBILITY TESTING

5.1 Antimicrobial susceptibility testing

Laboratory standards in the USA recommend the use of agar diffusion methods for susceptibility testing (Saiman & Siegel, 2003 [IV]). However, a survey of the laboratory protocols of 150 of the 190 laboratories providing support to US CF care centres found that only 52% of these were performing agar diffusion methods (e.g. disc diffusion, Etest) for susceptibility testing (Zhou et al, 2006 [III]). An external quality assessment scheme for German laboratories processing CF respiratory samples revealed a wide variability in methods used for susceptibility testing and inconsistent performance (Balke et al, 2008 [III]).

For UK laboratories, detailed methods on susceptibility testing are available from the British Society for Antimicrobial Chemotherapy (BSAC). Another useful source of guidance and information is the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST). There has been a recent move to standardise breakpoints across the countries of the European Union and a European disc diffusion method is being developed through EUCAST, which, if accepted, will introduce further standardisation.

All general national and international methods are based on recommendations for the treatment of organisms causing acute infection and PK/PD models using blood levels of antibiotics. These methods do not therefore address the particular challenges of treatment in the CF respiratory tract, where lower concentrations may be achieved when antibiotics are administered systemically or conversely where local deposition to lungs achieved by aerosolised therapy results in higher antibiotic levels. Nor are current methods designed to test organisms causing chronic infection that, in the case of P. aeruginosa, is associated with slow-growing bacterial populations within complex polymicrobial biofilms.

The BSAC guidelines are appropriate for testing organisms associated with acute infections that grow overnight without the need for special growth requirements. Once bacteria cause chronic infection in CF, mixtures of colonial morphotypes are observed. At this stage, the susceptibility of different morphotypes and even the same morphotype within a single specimen can vary greatly (Foweraker et al, 2005 [III]). Atypical forms can include slow growing variants. All these factors can make antimicrobial susceptibility testing difficult, particularly as an aid to the choice of antibiotic(s) and forecasting clinical efficacy.

Some bacterial species cultured from CF samples are rarely encountered in respiratory tract infections in non-CF patients. While guidance exists for Acinetobacter spp. using the BSAC method, it does not exist for others such as Bcc, other Burkholderia spp., Stenotrophomonas maltophilia, Achromobacter xylosidans, Bordetella spp., Ralstonia pickettii, Pandorea spp., or Inquilinus limosus in either BSAC or EUCAST recommendations. Although there are some data on the distribution of MICs in wild type strains of Bcc (Nzula et al, 2002 [IV]), very little has been published on the correlation of in vitro antibiotic susceptibility with the clinical efficacy of treatment. In practice, treatment of such infections, including life-threatening ‘cepacia syndrome’ is usually empirical, and based on combinations of 3-4 antibiotics delivered by a variety of routes (Cystic Fibrosis Trust, May 2009 [IV]; Grimwood et al, 2009 [IV]).
5.2 Susceptibility testing guidelines

5.2.1 Pseudomonas aeruginosa

A survey was conducted using 2,194 isolates of *P. aeruginosa* to assess the accuracy of the standardised BSAC disc diffusion method performed in 25 diagnostic laboratories compared to MIC determination in a central reference laboratory (Henwood et al, 2001 [III]). Ninety-eight of the isolates were from people with CF. While resistance was over-reported by the BSAC method, results obtained were more accurate than previously obtained with the non-standardised Stokes’ method.

The correlation between disc diffusion, Etest (AB Biodisk, Solna, Sweden) and a reference broth obtained from people with CF (Haussler et al, 2003 [III]). The system determines MICs using a broth microdilution method for susceptibility testing of 12 antimicrobials was evaluated using 597 isolates of *P. aeruginosa* obtained from CF sputum samples (Burns et al, 2000 [III]). Disc diffusion was performed using a standardised Clinical and Laboratory Standards Institute (CLSI) method. CLSI breakpoints were used to interpret Etest and broth microdilution results. Results of standardised disc diffusion and Etest both correlated well with MICs obtained by broth microdilution. Major errors (i.e. calling an organism resistant when susceptible) and very major errors (i.e. calling an organism susceptible when resistant) were 1.1 and 0.4% for standardised disc diffusion and 2.2% and 0.1% for Etest, respectively. Most problems were observed with mucoid strains, particularly when testing cefazidime or piperacillin-tazobactam.

The problems in measuring antibiotic susceptibility of *P. aeruginosa* were further illustrated by the results of the 2007 European Quality Assessment survey of diagnostic microbiology. An atypical *P. aeruginosa* from a CF patient was sent to 31 laboratories in Europe for susceptibility testing. Most laboratories used either disc diffusion or Etest. The results were described by the author as “close to random” for quinolones and tobramycin. For ciprofloxacin 36% of laboratories reported the organism as susceptible (S), 27% reported intermediate susceptibility (I) and 37% resistant (R). For tobramycin the results were 36% S, 27% I, and 36% R (Hoghardt et al, 2009 [III]).

The susceptibility results obtained by two automated devices were compared to those of the reference method (broth microdilution) using 498 isolates of *P. aeruginosa* obtained from people with CF (Burns et al, 2001 [III]). The devices used were Vitek (Bio-Merieux, Marcy l’Étoile, France) and MicroScan WalkAway (Dade-Behring, Sacramento, USA). More very major errors were seen using these devices than were previously encountered using broth microdilution, disc diffusion or Etest methods (Burns et al, 2000 [III]).

The Micronaut Merlin system was evaluated using 56 isolates of *P. aeruginosa* (22 were mucoid) obtained from people with CF (Haussler et al, 2003 [III]). The system determines MICs using a broth microdilution method. The results generated by the system were compared to an in-house broth microdilution method and agar dilution using CLSI breakpoints as standards. There were more very major errors (called susceptible when resistant) with the system compared to the in-house broth micro-dilution method, particularly for non-mucoid *P. aeruginosa*. A follow-up study further evaluated the system using 405 *P. aeruginosa* isolates (109 were mucoid) from 154 people with CF (Balke et al, 2004 [III]). The system generally reported lower MICs than were reported with a standard agar dilution method. This resulted in fewer major errors (calling resistant when susceptible) but the rate of very major errors (calling susceptible when resistant) was high (19.2%). In this study, these very major errors were more likely with mucoid strains.

A comparison was made between susceptibility testing of single *P. aeruginosa* morphotypes and a mix of all *P. aeruginosa* morphotypes using isolates obtained from 94 sputum samples from children with
CF (Morlin et al, 1994 [III]). Susceptibilities were performed using a broth microdilution method. Mixed morphotype testing correctly predicted susceptibility to a panel of seven agents on 90.4% of occasions but correctly predicted resistance on only 57% of occasions. Although the mixed morphotype method was quicker and cheaper than selecting individual morphotypes, it was less accurate.

A method of direct susceptibility testing on CF sputum samples was compared with testing of individual morphotypes using 316 sputum samples from people with CF (Zebouh et al, 2008 [III]). Etest strips for six different antibiotics (ticarcillin, ceftazidime, imipenem, aztreonam, ciprofloxacin and tobramycin) were placed directly onto agar plates inoculated with sputum. MICs were determined at 18h, 24h and 48h of incubation. P. aeruginosa was isolated from 303/316 (95.8%) samples, with a bacterial load of >10^5 CFU/ml in 276 (91%). Agreement between the direct method and testing of individual morphotypes occurred for between 90.4% (ticarcillin) and 96.3% (imipenem) of sputa. The direct susceptibility method was less able to detect P. aeruginosa if other Gram-negative bacilli (e.g. Bcc) or S. aureus were present in the sample and was particularly poor if the bacterial load of P. aeruginosa was <10^5 CFU/ml. No comment was made regarding the impact of this method on laboratory resources.

One study proposed revised breakpoints for tobramycin when testing P. aeruginosa isolates from people with CF receiving aerosolised tobramycin therapy (Morosini et al, 2005 [III]). The Spanish Antibigram Committee (The Mensura Group) advised the adoption of higher breakpoints for those receiving aerosolized rather than parenteral tobramycin. These breakpoints were susceptible <64 mg/l and resistant >128 mg/l, as compared to the normal CLSI breakpoints of susceptible <4 mg/l, intermediate 8 mg/l, and resistant >16 mg/l. Using the lower CLSI breakpoints susceptibility rates were 79% and 81% for agar dilution and Etest methods, respectively. These rates increased to 95% using the proposed Mensura breakpoints. It is not known if these higher breakpoints correlate better with clinical outcomes.

The reproducibility of the BSAC disc diffusion method in ascertaining the susceptibility of P. aeruginosa isolates associated with chronic infection in CF was studied using 101 sputum cultures (Foweraker et al, 2005 [III]). A mean of four different morphotypes of P. aeruginosa were identified in each sample, each morphotype yielding a mean of three different antibiograms. Wide variability in susceptibility test results was observed, whether performed by different workers in the same laboratory or between different laboratories testing the same sample. The authors questioned the role of antimicrobial susceptibility testing once chronic P. aeruginosa infection was established in the CF lung.

Culture conditions may have a significant impact on the results of susceptibility testing. Twelve multi-resistant isolates of P. aeruginosa were obtained from sputum samples of six people with CF and grown either under planktonic conditions, as an adherent monolayer, or as a biofilm (Aaron et al, 2002 [III]). MICs and multiple combination bactericidal antibiotic susceptibility testing (MCBT) were performed using CLSI criteria. MICs of ceftazidime and ciprofloxacin were significantly higher for adherent monolayer and biofilm-grown bacteria compared to planktonic bacteria but largely unchanged for aminoglycosides and meropenem. The MCBT tests were no different for planktonic, adherent, or biofilm-grown bacteria for three isolates but for the other nine isolates two- and three-drug regimens were less active against biofilm-grown bacteria than adherent bacteria, which in turn were less active than against planktonic bacteria. Antagonism was seen against biofilm-grown bacteria but not against planktonic bacteria.

A similar study was conducted using 16 P. aeruginosa isolates from the sputa of 16 people with CF (Hill et al, 2005 [III]). MICs and MCBT to ten different agents were tested using CLSI criteria under aerobic and anaerobic planktonic conditions and when grown in biofilms. However, some results
conflicted with those obtained by Aaron et al (2002). Colistin was bactericidal against 100%, 75% and 25% of isolates under aerobic, anaerobic, and biofilm conditions, respectively. By contrast, meropenem was bactericidal against 69%, 31%, and 19% of isolates under the same conditions, respectively. MCBT results indicated significantly more bactericidal regimens (25/37) under aerobic conditions than either anaerobic (14/37) or biofilm (13/37) conditions. Antagonism was seen with 38%, 26%, and 36% of regimens under aerobic, anaerobic, and biofilm conditions, respectively.

A more recent study of susceptibility testing under biofilm testing found that 29% of CF *P. aeruginosa* isolates were resistant to double-antibiotic combinations (Dales et al, 2009 [III]). Interestingly the addition of either azithromycin or rifampicin to effective antibiotic combinations frequently resulted in antagonism.

Biofilm and conventional susceptibility tests were compared using isolates of *P. aeruginosa* obtained from 40 people with CF (Moskowitz et al, 2005 [III]). Conventional susceptibility was determined using a broth microdilution method and biofilm susceptibility using biofilms grown on pegs in microtitre trays (modified Calgary method). For chronic *P. aeruginosa* infections the outcome of conventional testing would be the inclusion of a β-lactam antibiotic in all regimens. Conversely, if treatment was based on biofilm testing, a β-lactam would be included in only 42.5% of regimens, and 57.5% of regimens would include azithromycin. There was significant discordance between the two susceptibility testing methods, with conventional and biofilm testing indicating the same combination on only 20% of occasions.

The correlation between conventional susceptibility testing results and clinical response to intravenous antibiotics (tobramycin plus ceftazidime) was evaluated in 77 people with CF with an acute exacerbation of chronic *P. aeruginosa* infection (Smith et al, 2003 [III]). MICs were determined (method not stated) and isolates categorised as susceptible or resistant on the basis of CLSI breakpoints. Fifty-four patients improved on therapy, nine worsened, and 14 remained unchanged. There was no correlation between susceptibilities to tobramycin and ceftazidime and the clinical response to therapy for acute exacerbations of chronic *P. aeruginosa* infection in CF.

A prospective, randomised, double-blind controlled clinical trial assessed whether MCBT improved clinical outcomes in people with CF experiencing acute infective exacerbations with multi-resistant bacteria, including *P. aeruginosa* (Aaron et al, 2005 [I]). Two hundred and fifty-one people with CF, each infected with multi-resistant pathogens, were enrolled in the study. Each study participant submitted a sputum sample every three months for conventional culture and susceptibility testing and MCBT. Over 4.5 years, 132 participants experienced an acute infective exacerbation and were randomised to receive a 14-day course of two intravenous antibiotics chosen either on the basis of conventional testing results or MCBT. Forty-three (67%) of those randomised to the MCBT group and 39 (57%) of those randomised to the control group grew *P. aeruginosa*. There were no significant differences between the two groups in clinical response rates or time to next exacerbation. The authors concluded that the additional complexity of MCBT did not result in improved patient outcomes.

One study has analysed the correlation between clinical outcomes and biofilm susceptibility testing by MCBT (Keays et al, 2009 [III]). Isolates from 110 acute exacerbations in people with CF were retrospectively subjected to biofilm susceptibility testing in addition to conventional MCBT. They included people chronically infected with *P. aeruginosa* (50 cases), *Bcc* (33), *A. xylosoxidans* (5), *S. maltophilia* (3) or various combinations of these four organisms (19). Sixty-six (60%) of 110 people were treated with combinations to which all their isolates were susceptible on conventional testing but only 24 (22%) were treated with combinations to which all isolates were susceptible on biofilm testing. When those treated with a combination to which at least one isolate was susceptible on biofilm testing were compared to those treated with a combination to which none were susceptible
there was no significant difference in treatment failure rates or time to next exacerbation but there was a significant trend to shorter hospital stay (13.3 days versus 17.4, p = 0.04). However, there was no difference in any outcome measure between patients with all isolates susceptible and those with at least one organism resistant by biofilm MCBT. This potential contradiction may be explained by study design and the complex mix of different species involved. Further work is therefore needed to clarify the role of biofilm MCBT testing in chronic infections associated with CF.

MCBT does not measure classical synergy. Instead it simply looks at the effect in vitro of antibiotic combinations at single concentrations. Therefore classical synergy testing methods, such as checkerboard and time kill studies, have been proposed as a way to find antibiotic combinations that may be effective against multi-resistant *P. aeruginosa*. A recent study of 44 resistant isolates of *P. aeruginosa* from nine people with CF compared the results of synergy testing by checkerboard and time kill with MCBT. The authors found that isolates with the same colonial morphotype or PFGE pulsortype from single sputum samples could produce different synergy results. The results also varied depending on the method used with no one method able to predict the outcome of treatment of an acute exacerbation (Foweraker et al, 2009 [III]).

The impact of a new protocol reducing the number of routine susceptibility tests performed on isolates of *P. aeruginosa* associated with chronic infection in CF was assessed by comparing two time periods in 2005 and 2006 (Etherington et al, 2008 [III]). In the first period in 2005 susceptibility tests were performed on all *P. aeruginosa* isolates obtained from every sample. In the second time period in 2006 susceptibility tests were only performed if the sample had been taken at the commencement of intravenous antibiotics, if there was a clinical deterioration, or if no tests had been performed in the previous three months. The testing method was Stokes’ disc diffusion. The number of tests performed in 2006 was reduced by 56%, saving an estimated 170 hours of laboratory time and €10,000 in consumables and salary costs. No significant differences in a number of clinical parameters were observed after the introduction of the new testing protocol, suggesting the omission of susceptibility tests had had no detrimental effect on clinical outcomes.

**Recommendations**

- *Susceptibility testing should be performed on isolates of* *P. aeruginosa* *associated with early and intermittent colonisation [C].*
- *Susceptibility testing should be performed using a standardised and validated method (e.g. BSAC disc diffusion) [B].*
- *Susceptibility testing of* *P. aeruginosa* *using automated devices cannot be recommended at this time [B].*
- *There is no evidence to support the routine use of multiple combination bactericidal antibiotic testing [A].*
- *There is no evidence to support the routine use of biofilm testing methods [B].*
- *Conventional susceptibility tests on* *P. aeruginosa* *isolates associated with chronic infection in CF are poorly reproducible and may not predict clinical response. Their omission does not adversely affect short-term clinical outcomes [B].*

**5.2.2 Burkholderia cepacia complex**

There is little published evidence specifically relating to susceptibility testing of Bec isolates from people with Cystic Fibrosis.
An initial evaluation of the Micronaut Merlin system included 14 ‘Burkholderia cepacia-like organisms’ (Haussler et al, 2003 [III]). Species listed included B. multivorans and B. cenocepacia. The number of major and very major errors was comparable to the in-house broth microdilution methods.

Problems were also encountered in measuring antibiotic susceptibility of Bcc in the 2007 and 2008 European Quality Assessment surveys of diagnostic microbiology. Most participating laboratories used agar diffusion or Etest and reported varying susceptibility results for B. cenocepacia and B. vietnamiensis (Hoghardt et al, 2009 [III]). It is unclear if these differences were because the organism formed a heterogeneous population, or were due to variations in methodology or because the breakpoints divided the distribution of MIC for the natural population.

Isolates from sputa of 110 people with CF were subjected to MCBT of 94 double- and triple-antibiotic combinations (Dales et al, 2009 [III]). Biofilm-grown isolates were significantly less susceptible to combinations than planktonic-grown bacteria. Fifty-nine percent of Bcc isolates were resistant to all double-antibiotic combinations tested. Triple-antibiotic regimens were significantly more active.

A study using three different species from the Bcc revealed that biofilm inhibitory concentrations of meropenem and piperacillin-tazobactam were considerably higher than MICs for planktonic-grown isolates (Caraher et al, 2006 [III]). Such differences were not observed for either tobramycin or amikacin.

The prospective, randomised, double-blind controlled clinical trial that assessed whether selecting antibiotic therapy on the basis of MCBT improved clinical outcomes in people with CF also included a number of people infected with Bcc (Aaron et al, 2005 [Ib]). Twenty-five (39%) of those randomised to the MCBT and 29 (43%) randomised to conventional susceptibility testing were infected with Bcc. There were no significant differences in outcome between the two groups.

**Recommendation**

- There is insufficient evidence to make any specific recommendations regarding susceptibility testing of Bcc isolates. If laboratories do perform susceptibility testing a standardised method should be used (e.g. BSAC disc diffusion) [C]. There are however no published breakpoints specifically for Bcc – see 5.2.3.

**5.2.3 Other Gram-negative bacilli**

There are no CF-specific publications relating to susceptibility testing of S. maltophilia.

Disc diffusion and Etest were compared with an agar dilution method using 70 isolates of S. maltophilia from a variety of clinical sites (Nicodemo et al, 2004 [III]). Methods were conducted using CLSI standards and the isolates were tested against chloramphenicol, doxycycline, gatifloxacin, trimethoprim-sulfamethoxazole, ticarcillin-clavulanate, polymyxin B and colistin. Good correlation between disc diffusion and Etest with agar dilution was observed for chloramphenicol, doxycycline, gatifloxacin, trimethoprim-sulfamethoxazole and ticarcillin-clavulanate but not for polymyxin B or colistin. Current BSAC recommendations for conducting susceptibility tests suggest that only trimethoprim-sulfamethoxazole can be reliably tested by disc diffusion or Etest (Andrews, 2009 [IV]).

There are difficulties in interpreting susceptibility results for many of the other unusual oxidase positive Gram-negative bacilli associated with CF (e.g. Achromobacter xylosoxidans, Pandoraea spp., Ralstonia spp.) as there is limited data on the distribution of MICs in the wild-type population and few have had breakpoints set by BSAC or EUCAST. EUCAST has however listed non species-specific
“clinical” breakpoints based on pharmacokinetics (Monte Carlo simulation) that may be used to categorise the in vitro susceptibility of these bacteria.

**Recommendations**

- **Susceptibility testing of S. maltophilia isolates should be guided by published recommendations, such as BSAC, CLSI, EUCAST, etc [C].**

- **EUCAST non species-specific breakpoints may act a guide for interpretation of susceptibility results for other unusual GNBs associated with CF [C].**

### 5.2.4 Staphylococcus aureus, Haemophilus influenzae

There are no CF-specific publications relating to susceptibility testing of *S. aureus* or *H. influenzae*.

**Recommendation**

- **Susceptibility testing of *S. aureus* and *H. influenzae* isolates should be guided by published recommendations, such as BSAC, CLSI, EUCAST, etc [C].**

### 5.2.5 NTM

A multicentre study evaluated the inter-laboratory reproducibility of broth microdilution susceptibility testing of ten isolates of rapid-growing mycobacteria (four *M. fortuitum*, three *M. abscessus*, and three *M. chelonae*) (Woods et al, 1999 [III]). Isolates were distributed to four different laboratories and each one was tested three times on three different days. There was inter-laboratory variation as to when the MIC was read (ranging from after three to after five days of incubation). There was wide variability in the level of agreement regarding susceptibility, ranging from 91.7% to 100% for cefoxitin against all isolates to 27.8% to 88.9% for clarithromycin against *M. fortuitum*. The authors suggested that a lack of experience on the part of some laboratories contributed to the wide variability seen.

A similar study was conducted to assess the inter-laboratory variability of susceptibility tests using Etest (Woods et al, 2000 [III]). The same ten isolates of rapid-growing mycobacteria were used. Similarly isolates were tested three times on three different days. The Etest gave reproducible results for some drugs (e.g. co-trimoxazole, doxycycline) but not for others (imipenem). Of greater concern was the poor correlation of Etest results with those obtained by the broth microdilution method, with Etest giving higher MICs and falsely reporting resistance.

A further study assessed inter-laboratory variability of broth microdilution conducted using two different media and macrodilution using the BACTEC 460TB system for susceptibility testing of macrolides against ten well-characterised isolates of *M. avium* (Woods et al, 2003 [III]). Results were more variable with broth microdilution, caused mainly by reader error and inexperience.

The recent description of inducible macrolide resistance in *M. abscessus* may explain some of the inter-laboratory variability, as it can cause difficulties reading disc susceptibility and trailing end points in broth microdilution testing (Nash et al, 2009 [III]).

**Recommendation**

- **Susceptibility testing of NTM is a highly complex area and should only be carried out in laboratories**
6. POST-ANALYTICAL PROCESSES: INTERFACING WITH THE CLINICAL TEAM

There are a number of reasons for ensuring rapid communication of particular laboratory results to clinicians caring for those with Cystic Fibrosis.

6.1 Infection Prevention

There are a number of recognized Infection Prevention hazards in CF Centres. Members of the *Burkholderia cepacia* complex, particularly *B. cenocepacia* IIIA, can spread rapidly from patient to patient (Mahenthiralingam et al, 2002 [IV]). The number of people with CF colonised and infected with *MRSA* is rising (Molina et al, 2008 [III]). A number of transmissible strains of *Pseudomonas aeruginosa* have also been recognized in CF Centres in the UK, Australia and Europe (Scott & Pitt, 2004 [III]; Govan et al, 2007 [III]; Brimicombe et al, 2008 [III]). Provisional data suggests that some of these transmissible strains have been associated with worse clinical outcomes. In Australia, paediatric deaths dramatically drew attention to the spread and virulence of the widespread clone PAS 1 (Armstrong et al 2002 [IV]). People with CF chronically infected with the Liverpool Epidemic Strain of *P. aeruginosa* (LES) had significantly worse lung function and poorer nutritional status than those chronically infected with other strains (Al-Aloul et al, 2004 [III]). LES has been identified in many UK CF Centres (Scott & Pitt, 2004 [III]). People with CF chronically infected with the Manchester transmissible strain of *P. aeruginosa* suffered significantly more acute exacerbations, required more in-patient stays, and required more courses of intravenous antibiotics than those infected with other strains (Jones et al, 2002 [III]). Identification of transmissible strains of *P. aeruginosa* within a CF Centre may therefore be of prognostic, as well as Infection Prevention, value.

All of these have been subject to recent guidance from the Cystic Fibrosis Trust's Infection Control Group (CF Trust, September 2004 [IV]; CF Trust, November 2004 [IV]; CF Trust, 2008 [IV]). As well as Infection Prevention advice all three documents place emphasis on the importance of microbiological surveillance and genotyping, particularly in relation to *P. aeruginosa* and *Bcc*. Given the potential of these organisms to spread between people with CF it is vital that any significant changes in prevalence are detected early and new isolates are effectively identified to allow appropriate Infection Prevention measures to be initiated in order to reduce risks of transmission. Urgent communication by the microbiologist to the CF clinical team, even if results are provisional, either by telephone or e-mail according to local practice, will expedite this process.

Other situations may arise that similarly require rapid communication e.g. positive smears for acid-fast bacilli. Whilst these will almost always be caused by NTM the possibility of tuberculosis should be assessed.

6.2 Clinical intervention

There is evidence that treatment of initial colonisation with *P. aeruginosa* in CF will slow down the progression to chronic *P. aeruginosa* infection (Hansen et al, 2008 [III]). Although robust clinical evidence supporting the long-term clinical benefit of treatment for initial *P. aeruginosa* colonisation is lacking (Wood & Smyth, 2006 [IV]), almost all CF Centres will commence early treatment when it is identified. This approach was recommended by the CF Trust in their latest Antibiotic Treatment for CF document (CF Trust Antibiotic Working Group, May 2009 [IV]). It is therefore important to have systems in place to identify CF individuals with initial colonisation with *P. aeruginosa* who require eradication therapy. The use of criteria based on past respiratory culture results, such as the Leeds Criteria (Lee et al, 2003 [III]), may be useful in this respect.

The clinical impact of MRSA on people with CF is unclear but it is recommended that an eradication programme is commenced with sufficient knowledge and expertise e.g. mycobacteriology reference laboratory [B].

5.2.6 *Aspergillus* spp.

Correct identification of the species of *Aspergillus* is important as some are resistant to amphotericin e.g. *A. versicolor*, *A. nidulans*, *A. lentulus*. Itraconazole-resistant *Aspergillus fumigatus* has been described (Denning et al, 1997 [III]) and multiple triazole resistance has also been reported (Verweij et al, 2007 [IV]). If a patient has had prior treatment with azoles, susceptibility testing may therefore be warranted (Walsh et al, 2008 [IV]).

**Recommendation**

- **In people with CF, the repeated isolation of *Aspergillus* spp., in spite of long-term treatment with antifungal drugs (e.g. itraconazole or voriconazole as steroid sparing treatment for ABPA), may indicate the need for referral of isolates to a reference laboratory for susceptibility testing [C].**
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The clinical impact of MRSA on people with CF is unclear but it is recommended that an eradication
regimen is initiated after first isolation (CF Trust, 2008 [IV]). Although a number of different regimens have been reported, the optimum approach remains unknown. There are also anecdotal reports of successful eradication of initial colonisation with Bcc (Etherington et al, 2003 [III]) but this is not a universally applied standard of care.

6.3 Multi-disciplinary team working

A recent European consensus document highlighted the importance of microbiologists in multi-disciplinary teams caring for people with CF (Kerem et al, 2005 [IV]). The document recommends that, in addition to providing a laboratory service and advice on Infection Prevention, microbiologists should participate in regular multi-disciplinary meetings with other team members to discuss the management of individual patients. The nature and frequency of this input was not stipulated.

Recommendations

- CF clinicians and microbiologists should agree the structure, content and communication of laboratory reports [C].
- All new or suspect isolates of Bcc should be communicated urgently to the CF clinical team [C].
- All new isolates of MRSA should be communicated urgently to the CF clinical team according to local Infection Prevention policy [C].
- A reliable system of identifying those with initial infection with P. aeruginosa needs to be in place in order to commence timely eradication therapy [C].
- Regular surveillance of P. aeruginosa, including molecular typing, should be carried out as recommended by the CF Trust Infection Control Group [C].
- In addition to providing a laboratory service and advice on Infection Prevention, microbiologists should participate in regular multi-disciplinary meetings to discuss the management of individuals with CF. The local service should determine the nature and frequency of this input [C].
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