Review

Molecular detection of CF lung pathogens: Current status and future potential

Sally H. Pattison [a,b,*], Geraint B. Rogers [c], Martin Crockard [d],
J. Stuart Elborn [a,b], Michael M. Tunney [a,e]

[a] *CF and Airways Microbiology Research Group, Queen's University Belfast, United Kingdom*
[b] *Centre for Infection and Immunity, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, United Kingdom*
[c] *Institute of Pharmaceutical Science, Molecular Microbiology Research Laboratory, King's College London, London, SE1 9NH, United Kingdom*
[d] *Randox Laboratories Ltd, Crumlin, United Kingdom*
[e] *School of Pharmacy, Queen's University Belfast, United Kingdom*

Abstract

Molecular diagnostic tests, based on the detection and identification of nucleic acids in human biological samples, are increasingly employed in the diagnosis of infectious diseases and may be of future benefit to CF microbiology services. Our growing understanding of the complex polymicrobial nature of CF airway infection has highlighted current and likely future shortcomings in standard diagnostic practices. Failure to detect fastidious or slow growing microbes and misidentification of newly emerging pathogens could potentially be addressed using culture-independent molecular technologies with high target specificity. This review considers existing molecular diagnostic tests in the context of the key requirements for an envisaged CF microbiology focussed assay. The issues of assay speed, throughput, detection of multiple pathogens, data interpretation and antimicrobial susceptibility testing are discussed.
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Contents

 * Corresponding author at: CF and Airways Microbiology Research Group, Queen's University Belfast, United Kingdom.
  E-mail address: s.pattison@qub.ac.uk (S.H. Pattison).

1. Introduction

Over recent years nucleic acid based technologies have been increasingly exploited in human health, both in the analysis of human genes and in the detection and analysis of pathogenic micro-organisms. Assays targeting human genes and their expression can be used in screening to identify people at increased risk of disease, in the diagnosis of hereditary conditions, for detection of disease associated biomarkers and in predicting or monitoring response to treatment. Microbial nucleic acids can be targeted for the diagnosis of infectious diseases, to identify epidemic strains or pathogenicity traits, and for predicting or monitoring response to treatment. Molecular tests are promoted within clinical diagnostics due to their potential for rapid delivery of results, high sample throughput, accuracy, sensitivity and reproducibility. The focus of this review is the potential for using molecular tests in routine clinical practice to detect and identify respiratory pathogens associated with cystic fibrosis (CF).

2. CF lung health

Lung infection is the major cause of morbidity and mortality in CF patients [1] with improvements in antibiotic therapy contributing towards increased life expectancy. An improved ability to determine the presence of pathogens would allow more directed treatment, and may further improve outcomes for patients.

In the context of routine patient care and treatment of an infective exacerbation, the detection and identification of CF pathogens through diagnostic microbiology has proved beneficial in several ways. Monitoring people with CF from infancy promotes early discovery of newly acquired infections, thereby increasing the likelihood of eradication though timely treatment. Early eradication of *Pseudomonas aeruginosa* is recognised as contributing to better patient outcomes and reduced treatment costs [2,3]. Furthermore, it provides direct evidence of a response to antibiotic treatment where the goal and expectation is to completely clear an infection. This may have consequences clinically as failure to eradicate *P. aeruginosa* infection in children is linked to a higher risk of subsequent exacerbation and may be indicative of emerging chronic infection [4]. Routine outpatient clinics are also commonly segregated according to infection type to limit cross-infection between patient cohorts colonised with *Burkholderia cepacia* complex (Bcc), meticillin-

resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* [5,6]. Infection type also has implications for the organisation of inpatient care, such as admission to separate wards and the provision of individual rooms preferably with *en suite* facilities [5,6].

Regular microbiological surveillance to detect CF airway pathogens is recommended as best practice in CF patient care [5–7]. Depending on disease severity, the Cystic Fibrosis Trust and European Cystic Fibrosis Society (ECFS) advocate routine outpatient check-ups 4 to 6 times per year for clinically stable patients [5,6]. Additionally, respiratory samples are analysed following diagnosis of pulmonary exacerbation in order to guide choice of antibiotic therapy. Exacerbations are usually treated with intravenous antibiotics in a hospital setting or with homecare support [6,8] and at least 10–14 days of treatment has been recommended by the Cystic Fibrosis Trust [9]. Weekly microbiology culture is recommended by the ECFS for the duration of treatment [6].

3. Which microbes are clinically relevant in CF lung disease?

3.1. Historically recognised pathogens

Traditionally, the clinical impact of CF lung infections was attributed to the role played by a small number of pathogens, namely *P. aeruginosa*, *Haemophilus influenzae*, *S. aureus* and *B. cepacia*. Whilst these bacterial species have been associated with increased morbidity and mortality [10], their early identification as CF pathogens may have arisen, at least in part, from the relative ease with which they were detected by the culture practices employed at the time. Conversely, the same practices may not have detected more fastidious, anaerobic or slow growing pathogens. In recent years, the complexity of CF lung infection has been increasingly recognised. Conventional CF-related bacteria (*P. aeruginosa*, *H. influenzae*, *S. aureus*, *B. cepacia*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*) have been found to be absent from the sputum of 17% of patients with a pulmonary exacerbation [11], with enhanced culture [12–14] and molecular techniques identifying the presence of an unexpectedly diverse range of previously undetected bacteria, fungi and viruses. The role of many of these newly detected species in CF lung disease is unclear, with studies on-going to determine their pathogenicity and clinical relevance. The primary infectious agents, including bacteria, fungi and

viruses, currently considered most relevant to CF [6,15,16] are recorded in Table 1.

3.2. Recently detected diversity in the CF lung

Technological developments in the field of molecular microbiology have provided an opportunity to reassess the list of microbes traditionally associated with CF lung infections. In recent years, a range of DNA based techniques have exploited the 16S ribosomal RNA gene sequence for the detection and identification of bacterial species or taxa. Methodologies include terminal restriction fragment length polymorphism (T-RFLP) [17], phylogenetic hybridisation microarrays [18], clone library sequencing [19] and next generation sequencing [14,20,21]. Initially, microbiota studies tended to be qualitative, describing the wide diversity of bacteria detected. However, more recently quantitative pyrosequencing data have been published. The largest longitudinal study to date followed 6 patients for 8–9 years, with 13–20 samples collected from each [22], whilst the largest cross-sectional study analysed a single sample from each of 35 patients [23]. A third study analysed 63 sputum samples from 23 patients to monitor the effect of antibiotic treatment on community composition [24], whilst two other investigations analysed 2 samples from each of 4 patients [25] and 1 sample from each of 3 patients [20], respectively. Bacterial genera, detected at >1% abundance in at least one CF patient in these studies, are recorded in Table 2. Although high abundance of a species or genus does not necessarily signify pathogenicity, it identifies the potential for

clinical impact and may indicate an increased health risk if the species/genera were found to be harmful. The majority of genera reported at >1% abundance belong to the Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes phyla, with *Pseudomonas*, *Streptococcus*, *Prevotella* and *Rothia* genera being detected at >1% in at least one patient in all five studies. As these investigations vary greatly in their design and scope and involve heterogeneous patient groups, more data is required to give an accurate picture of the typical relative abundances of bacteria found in the CF lung. Furthermore, increasing evidence points to a reduction in diversity as lung disease progresses [18,22,26], thereby linking microbial community composition to clinical status. Thus, it may be more accurate to consider different microbial community profiles as typical at specific stages of patient health.

3.3. Clinical relevance of newly detected species

The detection of bacterial species in respiratory samples does not necessarily mean that they have a clinically important role in infection and determining their impact poses a major challenge to both researchers and clinicians. Ultimately a species can only be deemed clinically relevant if it is shown that clinical signs and symptoms are worse in patients where it is present as compared to patients where it is absent. However, a number of key factors can help predict the potential clinical impact of the different genera detected. These include —

(a) Are the organisms pathogenic in other infections?
(b) Do they possess virulence genes or exhibit virulence in *in vitro* or animal infection models?
(c) Are they present in significant numbers?
(d) Do they enhance or suppress the pathogenicity of other bacteria?
(e) Are they colonising or transient?

For example, no data currently exists demonstrating the clinical impact of any of the anaerobic genera found in the CF lung; however, some of the species detected include known oral pathogens which possess virulence genes, have demonstrated virulence *in vitro* or in a mouse lung infection model, are abundant and prevalent in the CF lung, and contribute to the core microbiota [12,14,20,27]. Additionally, 94% CF patients were found to have antibodies against two immunoreactive antigens of *Prevotella intermedia* [27]. Thus, application of the above criteria suggests that some anaerobic species may be significant in CF lung disease and warrant further investigation.

Antimicrobial therapy is generally targeted at new infections in non-colonised patients and the predominant pathogen in chronically colonised patients. Currently, the presence or acquisition of additional infections by a chronically colonised patient is unlikely to alter treatment as chronic infections tend to be treated with broad spectrum antibiotics targeting the predominant pathogen. These antibiotics are thought to have some effect on the majority of conventionally recognised CF pathogens [28]. Nevertheless, treatment may sometimes be used directly against existing, non-predominant pathogens, such as to target increases

Table 1
Bacteria, fungi and viruses considered clinically relevant in CF lung infection.

Infectious agents [a]	Cystic Fibrosis Trust [15]	ECFS [6]	Hauser et al. [16]
Staphylococcus aureus	✔	✔	✔
Haemophilus influenzae	✔	✔	✔
Streptococcus pneumoniae	✔	✔	
Pseudomonas aeruginosa	✔	✔	✔
Burkholderia cepacia complex	✔	✔	✔
Stenotrophomonas maltophilia	✔	✔	✔
Achromobacter xylosoxidans	✔	✔	✔
Non-tuberculous mycobacteria	✔	✔	✔
Other *Burkholderia* spp.	✔		
Ralstonia spp.	✔		✔
Pandoraea spp. / *P. apista*	✔	✔	✔
Anaerobic bacteria	✔		✔
Streptococcus spp.	✔		✔
Moraxella catarrhalis		✔	
Inquilinus limosus			✔
Aspergillus spp.	✔	✔	✔
Scedosporium apiospermum	✔		
Wangiella dermatitidis	✔		
Influenza virus	✔	✔	✔
Respiratory syncytial virus	✔		✔
Parainfluenza virus			✔
Adenovirus			✔
Rhinovirus			✔
Metapneumovirus			✔
Respiratory viruses		✔	

[a] As described in the references cited.

Table 2
Bacterial genera reported as detected at >1% abundance in the lungs of at least one CF patient.

Bacterial phylum/genus	Zhao et al. [22] (n=126)	Fodor et al. [24] (n=63)	Filkins et al. [23] (n=35)	Delhaes et al. [25] (n=8)	Guss et al. [20] (n=3)
Actinobacteria					
Actinomyces	✔	✔	✔	✔	
Atopobium	✔	✔			
Blastococcus		✔			
Bogoriella	✔				
Corynebacterium	✔	✔			
Curtobacterium		✔			
Micromonospora		✔			
Rothia	✔	✔	✔	✔	✔
Scardovia			✔		
Bacteroidetes					
Bacteroides	✔				
Capnocytophaga	✔	✔			
Cloacibacterium	✔				
Porphyromonas	✔	✔	✔	✔	
Prevotella	✔	✔	✔	✔	✔
Sphingobacterium	✔				
Deinococcus–Thermus					
Thermus		✔			
Firmicutes					
Abiotrophia	✔	✔			
Catonella		✔		✔	
Enterococcus				✔	
Gemella	✔	✔	✔	✔	
Granulicatella	✔	✔	✔		
Johnsonella	✔		✔		
Lactobacillus		✔	✔		
Megasphaera		✔	✔		
Mogibacterium			✔		
Moryella			✔		
Oribacterium	✔	✔	✔		
Paenibacillus		✔			
Parvimonas	✔	✔	✔		
Planomicrobium		✔			
Solobacterium		✔	✔		
Staphylococcus	✔	✔	✔		✔
Streptococcus	✔	✔	✔	✔	✔
Veillonella	✔	✔	✔	✔	
Fusobacteria					
Fusobacterium	✔	✔	✔	✔	
Leptotrichia	✔	✔			
Proteobacteria					
Achromobacter	✔	✔			
Acinetobacter	✔				
Actinobacillus	✔				
Aggregatibacter			✔		
Bradyrhizobium		✔			
Burkholderia		✔			
Campylobacter				✔	
Chryseomonas					
Devosia		✔			
Dickeya	✔				
Eikenella			✔		
Haemophilus	✔	✔	✔	✔	
Moraxella				✔	
Neisseria	✔	✔	✔	✔	
Pasteurella	✔				
Pseudomonas	✔	✔	✔	✔	✔
Sphingomonas		✔			
Stenotrophomonas	✔		✔		
Tenericutes					
Mycoplasma		✔	✔		

Table 2 (continued)

in *S. maltophilia* which are associated with the use of anti-pseudomonal antibiotics [29,30]. Nontuberculous mycobacteria may also warrant specific treatment [31]. As more knowledge is gained about the polymicrobial nature of CF infection, future treatment practices may change. Considerations might include prescribing antibiotics effective against anaerobes [13] or targeting bacteria which can increase the pathogenicity of the principal pathogen [32–35].

4. CF diagnostic microbiology

4.1. Current practice

In order to facilitate appropriate targeted treatment of infections by those species deemed pathogenic, there must first be effective strategies for their detection and identification. Current practice for diagnosis of CF infection depends on the microbial culture of a patient's sputum, cough swab, oropharyngeal swab or bronchoalveolar lavage (BAL) sample. The Cystic Fibrosis Trust guidelines [15] recommend the use of several selective and non-selective agars, and occasionally liquid culture, to maximise detection of pathogens commonly implicated in CF infections. Suspected pathogens are then isolated and subsequent identification is informed by a combination of visual characteristics, biochemistry tests, commercial phenotypic-based kits and instrumentation, with PCR amplification/sequencing of genes specific to individual pathogens and Matrix-Assisted Laser Desorption Ionisation-Time of Flight mass spectrometry (MALDI-TOF MS) also used. In some cases, consultation with a specialist reference laboratory may be required. *In vitro* antibiotic susceptibilities for the predominant pathogen are also typically determined.

4.2. Limitations

Standard diagnostic microbiology takes several days for a definitive report and requires highly specialised knowledge and experience gained over many years [6]. The challenge of applying such techniques effectively is highlighted by two recent quality assessment trials that observed significant shortcomings in laboratories across Europe providing microbiological services to large CF centres [36]. The trials assessed 31 laboratories in 2007 and 37 in 2008 for identification of microbial species from respiratory specimens, either as single isolates or in mixtures, with the same nine formulations being sent to each laboratory. Common pathogens such as *P. aeruginosa*, *S. aureus* and a *S. aureus* small colony variant, were correctly identified, whilst uncommon or newly emerging pathogens were frequently not detected or misidentified. For example, *A. xylosoxidans* and *Pandoraea pnomenusa* received incomplete/wrong identifications

or were undetected by 16/31 (52%) and 7/31 (77%) laboratories, respectively, in 2007, and *Inquilinus limosus* by 14/37 (38%) laboratories in 2008. In addition to the unexpected deficits uncovered by these trials, the authors suggest that as the participating laboratories had been specifically selected for their size and research activity, it might be expected that other smaller laboratories would have performed less well. Some identification failures may have been due to difficulties associated with commonly used phenotypic identification systems, such as the low biochemical reactivity of the bacteria tested, loss of its characteristic phenotypes during chronic colonisation or its absence from the commercial database [37,38]. As the number of species recognised as potentially pathogenic in this context increases, routine diagnostic CF microbiology is likely to become even more complex. This would present an increased challenge both with respect to detection by culture and subsequent correct identification.

4.2.1. Detection by culture

Pathogen detection through conventional culture is complicated by the problem of slow growing or fastidious microbes being overgrown by more abundant, faster growing organisms, and by a lack of routine culture conditions appropriate for the growth of some emerging pathogens. Both issues may result in clinically relevant microbes being overlooked. Whilst more extensive culture methods using multiple combinations of special media and growth conditions can detect the majority of CF bacteria identified by molecular methods in CF sputum [14], this approach is not practical for routine clinical laboratories. Continuing reliance on culture-based methods would necessitate the expansion of existing clinical culture procedures to include more laborious and potentially more demanding practices to enable reproducible detection of newly emergent pathogens. In the case of anaerobic bacteria, where clinical relevance can be demonstrated, the difficulties associated with effective anaerobic culture would have to be addressed. This was highlighted by a recent study evaluating culture protocols for pathogen identification in BAL samples from 12 CF patients [39]. The authors reported that anaerobic cultures were either negative (42%) or overgrown with aerobes (58%) and therefore diagnostically ineffective, yet other culture-based studies have been able to detect and isolate anaerobes from the majority of sputum samples [12,14,40]. Additionally, the incidence and abundance of potential fungal pathogens in CF sputum have been found to vary between geographical regions, most likely due to a lack of appropriate standardised culture practices [41]. Similar inconsistencies between 180 North American CF sites had also been reported for the detection of traditional CF bacterial pathogens prior to standardisation of clinical laboratory practices [42]. Furthermore, although viruses are recognised as contributing to patient poor health and are linked to pulmonary exacerbations [43], sputum samples are not routinely screened for their presence.

4.2.2. Species identification

Whilst fastidious growth and limited culture practices may contribute to failed detection of CF pathogens, misidentification and incomplete identification are also common [36]. In an investigation of molecular methods for the detection and identification of bacteria in CF sputum, conventional phenotypic methods incorrectly identified 5 out of 20 known pathogens isolated from children with CF and 3 out of 13 bacteria from adult patients [44]. Recently, MALDI-TOF MS has been applied to the identification of bacterial isolates by matching their protein mass spectra against a commercial library of pre-determined reference spectra. Two of these systems, the Bruker BioTyper and bioMérieux's VITEK® MS Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry System, have been evaluated against biochemical or molecular reference methods for identification of nonfermenting gram-negative bacilli isolated from CF patient sputa [45]. Both instruments performed well, with the Bruker Biotyper and Vitek MS agreeing with reference identifications at the combined species/complex/genus level for 194/200 (97%) and 179/200 (89.5%) isolates, respectively. Other trials also demonstrated the use of MALDI-TOF MS for definitive identification of Bcc isolates to the species level [46–48]. The Bruker instrument has been additionally tested directly on CF sputum for pathogen identification without prior culture, but was less suitable for this application [49]. This means that although MALDI-TOF MS can improve the identification of bacteria, its sensitivity is currently limited by the efficiency of routine sample culturing and pathogen isolation. Nevertheless, MALDI-TOF MS is increasingly being employed in clinical diagnostic laboratories and has distinct advantages over traditional phenotypic identification methods. A recent study found that 40/47 (85%) isolates from CF patients were identified by MALDI-TOF MS within 48 h of sample incubation compared to only 16/47 (34%) by conventional methods [46]. In particular, 15/21 (72%) of *P. aeruginosa* isolates were identified by day 2 and 17/19 (89%) of *S. aureus* isolates by day 1 with MALDI-TOF MS. In contrast, no *P. aeruginosa* isolates and 13/19 (68%) *S. aureus* isolates were identified by day 2 using conventional methods [46]. The authors concluded that adoption of this technology would reduce time to result reporting to the clinical care team and ultimately lead to improved patient outcomes. Additionally, switching to MALDI-TOF MS has been observed to be cheaper for routine bacterial identification [50].

4.2.3. In vitro antibiotic susceptibility

In addition to reporting on the presence of clinically important bacterial species, conventional diagnostic microbiology also provides information on the antibiotic susceptibility of isolates. This information is generally used in guiding therapy choice. However, these tests are not reproducible and are poorly predictive of clinical outcome in the management of pulmonary exacerbations caused by *P. aeruginosa* in both adult [51] and paediatric [52] CF patients. Furthermore, in a recent retrospective review of 452 pulmonary exacerbations occurring in our centre over a 4 year period in patients chronically infected with *P. aeruginosa*, 57% of pulmonary exacerbations were successfully treated with regimens containing antibiotics which were not deemed active against the cultured bacteria, as determined by *in vitro* susceptibility testing [53]. This lack of concordance between *in vitro* tests and clinical outcomes may be as a result of standard *in*

vitro culture methods not accurately reflecting the highly complex microbial community or environmental conditions found in the CF lung. Relevant issues include the inherent phenotypic diversity and instability within a chronically infecting *P. aeruginosa* population, biofilm growth, anaerobic/microaerophilic conditions, niche-specific nutrition and metabolism, and potential inter-species interactions [54–58]. Furthermore, attempts to develop more clinically relevant susceptibility tests through combination [59,60] or biofilm [56,61–63] directed testing have shown no consistent improvement in clinical or bacteriological outcome.

5. Molecular diagnosis of CF lung pathogens

5.1. Molecular diagnosis of infectious disease

Many molecular diagnostic methods do not require the growth and isolation of microorganisms, instead relying on the detection of target nucleic acids. There are three major approaches to microbial identification using nucleic acids extracted from clinical samples. The first uses sequence specific hybridisation of custom-designed primers and/or probes to detect the DNA or RNA of individual microbial targets, generally through PCR amplification. A range of hybridisation-based assays of varying design are already used in routine clinical practice for infectious disease diagnosis. The other two approaches, based on sequencing and PCR-mass spectrometry, are recently emerging technologies not yet adopted into routine clinical practice. Exceptions to this are two sequencing based assays, ViroSeq™ HIV-1 Genotyping System (Celera Diagnostics) and TruGene™ HIV-1 Genotyping and Open Gene DNA Sequencing System (Siemens Healthcare Diagnostics), which have been developed for HIV drug resistance testing [64].

Regulatory legislation governs whether a commercial diagnostic test can be marketed as an *in vitro* diagnostic for clinical use. For sales within Europe or the USA this entails CE marking or FDA approval, respectively, whilst regulation outside these regions varies nationally [65]. Approval requires evaluation of assay sensitivity and specificity, as well as proof of manufacturing consistency and the stability of test reagents, so guaranteeing reliability within the performance parameters claimed. To date, the FDA has approved just over 100 molecular tests from 27 manufacturers for the clinical detection and identification of bacterial, viral and fungal infections in various human biological samples based on hybridisation technologies [64]. Central to the majority of tests, is the amplification of a nucleic acid sequence unique to the target pathogen, with the amplified region (amplicon) being detected *via* reporter molecules either after or during the amplification step. The polymerase chain reaction (PCR) was the first and is still the most commonly employed method for amplification of a DNA target. It can be adapted for RNA targets, such as some viral genomes, by adding an initial reverse transcription step, known as RT-PCR. More recently, alternative amplification techniques have been developed and adopted into commercial molecular diagnostics due to their lower running costs and reduced reaction times [66–68]. Real-time PCR combines target amplification and detection in a single process. Approximately half of the manufacturers of FDA approved tests

for the detection of infectious agents employ real-time PCR technology in their assays [64]. Alternatively, in tests where target amplification and detection are separate, detection methods involve binding to target-specific probes or use of a secondary nested target-specific PCR.

5.2. Clinical requirements and considerations for a CF microbiology test

In order for a future molecular diagnostic assay to improve upon current CF microbiology practice, the defining requirements of the assay would be efficient detection and accurate identification of multiple CF-associated pathogens, directly from a patient sample, in a clinically beneficial timescale. Initially, the minimum specification should include the pathogens listed by expert bodies as clinically relevant (Table 1). However, assay flexibility would be required to allow future inclusion of other species as deemed clinically relevant. Additional desirable features not fully satisfied by current methodology may include the capability for quantitative reporting and antibiotic susceptibility measurements more predictive of *in vivo* response. Full automation within a contained environment would also be highly desirable as this would improve assay robustness by reducing the risk of operator error or sample contamination.

A number of considerations are applicable to any potential molecular diagnostic format to ensure delivery of good quality data which is truly representative of the *in vivo* microbiota. These issues have been discussed comprehensively by Rogers and Bruce in their review addressing the clinical application of next-generation sequencing to the human microbiome, including the detection of CF pathogens [69]. In particular, the authors emphasise that careful consideration should be given to (a) repeat sampling if maximum coverage of community diversity is required, (b) exclusion of extracellular DNA or DNA from dead cells from quantitative measurements, (c) development of a robust DNA extraction protocol equally efficient for all species present, and (d) exclusion of PCR contaminants, including exogenous DNA in commercial reagents.

5.3. Currently approved tests

Some FDA approved tests already exist which detect pathogens relevant to CF, such as *Streptococcus* spp., *S. aureus*, MRSA, *H. influenzae*, influenza viruses A & B and respiratory syncytial viruses A & B [64]. However, the majority of important CF airway pathogens are overlooked, including *P. aeruginosa* and Bcc. Thus, none of the currently available tests, or combinations of tests, is adequate for comprehensive CF microbiology for routine clinical purposes. Nevertheless, the technologies involved in existing tests and perhaps some of the existing tests themselves, could be adapted and validated for the detection of CF lung pathogens. The key aspects of molecular assay design most significant to clinical CF microbiology (multiplexing, throughput and automation, and data interpretation) and recent developments in molecular approaches to antimicrobial susceptibility testing are described below.

5.4. Molecular assay design

5.4.1. Multiplexing

As outlined above, CF respiratory samples may contain more than one pathogen of interest. To detect all clinically relevant pathogens, future molecular CF microbiology assays would have to detect a number of separate targets. One solution would be to run multiple single target tests consecutively or in parallel, delaying the speed of reporting or limiting sample throughput, respectively. Multiple reactions would also increase costs and require larger sample volumes. Alternatively, multiplexing could be used to combine multiple distinct tests in a single assay and allow the simultaneous detection and identification of each individual target present in a mixture. To date, six manufacturers sell FDA approved molecular diagnostic tests for infectious diseases which multiplex the detection of more than 3 targets. Half of these tests use real-time PCR/RT-PCR to multiplex 4 or 5 targets. The other three manufacturers employ highly multiplexed PCR or RT-PCR for amplification of 7–20 targets and differ in their modes of detection and identification. If detection and identification of all the pathogens listed in Table 1 are required for comprehensive diagnostic CF microbiology, a single highly multiplexed assay would be preferable to maximise throughput whilst minimising time-to-result.

5.4.2. Throughput and automation

One of the key benefits of molecular diagnostics is their suitability for automation, with clear potential benefits in terms of assay speed and sample throughput, and requiring minimal operator input. In practice, automation tends to be balanced against flexibility and instrument/running costs. The throughput of a test, *i.e.* the number of results generated within a defined time period, is governed by three parameters — the duration of a single test, the degree of target multiplexing per test (the number of tests that can be run in a single reaction), and the number of samples which can be tested in parallel, either as a batch or staggered at intervals. The priorities and throughput requirements of laboratories supplying diagnostic CF microbiology services would drive the degree of automation designed into a potential molecular CF microbiology diagnostic assay. Existing systems already FDA approved for diagnosis of other specified infectious diseases provide examples of automation strategies for multiplexed testing which could be applicable to CF. These include fully automated systems that operate independently sample-to-result, and semi-automated systems that frequently divide the workflow of a test into sample preparation including DNA extraction, assay set-up, the assay itself and data analysis/results reporting [64]. Each of these steps can be automated separately or performed manually, and typically manual intervention is required between steps. Examples of instrumentation approved by the FDA for defined purposes are provided in Table 3. When compared with those which are fully automated, semi-automated assays tend to have an increased overall test run time so making larger batch size and/or high multiplexing important for maximising throughput. Moreover, semi-automated assays may require significant input from skilled technical staff both for the test procedure and for data interpretation.

As already stated, there are currently no FDA approved molecular diagnostic assays suitable for complete CF microbiology analysis. However, several fully- or semi-automated instruments capable of multiplexed testing have been FDA-approved for the diagnosis of respiratory pathogens from sputum samples. This indicates that suitable technologies already exist which could be transferable to CF microbiology if appropriate assays were developed. In order to illustrate the potential of these technologies, the sample throughput rates and performance parameters for the existing multiplexed respiratory pathogen assays currently FDA approved for clinical *in vitro* diagnostic use are recorded in Table 4. All of the fully automated systems listed test single samples independently in completely closed systems with rapid time-to-results capabilities requiring less than 5 minutes of hands-on time per sample. Higher throughput can be achieved for the fully automated systems by using several stand-alone instruments or employing a modular setup where one central controller can run numerous assays independently in individual assay units.

5.4.3. Data interpretation

Current CF microbiology services inform the clinician of CF-associated pathogens detected in a sample and can to some extent provide semi-quantitative information in terms of relative abundance of the dominant pathogens. In order for a future molecular CF microbiology assay to be valuable clinically, the data gathered must similarly be interpreted and compiled into an actionable report. For the majority of FDA approved molecular assays, the output is qualitative in that target presence or absence is reported without indicating how much target is present. Research has shown that molecular assays employing target specific amplification are frequently more sensitive than culture [70]. This, combined with removal of the species bias introduced by standard growth conditions, would be expected to enable molecular reporting of microbes ordinarily overlooked by current practices. Whilst advantageous for patient health when enabling the earlier detection of pathogens of high clinical impact such as *P. aeruginosa*, the value of increased sensitivity might be more contentious if it resulted in the detection of a pathogen present at a constant level that may be too low to affect patient health. As yet, no minimum threshold levels associated with worse patient outcomes have been established for the majority of pathogens. Thus, the data output from any future molecular CF microbiology assays might initially be difficult to interpret until clinically significant thresholds are established and assay sensitivities are adjusted appropriately. It should be noted that the lack of definitive thresholds also applies to current culture-dependent methods and that the clinical significance of bacteria detected are usually judged subjectively by clinical microbiologists.

The availability of quantitative molecular reporting could be beneficial, particularly to indicate response to treatment of a chronic infection. However, this technology has not yet been widely adopted into infectious disease diagnosis and the only tests currently FDA approved are quantitative real-time PCR assays for hepatitis B & C viruses and human immunodeficiency virus, manufactured by Abbott, bioMérieux, Roche and Siemens [64]. Interestingly, Seegene recently launched their

Table 3
Examples of instrumentation approved by the FDA for specified purposes.

Instrument	Manufacturer	Function(s)	Product website
BD MAX[TM] System	BD Diagnostics–GeneOhm	Fully automated	http://www.bd.com/geneohm/english/products/max/
GeneXpert[TM] Real-time PCR System	Cepheid	Fully automated	http://www.cepheid.com/systems-and-software/genexpert-system
TIGRIS® DTS System	Gen-Probe	Fully automated	http://www.gen-probe.com/products-services/tigris-dts-system
FilmArray System	Idaho Technology	Fully automated	http://www.biofiredx.com/FilmArray/
Verigene® System	Nanosphere	Fully automated	http://www.nanosphere.us/product/reader-and-processor-sp
MagNA Pure Systems	Roche	Nucleic acid extraction	http://www.roche.com/products/product-details.htm?type=product&id=67
NucliSENS® easyMAG® System	bioMérieux	Nucleic acid extraction	http://www.biomerieux-diagnostics.com/servlet/srt/bio/clinical-diagnostics/dynPage?doc=CNL_PRD_CPL_G_PRD_CLN_70
QIAsymphony AS	Qiagen	Assay set-up	http://www.qiagen.com/products/qiasymphonysp.aspx
m2000sp System	Abbott Molecular	Nucleic acid extraction & assay set-up	http://www.abbottmolecular.com/us/products/instrumentation-automation/realtime-pcr/m2000-sp-rt.html
LX100/200	Luminex Molecular Diagnostics	Assay	http://www.luminexcorp.com/Products/Instruments/Luminex100200/
SmartCycler[TM] Real-time PCR System	Cepheid	Assay	http://www.cepheid.com/systems-and-software/smartcycler-system
m2000rt System	Abbott Molecular	Assay	http://www.abbottmolecular.com/us/products/instrumentation-automation/realtime-pcr/m2000-sp-rt.html

quantitative TOCE[TM] technology designed to return semi-quantitative results in real-time for up to 28 targets in a single multiplexed reaction [71]. Among their planned assays is the QuantPlex™ RV-16 Assay, aimed at detecting, differentiating and quantifying 21 respiratory viral pathogens associated with upper respiratory disease. If successful, this technology might in the future provide a useful basis for semi-quantitative molecular CF microbiology testing.

5.4.4. Antibiotic susceptibility

Whilst antibiotic susceptibility testing is typically practiced to guide choice of therapy [72], and is recommended by the Cystic Fibrosis Trust for early and intermittent *P. aeruginosa* isolates [15] and by the ECFS for determining the susceptibility of resistant pathogens to unusual antibiotics [6], its usefulness has been questioned [51,52]. However, if culture-independent assays are to replace conventional diagnostic microbiology, providing equivalent information represents a significant challenge.

Antibiotic resistance is common in CF pathogens, including the dominant pathogen *P. aeruginosa*. This resistance can be intrinsic to the bacteria or develop by means of spontaneous mutation or horizontal gene transfer, and manifests through a number of mechanisms including reduced outer membrane permeability, active efflux from the cell, target alteration to prevent binding, and enzymatic inactivation of the drug molecule [73]. The presence or absence of resistance genes relevant to CF could be determined molecularly through target specific amplification. Examples of the use of this technology in current FDA approved assays include the detection of vancomycin resistance gene *vanA*, and *mecA* which confers meticillin resistance in MRSA [64]. Conversely, the detection of gene mutations conveying resistance to a CF pathogen would be more complicated. The only examples of current FDA approved assays capable of detecting drug resistance acquired through gene mutation employ sequencing technologies.

If *in vitro* antibiotic susceptibility testing of CF pathogens continues to be required, detection of specific resistance genes

Table 4
Comparison of throughput and performance for multiplex respiratory pathogen assays FDA cleared for clinical *in vitro* diagnostic use.

Test (manufacturer)	Multiplexing	Samples per run	Run time	Sensitivity (%) [a]	Specificity (%) [a]
FilmArray Respiratory Panel [b] (Idaho Technology)	17 viral & 3 bacterial targets [c]	1	1 h	87.4–100 (95.9)	89.1–100 (98.9)
Verigene® Respiratory Virus Plus Test [b] (Nanosphere)	7 viral targets [d]	1	2.5 h	99.1–100 (99.7)	99.9–100 (100)
Xpert™ Flu [b] (Cepheid)	3 viral targets [e]	1	1 h	99.4–100 (99.8)	99.3–100 (99.8)
xTAG Respiratory Viral Panel *FAST* (Luminex)	8 viral targets [f]	≤96	5 h	85.7–97.2 (93.8)	92.5–99.3 (97.4)

[a] The highest and lowest sensitivities and specificities for an individual target within each test are recorded followed by the mean for all test targets in brackets.

[b] Fully automated test.

[c] Adenovirus, coronavirus 229E, coronavirus HKU1, coronavirus NL63, coronavirus OC43, human metapneumovirus, influenza A, influenza A subtype H1, influenza A subtype H3, influenza A subtype H1 2009, influenza B, parainfluenza virus 1, parainfluenza virus 2, parainfluenza virus 3, parainfluenza virus 4, rhinovirus/enterovirus, respiratory syncytial virus, *Bordetella pertussis*, *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*.

[d] Influenza A, influenza B, respiratory syncytial virus A, respiratory syncytial virus B, influenza A subtype 2009 H1N1, influenza A subtype H3 and influenza A subtype H1.

[e] Influenza A, influenza B and influenza A subtype 2009 H1N1.

[f] Influenza A, influenza A subtype H1, influenza A subtype H3, influenza B, respiratory syncytial virus, human metapneumovirus, rhinovirus and adenovirus.

would be insufficient to predict *in vivo* response given the many modes of resistance exploited. Additionally, gene sequencing may be impractical due to the number of genes potentially involved and their additive effects [74]. An alternative approach could be based on a semi-molecular method which has been described in several formats for the antibiotic susceptibility testing of blood cultures [75–77], *Bacillus anthracis* [78] and bacteria commonly isolated in clinical laboratories, including *S. aureus*, *H. influenza* and *P. aeruginosa* [79]. In essence, bacterial isolates or blood cultures are grown with and without antibiotics and quantitative real-time PCR detection of 16S rRNA genes or other targets used to measure differences in growth, so indicating antibiotic susceptibility or resistance. This methodology has been reported as accurate in the scenarios tested and generally more rapid than conventional testing methods.

5.5. Emerging technologies for infectious disease diagnosis

The considerable advances in next-generation sequencing technologies over the past few years have greatly enhanced the quantity and speed of data output. This, along with an appreciable reduction in costs, makes sequencing an increasingly realistic option for molecular diagnosis. Emerging technologies and existing commercial platforms have been comprehensively reviewed and their performances compared [80,81]. High-throughput sequencing of the 16S ribosomal RNA gene has been enthusiastically adopted by the research community for analysing polymicrobial populations, including those found in the CF airways [14,20,22,24]. A recent performance comparison of the high-throughput benchtop sequencers 454 GS Junior (Roche), MiSeq (Illumina) and Ion Torrent PGM (Life Technologies) reported run times ranging from 3 h to >27 h, generating data outputs of 70–300 Mb/run [81]. Although research studies have demonstrated the considerable potential of next-generation sequencing, data management and processing currently present significant bottlenecks. Following run completion, subsequent bioinformatic analysis of the output data requires significant time and expertise which would not be routinely available in a clinical laboratory setting. Additionally, the discriminatory power of 16S rRNA gene sequencing is not always sufficient for identification of bacteria to the species level, yet this information may be required for effective clinical treatment, for example identification of Bcc or *Mycobacterium* spp [82]. Furthermore, the migration of sequencing into clinical practice would involve an extra degree of data interrogation. Metagenomic sequencing provides a comprehensive shotgun view of individual microbial communities, but interpretation of the biological data to determine clinical relevance is highly complex requiring expert judgement. Therefore, in order to fully realise the potential of this technology for pathogen identification in polymicrobial infections, schemes for translating large-scale data output into a clinically useful report need to be developed. In particular, as mentioned previously, the clinical relevance of each species would have to be determined including a quantitative threshold indicating treatment and possible species interactions likely to increase pathogenicity.

The molecular diagnosis of infections using mass spectrometry incorporates a multiplex PCR/RT-PCR step for target amplification. This is followed by mass spectrometry analysis of the amplicons to identify which microbe targets were present in the sample. Current examples are Abbott's PLEX-ID^{TM} and Agilent's MassCode PCR systems. Recently, Abbott received CE marking for use of PLEX-ID^{TM} with three diagnostic assays intended for viral and bacterial identification: PLEX-ID Viral IC Spectrum, PLEX-ID BAC Spectrum BC and PLEX-ID Flu [83]. The PLEX-ID^{TM} system works by determining the mass of each PCR amplicon and then using software to calculate the nucleotide composition of the sequence through reference to the known molecular masses of the four nucleotides. Identification is by comparison with a database of reference sequences. The MassCode system uses PCR primers labelled with unique MassTags which are distinguishable by their molecular weight and can be cleaved from the primer by UV irradiation. Following target amplification, unincorporated primers are removed and the MassTags are retrieved from their amplicons for analysis by mass spectrometry to identify which targets had been amplified. MassCode technology has demonstrated the potential for detection of viruses and bacteria in respiratory samples [84], but is at present restricted to research applications.

6. Conclusions

Current CF diagnostic microbiology relies on culture-dependent methods for the detection and identification of microbes associated with CF airways infection. This significantly delays results delivery and biases diagnosis towards the most numerous and easily cultured microbes, which may not necessarily be responsible for poor patient health. Such an approach also requires highly specialised microbiological expertise and judgement, leading to inconsistencies between individuals or laboratories. Thus, culture-independent molecular diagnostics for the direct detection of multiple infectious agents in sputum presents an attractive prospect for improving CF patient care.

Transition from traditional culture-dependent methods to molecular methods would require the benefits incurred to outweigh any disadvantages. The most likely criticism of molecular assays is that lack of culture prevents immediate
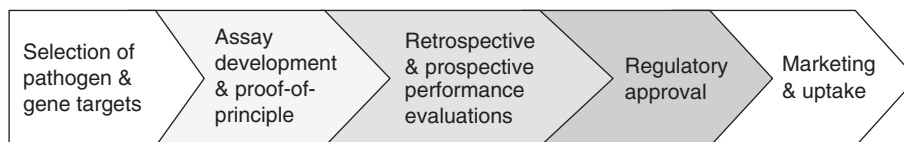

Fig. 1. Outline of the developmental process for a molecular diagnostic test from assay design to implementation.

further characterisation of an identified pathogen, such as antibiotic susceptibility testing or strain genotyping for the identification of epidemic clones of *P. aeruginosa* or Bcc. However, it could be argued that the usefulness of current *in vitro* antibiotic susceptibility tests in predicting clinical response is questionable and that the potential of molecular methods for predicting susceptibilities has not yet been thoroughly explored. Moreover, in cases where strain typing of a cultured isolate is indicated, rapid molecular testing would allow for subsequent follow-up culture with minimal time loss.

As yet no molecular tests suitable for comprehensive CF microbiology are commercially available, although potentially suitable technologies and instrumentation already exist. Thus, assay development and validation would have to be undertaken. The process involved in bringing a molecular assay through from design to deployment is outlined in Fig. 1, with regulatory approval such as CE marking and FDA approval essential for marketing as an *in vitro* diagnostic for clinical use. For a complex assay this process would take time. However, the success of other multiplexed molecular assays for infectious disease diagnosis demonstrates the considerable potential of a future CF assay for rapid, robust testing in automatable formats with a range of throughput levels. If judiciously applied to diagnostic CF sputum microbiology in a cost-effective manner, molecular technologies may in future help circumvent the bottle-neck and bias associated with conventional culture and improve detection of clinically relevant pathogens.

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