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Review

Culture-based diagnostic microbiology in cystic fibrosis: Can we simplify the complexity?

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Abstract

Cystic fibrosis (CF) diagnostic microbiology has evolved from a focus on *Staphylococcus aureus* as primary pathogen to identification of the contribution of *Pseudomonas aeruginosa* and other non-fermenting gram negatives; studies of the lung microbiome have added new complexity. This review summarizes state-of-the art culture methods and makes recommendations for addition of non-culture based methods in the diagnostic laboratory. Plating on selective media is recommended, with organism identification by matrix assisted laser desorption-time of flight mass spectroscopy and real-time polymerase chain reaction (PCR) supplanting both biochemical identification and other less accurate and more time-consuming molecular methods. Conventional antibiotic susceptibility testing, possibly at less frequent intervals, remains the standard but more CF-relevant methods may arise in the future. There is a role for direct identification of organisms in clinical samples using quantitative real-time PCR, next generation sequencing, and metagenomic studies for the re-examination of samples that do not yield traditional CF pathogens.
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1. Introduction

Cystic fibrosis (CF) is characterized by chronic airway infections with intermittent pulmonary exacerbations. The microbiology of CF airway infections has for many decades been determined by bacterial and fungal culture methods, focusing on the most commonly isolated organisms including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* [1]. However, many other opportunistic organisms not commonly recovered from healthy individuals have been associated with evolution of the airway disease in CF individuals over the last decade including gram negative non-lactose fermenting bacilli such as *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, *Achromobacter* spp., and *Inquilinus limosus*; non-tuberculous mycobacteria; and fungal organisms, both yeasts and molds [1–3]. In addition, respiratory viruses have been examined, although a specific role for viruses in CF pulmonary disease has not been clearly defined.

More recently, non-culture based methods have added to our understanding of the polymicrobial nature of CF airway infections. Many organisms not previously recovered from the CF airway have been reported from patient samples using polymerase chain reaction (PCR) and deep sequencing [2,4–6]. These include viridans streptococcus, *Prevotella* spp., *Veillonella* spp. and other anaerobic organisms, none of which would have been detected using currently recommended diagnostic culture methods for CF respiratory samples. The role of anaerobes and microaerophilic organisms in the progression of CF airway disease is not clear. While *Streptococcus milleri* has been reported to be a clinically relevant pathogen in CF because of its association with pulmonary exacerbations [7], studies by Zemanick and others have reported that anaerobes identified from sputum by sequencing are associated with less inflammation and higher lung function than *P. aeruginosa* [7,8].

Studies of the CF lung microbiome will not be covered in this review and non-culture based methods for direct examination of CF airway samples by PCR and next generation sequencing will only be reviewed briefly, because these strategies are not yet recommended for routine use in CF clinical diagnostic laboratories. However, non-culture based methods that are available in clinical laboratories including mass spectroscopy, PCR, and 16S rRNA sequencing for identification of isolated organisms will be addressed.

Although studies of the lung microbiome offer insight into the polymicrobial nature of CF airway infections, they also add a significant degree of complexity to an already multifaceted biological system. There are many unanswered questions: 1) Which organisms or combinations of pathogens and colonizing organisms are pathogenic in CF? 2) Are available culture methods adequate for the isolation and identification of these organisms? 3) How best can we characterize the interaction between different species? 4) What is the biological impact of antimicrobial therapy on CF airway infections? 5) What is the best method to determine antimicrobial susceptibility *in vitro*?

The overall goal of this manuscript is to summarize the current status of culture-based methods for CF samples and to make recommendations for the use of specific non-culture based methods in conjunction with CF-specific specimen processing and culture in order to more clearly understand CF airway infections.

2. Bacterial cultures of CF respiratory samples

2.1. Optimum respiratory sample for CF airway infection

A recent study has demonstrated that the site of sampling is critical for an accurate overview of the bacterial population. Identification of different organisms based on 16S amplicon pyrosequencing analysis was reported comparing direct lung samples obtained at the time of transplantation with oropharyngeal swabs and expectorated sputum samples. Oropharyngeal swabs in particular poorly reflected the lung microbiota [9]. Selection of the optimum sample for culture is a balance between accuracy and convenience; patient safety and preference must also be considered. Possible samples for culture-based microbiological diagnosis in CF airway infections include bronchoalveolar lavage fluid, expectorated or hypertonic saline-induced sputum, cough swabs and oropharyngeal swabs. Each has advantages and disadvantages. The deepest possible sample and presumably the most accurate is from a bronchoalveolar lavage. However, obtaining this sample is invasive, particularly in children for whom general anesthesia is usually required, and has a low but quantifiable risk of contamination by oral flora. Traditionally, sputum samples have been used as a surrogate for lower airway cultures. Expectoration of sputum for culture is far less invasive than bronchoalveolar lavage; however, not all individuals with CF are expectorators. Although induction of sputum can be useful in non-expectorating patients, this technique is not routinely performed at all CF centers and requires clinical expertise and patient proficiency. In addition, some patients experience bronchospasm with the use of hypertonic saline during sputum induction. When obtaining either expectorated or induced sputum, there is a higher likelihood of oral contamination than with bronchoalveolar lavage. More superficial samples include swabs of the oropharynx and cough swabs. While convenient and non-invasive, oropharyngeal and cough swabs are more likely to contain oral flora and are far less sensitive for *S. aureus* and *P.*

aeruginosa compared with cultures of lower airway samples (sputum or bronchoalveolar lavage fluid). The negative predictive value for *P. aeruginosa* is high in young children, but declines with age [10]. In addition, swabs are considered inadequate for the culture of mycobacteria. Overall, the most commonly cultured CF respiratory sample is expectorated sputum.

2.2. Role of the gram stain and other observational methods for evaluation of CF respiratory samples

Gram staining is a procedure that is generally recommended for processing all sputum samples. However, its utility in CF microbiology has been questioned. Nair et al. [11] found that the use of standard gram staining criteria would have resulted in rejection of 41% of samples, the majority of which were both purulent, based on examination of the sample, and culture positive for gram positive and gram negative CF pathogens. In that study each sample was visually inspected and areas of purulence were selected for culture. Results suggested that routine gram staining is not necessary for evaluating the quality or results of CF sputum samples.

Another technique that has been suggested for the evaluation of sputum quality is the use of a colorimetric card (BronkoTest®; Williams Medical), which is a visual method to assess sputum color. The BronkoTest® has been validated for use for the management of chronic obstructive pulmonary disease and a recent study from Elborn et al. used it for a study of biomarkers in CF [12].

2.3. Use of selective media and dilutional methods for bacterial and fungal cultures of CF samples

CF airway infections are polymicrobial and contain both relatively rapidly growing organisms and those that are auxotrophic and/or slow growing, making it possible for some organisms such as a mucoid *P. aeruginosa* to overgrow or obscure slower growing organisms or those present in lesser numbers. In addition, CF sputum samples are often viscous and non-homogenous with areas of purulence and mucus plugs that might trap organisms. Thus, special processing is recommended to improve the accuracy of CF culture results. Selective medium that inhibits the overgrowth of non-target organisms can be very helpful in the recovery of fastidious organisms and the use of solubilizing agents such as dithiothreitol with subsequent specimen dilution can improve detection of organisms present at a lower density.

Selective medium specific for *P. aeruginosa* is not recommended; it is unnecessary and may actually inhibit organism growth [13]. However, the use of a selective medium is strongly recommended for *B. cepacia* complex, because the organism has a high clinical impact, a high risk of transmissibility, and a relatively slow growth [13,14] (Table 1). Numerous media have been developed for this purpose including OFPBL, PC agar, MAST selective agar and BCSA. The latter two have the highest sensitivity and specificity for recovery and are recommended. It is also advised that these plates be incubated at 37 °C for a prolonged period and subsequently held at room temperature to complete a week of incubation. Although BCSA is highly selective for *B. cepacia* complex, other colistin-resistant organisms including *Achromobacter* spp., *S. maltophilia* or *I. limosus* would not be detected [15]. With the emergence of colistin-resistant bacteria among CF patients, the use of a less selective medium with rapid identification using matrix assisted laser desorption-time of flight mass spectrometry (MALDI-TOF MS) may actually be beneficial in laboratories with the ability to perform MALDI-TOF MS identification (see Organism identification by MALDI-TOF MS).

Table 1
Current recommendations for the use of selective media in CF clinical microbiology.

Organism	Recommended media
Pseudomonas aeruginosa	None
Burkholderia cepacia complex	BCSA
	MAST selective agar
Staphylococcus aureus	CHROMagar Staph aureus selective and differential agar
	Mannitol salt agar
Haemophilus influenzae	Blood agar supplemented with hemin, plus bacitracin [a]
	Chocolate agar, plus cefsulodin

[a] Anaerobic incubation is also recommended with this medium.

Selective media for *H. influenzae* and *S. aureus* can be very helpful in recovery of these organisms from CF samples [13]. *H. influenzae*, in particular, is fastidious and easily overgrown by *P. aeruginosa*. Recommended media include blood agar supplemented with hemin, containing bacitracin and incubated anaerobically, or chocolate agar containing cefsulodin (Table 1). The use of a selective medium such as mannitol salt agar or CHROMagar Staph aureus selective and differential agar significantly increases the yield of *S. aureus* from CF samples compared with non-selective medium (Table 1). Although Staphylococcal selective media are somewhat more expensive to use, the cost is offset by a decrease in laboratory technologist time for subcultures and confirmatory testing, because the chromogenic markers in the media help distinguish *S. aureus* from coagulase negative staphylococcus. Another selective medium that has been proposed for CF samples is McKay agar for the routine quantification of the *S. milleri* group [7].

A recent study of the CF airway microbiome enriched by culture-dependent techniques suggests that additional media, anaerobic cultivation and extended incubation times may help in the recovery of previously overlooked species [4]. However, until the clinical relevance of these species has been clarified, we do not recommend the routine use of additional culture conditions for CF clinical samples.

Increasing attention is being paid to the role of fungal species in older children and adults with CF. However, at this time the clinical relevance of these fungi in CF has not been fully delineated. *Aspergillus fumigatus* has been associated with allergic bronchopulmonary aspergillosis in CF, but invasive disease is rarely seen and the use of antifungal therapy remains uncommon. Other filamentous fungi, including members of the *Pseudallescheria*/*Scedosporium* group, are of concern and may

chronically colonize CF airways. However, the presence and prevalence of fungal pathogens in CF samples vary among different geographical areas; this may be related to a lack of standardization in the methods used to evaluate CF sputum samples for filamentous fungi [16]. Sabouraud dextrose medium with or without selective antibiotics is useful in the recovery of fungi from CF samples and may be enhanced by prolonged incubation [13]. Solubilization of CF samples using dithiothreitol reduces disulfide bonds and is helpful for disruption of mucus plugs and homogenization of sputum. In some laboratories, samples are subsequently serially diluted prior to plating on selective media to facilitate detection of organisms present at a low density. Although the density of organisms in respiratory samples is frequently reported semi-quantitatively (by arbitrary scores *e.g.* 1+, 2+, 3+, 4+), actual quantitation of bacterial density in CF sputum has been used as a microbiological endpoint in clinical trials testing the efficacy of new antimicrobial therapies [17,18]. Quantitative cultures are also used clinically in a number of laboratories that process CF samples (personal communication, J. Burns; personal communication F. Accurso), but the clinical utility of this method is controversial.

2.4. Mycobacterial culture methods

As the clinical and epidemiologic importance of nontuberculous mycobacteria especially members of the *Mycobacterium abscessus* complex is increasingly recognized in CF, the CF Foundation has recommended that mycobacterial cultures be performed annually especially in individuals receiving chronic azithromycin therapy. Acid fast staining of sputum smears using a fluorochrome method such as auramine–rhodamine is recommended in preference to the Ziehl-Neelsen and Kinyoun stains as the latter are reported to be less sensitive for non-tuberculous mycobacteria [13]. Sputum smears can be very useful in evaluating the burden of organisms in the lung and the results may impact treatment decisions.

To enhance isolation of mycobacteria from CF samples, decontamination with N-acetyl-L-cysteine, sodium hydroxide and oxalic acid [19] is recommended to minimize the overgrowth of *P. aeruginosa* and other rapid-growing, non-fastidious gram negative organisms. Bacterial density may decrease with these decontamination procedures, so only sputum (expectorated or induced) and bronchoalveolar lavage fluid are considered adequate samples for mycobacterial culture. CF samples should both be inoculated into a liquid medium and plated onto a solid medium such as the Middlebrook or Lowenstein–Jensen medium. Further recommendations will be forthcoming from the CF Foundation/European CF Society consensus committee on non-tuberculous mycobacteria, expected to be completed later this year (personal communication, C. Haworth). Culture on sheep blood agar medium following decontamination may also be a useful methodology (personal communication, J-M Rolain). In addition, culture on BCSA with extended incubation (5 to 14 days) may improve the recovery rate of rapidly growing mycobacteria in CF sputum samples [20].

3. Organism identification

3.1. Biochemical identification of gram negative organisms

Once organisms are isolated in pure culture, some may readily be identified by appearance (colonial morphology, pigment production, beta-hemolysis on sheep's blood agar, growth temperature) combined with a few simple assays (oxidase, catalase). However, because of the many phenotypic changes that may be induced in the CF airway related to selective pressure and hypermutability, gram negative CF isolates often require additional testing [1]. These traditional identification methods use a much larger panel of biochemical and chromogenic assays and may take up to 72 h after pure growth on a subculture plate to confirm an identification.

Multiple studies have identified the poor performance of commercially available biochemical identification systems for the identification of gram negative CF pathogens [2,3]. Difficulties with these systems arise for several reasons including relatively slow organism growth rates, phenotypic variability among CF isolates, and inadequate system databases.

3.2. Organism identification by polymerase chain reaction (PCR)

The clinical importance and infection control implications of specific bacterial organisms such as *P. aeruginosa*, *B. cepacia* complex and *M. abscessus* complex in CF make rapid and accurate identification imperative. The use of 16S rRNA PCR amplification and sequencing, and real-time PCR amplification of multiple gene targets can significantly improve the accuracy of identification. The former is considered a reference standard for organism identification. However, because of the (at times) limited interspecies discriminatory ability of 16S rRNA sequencing, the amplification of multiple gene targets may be necessary and can be substituted for the less rapid and more expensive 16S sequencing method. Many clinical laboratories perform real time PCR identification on a daily basis and in a significant number it has supplanted all but the most basic biochemical testing.

Multiple potential gene targets have been recognized for PCR identification of *P. aeruginosa*, *B. cepacia* complex, *S. maltophilia*, and *Achromobacter* spp. [2,21]. However, in order to accurately discriminate between *B. cepacia* complex, *Pandoraea* spp., *Achromobacter* spp. and some non-aeruginosa *Pseudomonas* spp. single or multiple gene sequencing is often necessary [22].

Accurate identification of non-tuberculous mycobacteria is important to distinguish organisms with potential for increased transmissibility and virulence and decreased antimicrobial susceptibility. Molecular methods hold the most promise for accurate identification. *Mycobacterium avium* complex includes *M. avium*, *Mycobacterium intracellulare* and several other related identified and unidentified species. Similarly, *M. abscessus* has been recognized as a complex including two subspecies, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *abscessus* [23]. Again, multiple gene sequencing is necessary [24].

3.3. Organism identification by MALDI-TOF MS

MALDI-TOF MS is a more recent and rapid method for bacterial identification and is being used routinely in many clinical microbiology laboratories. The rapidity of this method for bacterial identification has been a revolution in clinical microbiology laboratories because it is now possible to identify the majority of the colonies growing on agar plates [21]. It is an accurate and very rapid technique for identification of many organisms and has been evaluated for identification of CF pathogens [25]. MALDI-TOF MS relies on generation of a unique peptidic spectrum for each isolate which can then be compared with a database for organism identification. A recent study by Desai et al. demonstrated that the use of a bacterial database that was not supplemented with CF-specific reference strains yielded complete genus and species agreement in 92% of isolates from 24 CF samples, with genus agreement in 98% [25]. Organisms only identifiable to the genus level included *Achromobacter xylosoxidans*, *Acinetobacter* spp., and *Ralstonia pickettii*; the only member of the *B. cepacia* complex that can be reliably identified (greater than 95%) by MALDI-TOF MS is *Burkholderia multivorans*. Thus, identification of *B. cepacia* complex organisms by MALDI-TOF MS is not recommended. As noted above in the section on PCR identification, these species and others including non-aeruginosa *Pseudomonas* often require sequence-based methods of identification.

MALDI-TOF MS can be used for the rapid identification of other organisms, as well. Del Chierico et al. have reported its use for the identification of filamentous fungi from CF patients with good accuracy [26]. The use of MALDI-TOF MS for identification of mycobacteria is also being explored. For mycobacterial identification, Saleeb et al. have reported an optimized protein extraction protocol and created a mycobacterial database that permits identification of most strains of mycobacteria isolated from solid growth media [27]. While not yet in routine use, this technique offers the possibility of accurate and more rapid identification of these emerging CF pathogens. Finally, because an exhaustive analysis can be done with MALDI-TOF MS, identification of putative new and/or emerging bacteria is now possible in the context of CF. For example this technique allowed identification of a cluster of CF patients colonized with *Corynebacterium pseudodiphtheriticum*, an emerging respiratory pathogen that had not previously been reported in CF patients [28].

3.4. Detection and identification of small colony variant S. aureus

Genetic variants of *S. aureus* called small colony variants (SCVs) emerge during chronic CF infections and are associated with poor clinical outcomes in a pediatric population [29]. SCVs grow slowly on many culture media because they have metabolic defects, so they may be very difficult to identify in polymicrobial CF respiratory cultures; use of a Staphylococcal selective agar is necessary to avoid overgrowth of other organisms and prolonged incubation is critical to detect growth. To detect *S. aureus* SCVs, it is useful to subculture each *S. aureus* morphotype from primary isolation medium onto both a supplemented medium that will readily support normal colony growth and a medium on which small colony growth is demonstrated. A simple method using media routinely available in all clinical laboratories that meets these requirements was recently published by Wolter et al. [29]. All *S. aureus* isolates were subcultured onto blood agar from primary isolation plates with incubation in ambient air at 35 °C for 24–48 h; fastidious small colonies were subcultured to blood, chocolate and brucella agars, again with incubation in ambient air at 35 °C for 24–48 h. SCVs were identified by slow growth on the blood agar plate compared with robust growth on the brucella and chocolate agars. They were typically non-hemolytic and lacked pigmentation when grown on blood agar.

An important finding in that study was that only 53 of the 81 cultures positive for *S. aureus* SCVs had wild-type *S. aureus* isolated from the same culture [29]. If the SCVs had not been detected using enhanced culture techniques, 1/3 of *S. aureus* positive cultures might have been missed (personal communication, L. Hoffman). With increasing recognition of the clinical significance of *S. aureus* SCVs, adoption of a clinical laboratory protocol for their detection becomes increasingly relevant.

4. Antibiotic susceptibility testing

4.1. Standard susceptibility testing

For a number of years, the accuracy of antibiotic susceptibility testing for CF isolates of *P. aeruginosa* was questioned, primarily because of concerns regarding slow growth, auxotrophy and the impact of mucoid exopolysaccharide. Subsequent testing demonstrated that the agar diffusion methods including disk diffusion susceptibility testing and the agar-based stable gradient methods (Etest®) compared favorably with reference broth microdilution methods, while for several automated commercial systems correlation to reference methods was unacceptably low [30].

More recently, the validity of antimicrobial susceptibility testing by any method has been scrutinized. A laboratory study to evaluate the effect of phenotypic variability on the reproducibility of susceptibility testing found a mean of four morphotypes per sputum and four antibiograms per morphotype. In addition, there was a poor correlation of the results both within and between laboratories. Susceptibility of pooled cultures did not correlate with the results for individual organisms, with the pooled cultures missing resistant organisms [31]. Very few studies have been done to evaluate the importance of susceptibility testing in CF. However, clinical trials using inhaled antimicrobials have not seen any correlation of clinical response with susceptibility breakpoints [17,18] and minimal clinical impact was demonstrated when routine susceptibility testing of *P. aeruginosa* in chronic CF infections was decreased by 56% at an adult CF clinic in the UK [32].

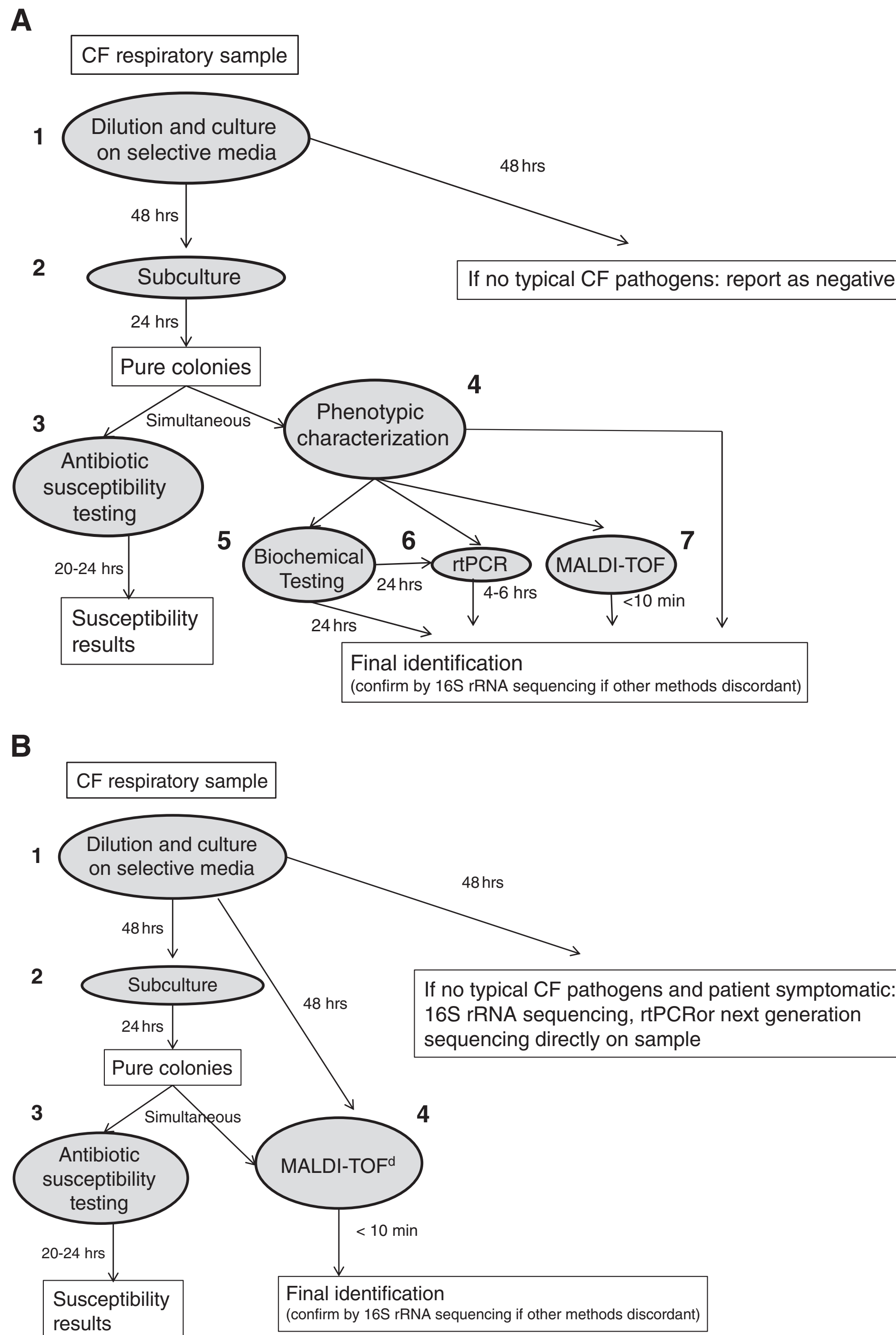
These data bring into question the relevance for CF of the susceptibility testing recommendations and guidelines of the European Committee on Clinical Laboratory Standards (ECCLS), the Clinical Laboratory Standards Institute (CLSI) and other regulatory bodies in microbiology. Standard testing methods and breakpoints were developed specifically for acute bloodborne and urinary tract infections caused by a single organism. This is in distinct contrast with the situation in CF

where infections are chronic, slow-growing, sequestered in the lung and airways, and frequently polymicrobial; among CF isolates there is also marked phenotypic variability within species. Thus novel, CF-specific methods of susceptibility testing may be more clinically relevant.

4.2. Novel methods of susceptibility testing in CF

Several novel methods of susceptibility testing have been developed for CF isolates, but none so far has demonstrated a clear-cut correlation with clinical outcomes. These methods have attempted to look at conditions more relevant to CF respiratory tract infections including antimicrobial combination testing, biofilm testing, and testing under anaerobic and microaerophilic conditions.

Drug combination testing has included both synergy testing and multiple combination bactericidal testing. A Cochrane review by Waters and Ratjen [33] evaluated available data on combination antimicrobial susceptibility testing in CF and determined that there is insufficient evidence to determine the effect of choosing antimicrobials based on combination testing.



Standard antimicrobial susceptibility testing is performed on planktonic bacterial cultures while organisms in the CF airways grow as biofilms. Thus, a number of investigators have examined the role of antibiotic susceptibility testing of biofilm-grown *P. aeruginosa* in directing treatment of CF patients. Moskowitz et al. [34] performed a prospective randomized trial of stable CF patients, comparing therapy directed by standard susceptibility testing with therapy directed by biofilm testing. Both patient groups improved and no significant difference was seen in microbiological or clinical analyses.

Other conditions that may be more relevant than the use of current ECCLS or CLSI standards for antimicrobial activity in CF airway infections include the use of an artificial sputum medium, growth under microaerophilic conditions, and prolonged incubation. Another approach with potential for revolutionizing susceptibility testing in CF isolates is analysis of antimicrobial resistance genes by metagenomics or whole genome sequencing. This was recently demonstrated in a series of 200 isolates with high concordance between phenotypic and predicted antimicrobial susceptibility based on gene sequence [35].

5. Next generation sequencing methods

Next generation sequencing is the term applied to sequencing strategies such as pyrosequencing and dye termination and ligation methods that enable sequencing of long stretches of DNA and analysis of whole genomes. These methods have revolutionized sequencing by increasing throughput, affordability and simplicity. There have been many recent developments that have decreased the cost and increased the efficiency of DNA sequencing and these hold the potential for routine diagnostic use in the near future.

While PCR and DNA sequencing of individual genes are used as adjunct methods for organism identification and demonstration of antimicrobial resistance in most diagnostic laboratories, the use of next generation high-throughput sequencing methods directly on patient samples is primarily a research tool at this time. However, as sequencing costs decrease and the technology becomes increasingly available in laboratories, the opportunity presents itself to provide real-time culture-independent results to clinicians [36].

The areas in which next generation sequencing seems poised to contribute the most to clinical care at this time include rapid detection of multiple antibiotic resistance determinants, improved taxonomic identification and real time outbreak investigation. The utility of sequencing methods to detect antibiotic resistance has been demonstrated by Zankari [35] and offers the advantage of being able to test for hundreds of beta-lactamases and aminoglycoside modifying enzymes. With the marked phenotypic variability of CF clinical isolates from chronic infections, the use of whole genome sequencing for taxonomic identification of all of the organisms in a sputum sample offers potential to rapidly detect both traditional pathogens and also anaerobic and fastidious organisms that might otherwise be missed using current culture methods. Although molecular epidemiology is not traditionally the purview of clinical diagnostic microbiology laboratories, once the equipment is available and protocols are in place whole genome sequencing performed as a part of resistance investigation and organism detection may lead to earlier discovery of highly transmissible organisms.

The routine use of next generation sequencing methods in clinical microbiology laboratories for direct detection of organisms in CF patient samples is currently limited by lack of equipment and expertise. In addition with our limited understanding of the clinical importance of many organisms that we can currently detect in the CF microbiome, the analysis of the data generated by whole genome sequencing remains prohibitive in many clinical microbiology laboratories. Analysis of sequencing data from highly diverse polymicrobial samples requires the organization and interpretation of large amounts of data and not all clinical diagnostic laboratories are staffed with technologists with the expertise needed.

Like all methods, next generation sequencing can have drawbacks. For example, sequence data is only as reliable as the sequence databases that are available. And although the price of sequencing is markedly decreased from a decade ago, equipment and reagents are generally much more expensive than culture-based microbiology. Most important, diagnostic laboratories processing patient samples are highly regulated and the clinical validation and approval of molecular assays are required. As technology changes rapidly, regulatory bodies such as CLSI and ECCLS will need to be very responsive to these changes. Despite

Fig. 1. Comparison of current and proposed methods for CF respiratory sample processing for bacteria. A: Current procedures. *Step 1*: Dilution and culture on selective medium (48 h for the majority of organisms to be detectable). *Step 2*: Subculture to recover pure colonies (24 h). *Step 3*: Susceptibility testing (20–24 h). *Step 4*: Phenotypic characterization may include examination for beta hemolysis, colony morphology including pigmentation, mucoid phenotype, and metallic sheen, and oxidase activity. This is done by examination of a purified colony and does not require additional incubation. This can lead to immediate final identification based on phenotype (minutes). *Step 5*: Biochemical testing for non-lactose fermenting gram negative bacilli may include: arginine dihydrolase, lysine decarboxylase, and urease, oxidation–fermentation reactions with various sugars including glucose, maltose, sucrose, mannitol and xylose, and growth at 42 °C. Biochemical testing may lead to final identification or further testing (24 h). *Step 6*: Real-time polymerase chain reaction (rtPCR) using single or multiple targets may be done immediately from pure colonies or may be delayed until after biochemical testing (4–6 h). Step 7: MALDI-TOF MS identification (less than 10 min). In the current schema, the most straightforward sample with an easy to identify *P. aeruginosa* would take a minimum of 72 h from start to finish (Steps 1, 2, 3 and 4). The most complicated sample with multiple atypical organisms could take as long as 102 h to final identification (Steps 1, 2, 3, 4, 5 and 6). B: Proposed method. *Step 1*: Dilution and culture on selective agar are still required (48 h). *Step 2*: Subculture to recover pure colonies (24 h). *Step 3*: Susceptibility testing (20–24 h). *Step 4*: MALDI-TOF MS identification may be performed on isolates from the primary culture plate when isolated and easily visible individual colonies are present, especially if immediate identification is important for patient management (Steps 1 and 4 in the proposed schema; Step 3, susceptibility testing, would require an additional 20 to 24 h). In this situation, a final result would be available within 48 h. However, pure colonies from subculture are more frequently used (Steps 1, 2, 3 and 4). In this scenario, final results would be available at 72 h. The technologist time and supply costs are much lower with MALDI-TOF identification compared with other identification methods.

these current limitations, for culture negative samples, direct sequencing strategies hold great potential as adjunctive methods.

6. Combined molecular and culture-based methods for the clinical evaluation of CF respiratory samples

6.1. Comparison of culture-based microbiology and molecular analysis in CF

The advent of non-culture methods for CF microbiology has transformed our understanding of the microbial ecology of CF airway infections [4,5,8,37]. The airway microbiome has been found to be much more diverse than previously thought based on bacterial culture, and a relationship between that diversity and pulmonary function has been identified [38]. Data from these and other molecular analyses have added several layers of complexity to our understanding of an already multifaceted system. While it is too early for this knowledge to have broadly impacted clinical management, this data is invaluable to our long term progress.

It is clear that both culture-based and culture-independent microbiology methods can contribute to the clinical management of CF airway infections and, in many ways, the two methods are complementary. Benefits of culture-based microbiology include the relatively inexpensive cost and standardized methods that allow portability from lab to lab, the ability to identify only viable organisms, and the easy phenotypic characterization of organisms (*e.g.* antibiotic susceptibility, mucoidy, small colony variants). The addition of MALDI-TOF MS has improved the accuracy of identification and shortened the time to final results. Cultures also yield individual organisms that can be archived for further study including epidemiological investigations when outbreaks occur, the testing of new antimicrobials, and research examining the progression of airway disease. On the other hand, culture-independent methods allow precise identification based on sequencing or RFLP patterns, detection of organisms present in low numbers, and characterization of the entire community of organisms present. Quantitative real-time PCR performed directly on CF oropharyngeal and sputum samples appears to offer advantages for detection of both *H. influenzae* and anaerobes [39]. Molecular methods also have the potential to yield results much more rapidly than culture. Both strategies may be performed quantitatively and both appear to have the ability to identify the majority of known pathogens present [9,37]. As clinicians and microbiologists we need to determine how best to use these tools to manage CF patients.

In CF, current practice of diagnostic microbiology uses primarily culture-based methods while recent ecological research describing the microbiota and its diversity relies primarily on non-culture methods. We propose a model for clinical CF microbiology that combines culture-based and molecular methods. In Fig. 1 we compare the current strategies with our proposed model, which has comparable accuracy and improved efficiency. Many clinical laboratories already use both culture based and non-culture based methods to some extent with their utilization of real-time PCR, sequencing and MALDI-TOF MS for identification of organisms recovered from culture. Each has advantages, but we endorse the use of MALDI-TOF MS for routine identification of organisms isolated from CF respiratory samples, because it is very rapid and is accurate for a large number of organisms. Some isolates including *Burkholderia* spp. and non-aeruginosa pseudomonads may require additional workup using real-time PCR or sequencing for identification to the species level. Although at this time routine direct identification of the organisms present in a CF sample prior to culture using terminal restriction fragment length polymorphism, PCR or next generation sequencing is not practical in the majority of clinical diagnostic laboratories, it may be very beneficial to reexamine samples that do not yield traditional CF pathogens on culture in reference laboratories that perform more complex molecular techniques.

Antibiotic susceptibility testing using traditional culture-based techniques and approved methods for CF isolates [30] is still recommended, although defining the relevance of these standard test methods to CF will be important. Possible changes in the future include performance of conventional antimicrobial susceptibility testing at less frequent intervals, revision of susceptibility breakpoints for CF isolates, the use of more CF-relevant media and growth conditions, and the application of molecular methods for detection of resistance.

7. Conclusions

Clinical microbiology is changing rapidly and ongoing research into the CF airway microbiome is adding both to our knowledge and to our uncertainty about which organisms should be evaluated. Clinicians and microbiologists need to be open to trying new methods and strategies to help us better understand the complexity of airway infections in CF and their impact on patient clinical status.

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