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Review

Molecular methods for pathogen and microbial community detection and characterization: Current and potential application in diagnostic microbiology

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ABSTRACT

Clinical microbiology laboratories worldwide have historically relied on phenotypic methods (i.e., culture and biochemical tests) for detection, identification and characterization of virulence traits (e.g., antibiotic resistance genes, toxins) of human pathogens. However, limitations to implementation of molecular methods for human infectious diseases testing are being rapidly overcome allowing for the clinical evaluation and implementation of diverse technologies with expanding diagnostic capabilities. The advantages and limitation of molecular techniques including real-time polymerase chain reaction, partial or whole genome sequencing, molecular typing, microarrays, broad-range PCR and multiplexing will be discussed. Finally, terminal restriction fragment length polymorphism (T-RFLP) and deep sequencing are introduced as technologies at the clinical interface with the potential to dramatically enhance our ability to diagnose infectious diseases and better define the epidemiology and microbial ecology of a wide range of complex infections.

Contents

1. Introduction

Defining the etiological agent(s) of infection facilitates more effective patient management by tailoring specific targeted antibiotic therapy. Clinical microbiology laboratories worldwide have historically relied on phenotypic methods (i.e., culture and biochemical

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tests) for detection, identification and characterization of virulence traits (e.g., antibiotic resistance genes, toxins) of human pathogens. However, limitations to implementation of molecular methods for human infectious diseases testing are being rapidly overcome allowing for the clinical evaluation and implementation of diverse technologies with expanding diagnostic capabilities. Molecular methods have been increasingly exploited to diagnose classic and fastidious pathogens, since the advent of the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). Some infectious agents identified and largely characterized in the past two decades using molecular methods include; Hepatitis C virus (Choo et al., 1989), *Tropheryma whipplei* (Relman et al., 1992), *Mycobacterium genavense* (Bottger et al., 1992), *Bartonella* (previously *Rochalimaea*) *henselae* (Matar et al., 1993), Hantavirus (Sin Nombre virus) (Chapman and Khabbaz, 1994), *Chlamydia pneumoniae* TWAR agent (Kuo et al., 1995), Herpesvirus 8 (Huang et al., 1995), the SARS Coronavirus (Drosten et al., 2003; Ksiazek et al., 2003), Metapneumovirus (Mackay et al., 2003), and Influenza A H1N1 (Renesto et al., 2000; Dawood et al., 2009).

Commercial suppliers have been steadily expanding the number of molecular diagnostic assays for infectious diseases testing (http://www.fda.org). Despite these continuing advances, a microbial etiology remains to be defined in a number of cases of human disease that have epidemiological, clinical and/or histological features of infection. Worldwide populations are increasingly being put at risk for novel contagions, particularly zoonotic agents or bacterial pathogens carrying acquired antimicrobial resistance genes (Church, 2004). Broad application of highly sensitive molecular technologies to common infections is also revealing that these diseases are not mono-microbial as previously thought, but rather, due to a disorder in complex microbial communities.

The human microbiome comprises 100 trillion microbial cells, outnumbering the somatic cells in the body by at least an order of magnitude (Savage, 1977). It was suggested early on that in order to properly appreciate human biology the 30,000 genes encoded in the human genome must be placed in the context of the millions of genes encoded by our microbial counterparts that inhabit the gastrointestinal tract, the genitourinary tract, the oral cavity, the nasopharynx, the respiratory tract, and the skin (Davies, 2001). The human microbiome project was designed to sequence 1000 novel bacterial reference genomes and the entire collective of microbial genomes from 250 healthy individuals (Turnbaugh et al., 2007; Peterson et al., 2009). A vast number of human commensal microbial species have yet to be identified and characterized. The composition of the human microbiota is unique and varies significantly between body sites. A recent study showed that 3.3 million non-redundant genes (150 fold greater than the human genome) were assembled from 1000 to 1150 distinct microbial species from 124 human gut samples (Qin et al., 2010). The diversity of the human microbiome is also affected by environmental factors, diet, and the host immune system (Ley et al., 2006). The essential role that commensal micro-organisms play in human health is becoming increasingly recognized for its functional involvement in normal development, nutrition, immune regulation and colonization resistance (Brook, 1999; Hooper, 2004; Mazmanian et al., 2005; Li et al., 2008; Clarke et al., 2010). There is also evidence accruing that the functional capacity of the human microbiome may be altered in patients with obesity and other chronic conditions (Qin et al., 2010).

The molecular methods for diagnostic microbiology are diverse. The advantages and limitation of techniques including real-time polymerase chain reaction, partial or whole genome sequencing, molecular typing, microarrays, broad range PCR and multiplexing will be discussed. Finally, terminal restriction fragment length polymorphism (T-RFLP) and deep sequencing are introduced as technologies at the clinical interface with the potential to dramatically enhance our ability to diagnose infectious diseases and better define the epidemiology and microbial ecology of a wide range of complex infections.

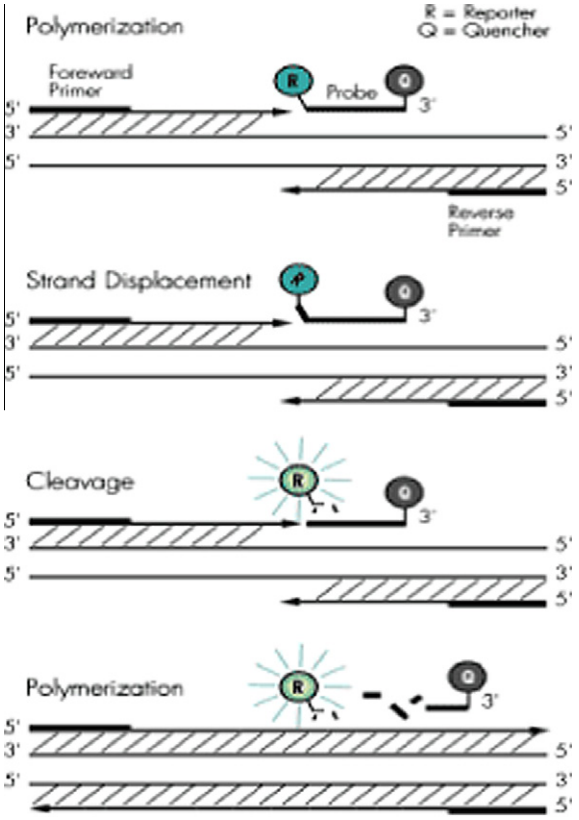

Fig. 1. Principles of real-time PCR Probes**. **Illustration from Heid et al. (1996).

2. Pathogen detection, surveillance and discrimination

Diagnosis of infectious diseases by using PCR involves target amplification methods and often nucleic acid sequence analysis of the amplicon. Unlike phenotypic characteristics that may be variable, molecular amplification methods take advantage of the use of stable genotypic characteristics. Another major diagnostic advantage of PCR is that the detection of nucleic acid does not require the labor intensive cultivation of microbial biomass. The bacterial genome provides variable regions for species identification and virulence factors or toxins loci can be useful for defining the pathogenicity of an organism. In addition to standard PCR, complete genome sequences have greatly facilitated the rapid development of other molecular identification methods for pathogen detection including nucleic acid hybridization (probes) (Procop, 2007), ligase chain reaction (Drancourt et al., 2000), strand-displacement amplification (SDA) (Walker et al., 1992a,b), transcription-based amplification (TAS, TMA, NASBA and 3SR) (Kwoh et al., 1989; Compton, 1991; Fahy et al., 1991) and loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000). The enormous amount of nucleic acid sequence data from human pathogens has enhanced the diagnostic capabilities of sequenced-based identification and genotyping methods of both common and uncommon micro-organisms (Fournier and Raoult, 2011).

2.1. Real-time PCR

Despite the development of alternative amplification technologies, PCR remains the most widely used method in both research and diagnostic laboratories for pathogen detection (Lisby, 1998;

Mothershed and Whitney, 2006; Procop, 2007). Conventional PCR assays designed for single target detection have been broadly utilized by most large diagnostic microbiology laboratories for the past 15 years (Lisby, 1998). Targets for human pathogens are based on specific loci for taxonomic identification, important phenotypic traits (such as antimicrobial resistance determinants), or virulence factors (Mothershed and Whitney, 2006).

Real-time PCR detection using probes is illustrated in Fig. 1 (Heid et al., 1996). The targets are detected in real time from the sealed PCR plate and there is no post-PCR processing required (Whitcombe et al., 1998), therefore, the risk of false-positive results due to amplicon carryover is substantially decreased compared to conventional PCR (Barken et al., 2007). In addition, most real-time PCR formats offer the option of melting curve analysis so that the amplicon can be distinguished from nonspecific product or primer-dimers (MacKay 2007; Tevfik Dorak, 2006). The performance of real-time PCR assays therefore has increased sensitivity and reliability of the reported results compared to those obtained by conventional PCR (Espy et al., 2006; Mothershed and Whitney, 2006; Procop, 2007). Real-time PCR assays can be designed to allow the detection and simultaneous quantification of the PCR products over a wide dynamic range of quantitative linearity, which is an important requirement of many diagnostic applications (Croagh et al., 2010; Itakura et al., 2011; Luft et al., 2011). Because real-time PCR assays are amenable to automation, most of these detection assays are run and completed in the clinical laboratory setting in a shorter time than conventional PCR, therefore providing a more rapid test cycle turnaround time. Real-time PCR has truly become 'walk-away' molecular testing in the clinical laboratory setting. Despite this benefit, cost is a limitation of implementation in smaller laboratories with limited resources because of instrument cost and the price of a single real-time PCR reaction including DNA extraction can be more than three times of the cost of conventional PCR (Mothershed and Whitney, 2006).

While commercially available PCR assays (kits) initially focussed on the detection of sexually transmitted infections and blood-borne viruses, the conversion to fluorescence based methods is resulting in rapidly expanding menus of human diagnostic real-time PCR assays developed in-house as well as commercially (http://www.fda.org) (Renesto et al., 2000; Edberg et al., 2008; Heymans et al., 2010; Scott et al., 2010; Diene et al., 2011; Hadfield et al., 2011; Kruttgen et al., 2011; Lehours et al., 2011; Luo et al., 2011; Luong et al., 2011; Nakauchi et al., 2011; Polley et al., 2011; Thurman et al., 2011; Touinssi et al., 2011; Wilson et al., 2011; Wolffs et al., 2011; Xu et al., 2011). Many more real-time PCR assays for detecting pathogens are currently in development and include detection of vancomycin-resistant enterococci and *Clostridium difficile*. In the near future, clinical laboratories will be able to perform many different types of monoplex and multiplex real-time PCR assays for screening, detection or nosocomial surveillance on the same automated instrument system akin to the performance of high-volume infectious diseases serological testing on automated instruments that perform enzyme immunoassay testing.

2.2. DNA target sequencing

Nucleic acid sequencing has emerged as important tool for pathogen identification and discovery. Identification of human pathogens by partial or whole gene sequencing had not been widely adopted by clinical microbiology laboratories even a decade ago due to the labor and expense of bringing this technology in-house, the lack of standardized methods for conducting analyses, and interpreting the results. Prior to 2000, DNA target sequencing had been primarily used for identification of organisms that were difficult to identify by using conventional phenotypic methods or recover by cultivation (Woo et al., 2008; Pareek et al., 2011). DNA target sequencing for the rapid identification of bacterial, viral and fungal pathogens has since been increasingly implemented as a routine procedure by many clinical laboratories. In the last decade, DNA sequencing technologies have improved throughput capability and cost. Important guidelines have been published that provides interpretive criteria for identification of a wide range of clinically important bacteria and fungi to the species and genus levels (Petti et al., 2008a). Commercial software is available that provides a quality assured database of known bacterial and fungal sequences for human pathogens (SmartGene® IDNS, Lausanne, Switzerland) (Simmon et al., 2006). Some diagnostic laboratories also perform sequencing analysis of viral cDNA generated by reverse transcription (Fournier and Raoult, 2011).

Sanger-based sequencing technology remains the most commonly used method, although newer non-electrophoresis methods (i.e., pyrosequencing, ion-torrent sequencing) are increasingly being used for clinical applications because of their improved efficiency and decreased cost (discussed later). DNA target sequencing for routine identification utilizes conserved genes for both bacteria and fungi that have been widely used for taxonomic purposes (Bottger, 1989; Doolittle, 1999; Atkins and Clark, 2004; Petti, 2007). In bacteria, the rRNA genes (5S, 16S, and 23S) and their intergenic regions have been commonly used for taxonomic purposes. These genes are universally present, are only minimally affected by horizontal gene transfer and contain hypervariable regions flanked by high sequence conservation. These characteristics have made the 16S rRNA gene the most widely used region for bacterial taxonomy and identification (Doolittle, 1999). The sequence information obtained from the 5′ portion of the 16S rRNA gene often provides sufficient data for identification purposes (Petti, 2007; Woo, et al., 2008). Much of the data in public databases also correspond to this region of the 16S rRNA gene (e.g., GenBank). Occasionally, the entire 1540 bp region is required to distinguish between particular taxa or strains, or alternative DNA gene targets may also have to be interrogated because some species may share 99–100% 16S rRNA sequence identities (Facklam, 2002; Janda and Abbott, 2002). Alternative gene targets for speciation have been used including *recA*, *rpoB*, *tuf*, *gyrA*, *gyrB* and *cpn*60 family proteins because of functionally conserved regions with intervening variability. In fungi, the eukaryotic rRNA gene complex consists of four ribosomal genes, 18S (small subunit), 5.8S, 28S (large subunit), and 5S genes. Within this region, the internal transcribed spacer (ITS) (White et al., 1990) and an approximately 600 bp D1/D2 region of the 28S subunit (Kurtzman and Robnett, 1997) are the most phylogenetically variable regions and have been widely used for fungal taxonomy and identification (Atkins and Clark, 2004; Petti et al., 2008a).

Several investigators have compared the performance of 16S rRNA gene or fungal ITS gene sequencing with conventional phenotypic methods and commercial automated systems for the identification of human pathogens. Overall, DNA target sequencing provides a somewhat higher rate of species identifications than phenotypic methods with an accuracy between 62% up to 92% depending on the group of bacteria studied and the criteria used to define a species (Bosshard et al., 2003a, 2004, 2006; Heikens et al., 2005; Simmon et al., 2006, 2008b). The 16S rRNA gene target will not however discriminate many species within the Enterobacteriaceae or closely related Staphylococcus, Enterococcus or Streptococcus species (Petti, 2007; Petti et al., 2008a,c). Similarly, fungal sequencing using the ITS or D1/D2 gene regions provides excellent identification compared to phenotypic methods and allows much more rapid identification of non-sporulation molds and rare yeasts that are difficult to identify using classical methods (Ciardo et al., 2007, 2010; Borman et al., 2008, 2010; Pounder et al., 2007).

2.3. Molecular typing

Molecular typing of bacterial isolates is required for infectious diseases epidemiological surveillance, to detect laboratory cross contamination, distinguish relapse from re-infection, and for surveillance during infectious outbreaks (van Belkum et al., 2007). Molecular typing is therefore crucial for source tracking and epidemiology (van Belkum et al., 2007; Fournier and Raoult, 2011). Van Belkum and colleagues published a comprehensive review and guidelines for the validation and application of molecular typing methods for use in bacterial epidemiology (van Belkum et al., 2007). Among non-sequence-based methods, pulsed-field gel electrophoresis (PFGE), which separates macro-restriction DNA fragments has been the "gold standard" technique used by clinical laboratories to examine the molecular relatedness of bacterial isolates as well as yeasts and fungi (Beadle et al., 2003). Provided that the PFGE method and interpretation are carefully standardized for a given microorganism, this method has robust reliability and intra- and inter-laboratory reproducibility (Goering, 2010). Similar DNA fragment-based methods have also been developed and used for clinical analyses including; restriction endonuclease fingerprinting analysis (REA) (Falk et al., 1985), plasmid typing (Farrar, 1983; Mayer, 1988; Nicolle et al., 1992), ribotyping (Thomson-Carter et al., 1989), PCR fingerprinting (Welsh and McClelland, 1990), restriction fragment length polymorphism (RFLP) (Goh et al., 1992; Metzgar et al., 1998), and amplified fragment length polymorphism (AFLP) (Vos et al., 1995; Meudt and Clarke, 2007). Multi-locus variable number tandem repeat analysis (MLVA) is another PCR-based method, which is used to determine the number and length of variable number tandem repeats (VNTRs) present in a genome due to slipped mispairing (van Belkum et al., 1998; Fournier et al., 2007). Several MLVA systems have been developed for interpretation of a variety of species (van Belkum et al., 2007) but few multicentre studies have been done (Francois et al., 2005; Kremer et al., 2005).

Due to the dedicated expertise, labor and specialized equipment needed to perform PFGE and other fragment-based methods, clinical laboratories are increasingly turning to more rapid, semi-automated, PCR-based or sequence-based methods to perform either rapid initial or routine genotyping analyses. The Diversilab system performs semi-automated repetitive-PCR analysis (Healy et al., 2005) and has good to excellent agreement compared to several other typing methods for distinguishing several different types of micro-organisms (Fluit et al., 2010; Overdevest et al., 2011; Deplano et al., 2011) including methicillin-resistant *Staphylococcus aureus* (MRSA) (Tenover et al., 2009; Babouee et al., 2011; Church et al., 2011a), vancomycin-resistant Enterococcus (Chuang et al., 2010; Bourdon et al., 2011), and commonly encountered dermatophytes dematophytes, Candida and fungi (Pounder et al., 2005,2006; Wise et al., 2007; Hansen et al., 2008). This method has also been used to genotype Acinetobacter baumanii outbreaks strains, and extended-spectrum B-lactamase producing Escherichia coli and Klebsiella pneumoniae isolates (Pitout et al., 2009; Brolund et al., 2010; Higgins et al., 2012). Semi-automated rep-PCR (Diversilab) however does not always provide the same discrimination power as PFGE or other typing methods (Babouee et al., 2011; Bourdon et al., 2011; Brolund et al., 2010, Chuang et al., 2010; Church et al., 2011a,b; Tenover et al., 2009). Recognizing this limitation of this semi-automated platform, Diversilab may nevertheless be a useful tool for the rapid identification of nosocomial outbreaks (depending on the type of bacteria involved) when used in parallel with confirmatory analysis with greater discriminatory power.

Several sequence-based genotyping methods have been used for infection surveillance and the tracking of strain evolution within a given geographic location. Single-locus sequence typing (SLST) uses the DNA sequence obtained from a highly variable single genetic locus to provide strain discrimination. Genotyping of *Streptococcus pyogenes* using the *emm* locus relies on sequencing of 150 bps coding for the N-terminal of the M protein (Simmon et al., 2008a). Interestingly, SLST typing of *S. pyogenes* has identified more than twice as many M-types as previously shown by using antisera (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Many clinical laboratories also now rely on SLST typing of *S. aureus* according to the *spa* gene that encodes protein A. Rapid sequence determination of the *spa*-type may now be automated because of the development of dedicated software for sequence interpretation (Harmsen et al., 2003a,b). The clinical appeal of SLST systems such as the one developed for *S. aureus* are the ability to use existing equipment (many laboratories have automated sequencers) and the excellent site to site reproducibility.

Other sequence-based genotyping methods that incorporate genome sequence data or were designed based on genome sequences, include single-nucleotide polymorphisms (SNPs) typing and multilocus sequence typing (MLST) (Maiden et al., 1998; Chaloner et al., 2011; Hayford et al., 2011; Lilliebridge et al., 2011; van Gent et al., 2011). However, these more expensive, laborious and complex methods have mainly been used for specific population-based microorganism diversity or population-based epidemiological studies, and currently are not widely performed as a diagnostic technique. The identification of single nucleotide polymorphisms (SNP), which is based on identifying point mutations at various genomic loci, has been used for genotypic characterization of several bacterial pathogens (http://www.ncbi.nlm.nih.gov/projects/SNP). MLST is based on determining the DNA sequence variation of several housekeeping genes (5–10) and has proven its taxonomic utility largely due to standardized interpretation, publically available software (i.e., eBURST) and the accessibility of databases (http://www.mlst.net). Multi spacer typing (MST) relies on a combination of the most variable intergenic spacers between strains (Fournier et al., 2007). Reports using this method are restricted to *Rickettsia* isolates (Fournier and Raoult, 2007).

3. Pathogen discovery and characterization

Clinical microbiology laboratories use molecular methods to enhance the detection of pathogen(s) in critical clinical situations whereby phenotypic methods have failed to identify an etiological agent usually because the pathogen is fastidious or uncultivable and/or the patient has been given empiric antibiotic coverage prior to the collection and submission of clinical specimens. The organism may be cultivatable in the later situation but present in such low numbers that phenotypic methods are not sensitive enough for detection. Currently, the two most commonly used methods to detect such organisms include a microarray-based assay and a universal broad-range 16S PCR which utilizes species-specific hybridization probes, sequencing or electro-mass spectrometry for subsequent analysis of the product(s). Next-generation sequencing technologies may also be routinely employed in the future for pathogen detection and discovery in the clinical laboratory.

3.1. Broad-range PCR

Although universal broad-range 16S rRNA gene PCR was originally developed for determination of microbial community diversity within complex bacterial populations in extreme environments, it has subsequently been used for pathogen detection where conventional bacteriological techniques fail to produce a diagnostic result (Sontakke et al., 2009). Broad-range PCR offers an important clinical benefit for analysis of critical culture-negative samples as it can detect bacterial nucleic acid present in a sample through targeting

conserved bacterial sequences (described in Section 2.2). Nucleic acid sequencing of amplicon generated by broad-range PCR allows identification of the organism(s) by comparison with known sequences deposited at Genbank or other databases (Drancourt et al., 2000; Janda and Abbott, 2002; Petti, 2007). Previously uncharacterized microbes (novel pathogens) may also be detected because of the universal nature of primers/probes and the high sensitivity for detecting the presence of low copy number targets (Bottger, 1989; Relman and Falkow, 1992; Greisen et al., 1994; Monstein et al., 1996; Drancourt et al., 2000; Mothershed and Whitney, 2006; Procop, 2007).

Several investigators initially used amplification of conserved regions of bacterial ribosomal RNA and amplicon sequencing to identify human pathogens (Bottger, 1989; Wilson et al., 1990). Subsequently broad-range PCR has been extensively used non-selectively to detect bacterial DNA in clinical samples (Monstein et al., 1996; Corless et al., 2000; Nadkarni et al., 2002; Nikkari et al., 2002; Harris and Hartley, 2003; Saravolatz et al., 2003; Xu et al., 2003; Ott et al., 2004; Jordan et al., 2005; Zucol et al., 2006; Fenollar and Raoult, 2007; Rosey et al., 2007; Reier-Nilsen et al., 2009; Chang et al., 2011). Rather than perform multiple monoplex PCR assays on a given sample, broad-range assays are designed such that a single clinical sample can be simultaneously screened for multiple organisms (i.e., any bacterial species); however, amplicons may require cloning prior to sequencing in order to get an accurate result with traditional Sanger-based methods due to the potential mixed nature of amplified products (Grahn et al., 2003). This approach has been successfully applied to clinical samples from normally sterile sites to diagnose invasive bacterial or fungal infections, including culture-negative meningitis, pneumonia and endocarditis. Increasingly, this approach is also being used to identify the putative pathogen(s) in a number of inflammatory diseases, and for detection of known pathogens that are not easily diagnosed using standard methods. *Tropheryma whippelii* was identified as the cause of Whipple's disease using a broad-range molecular PCR (Lepidi et al., 2004). *Helicobacter pylori*, the cause of peptic ulcer disease, has been found to be the dominant microbiota in the human stomach using broad-range molecular analysis (Bik et al., 2006). Broad-range 16S PCR has also been used to detect clinically relevant *Chlamydia* spp. infecting humans and animals (Goldschmidt et al., 2006).

A significant advantage of real-time broad-range PCR assays are the ability to quantify the bacterial load in the sample and the higher analytical sensitivity compared to conventional PCR (Millar et al., 2001; Zucol et al., 2006; Ammann et al., 2007; Fenollar et al., 2008a). The minimal detection limits for conventional broad-range PCR has been determined to be in the range between ten and 10^3 CFU or CFU equivalents per PCR (Harris and Hartley, 2003), whereas the reported detection limits for real-time broad-range assays is 10–100X lower with improved sensitivity. Zucol and colleagues developed a broad-range real-time PCR assay method that was able to detect $\leqslant 10^2$ CFU in water per reaction but was not tested on clinical samples (Zucol et al., 2006).

Overall sensitivity and detection limits of broad-range PCR assays may also be influenced by several factors including the prior culture enrichment of the sample, the use of good molecular laboratory practices and decontamination procedures, the extraction methods, the choice of universal primers and probes, the assay cycle time and melting temperature, concentration of the amplified products prior to sequencing, and the appropriate use of controls throughout the procedure (Harris and Hartley, 2003; Sontakke et al., 2009). Several studies have shown substantial increases in the sensitivity of broad-range PCR reactions when clinical samples undergo prior culture enrichment in broth or shell vials rather than direct plating prior to molecular analysis (Harris and Hartley, 2003; Gouriet et al., 2005; Saglani et al., 2005). Enrichment culture of 382 pediatric fluid and tissue samples in a broth medium prior to 16S rDNA PCR identified several pathogens in these samples that were not previously detected by primary plating or direct PCR of extracted DNA from the sample (Harris and Hartley, 2003). Saglani and colleagues also performed 16S broad-range PCR on pleural fluid samples from 32 children with empyema and detected bacterial DNA in 75% of the samples following enrichment culture versus only 18.7% after direct plating (Saglani et al., 2005). Culture-enrichment in parallel with broad-range PCR has also been shown to be an effective means of bacterial recovery and identification from cystic fibrosis sputum (Sibley et al., 2011). Shell vial culture has also been used as an enrichment technique for the growth of fastidious or previously uncharacterized micro-organisms from clinical samples (Gouriet et al., 2005). Human embryonic lung fibroblast and human endothelial cell lines have been used most often followed by 16S broad-range PCR and sequencing to identify the organism.

Initial clinical evaluations of broad-range PCR used conventional methods. However, DNA contamination from other sources including environmental or commensal organisms, PCR reagents including *Taq* polymerase, or even the disposables have been identified as a major concern for broad-range PCR due to the sensitivity of the assay (Corless et al., 2000; Vandecasteele et al., 2002; Mohammadi et al., 2005; Chang et al., 2011). Universal primers and probes that target the 16S rRNA gene have therefore been recently adapted to a real-time broad-range PCR in order to minimize this problem (Kobayashi et al., 2005; Goldschmidt et al., 2006; Zucol et al., 2006; Deutch et al., 2007; Schabereiter-Gurtner et al., 2008).

The universal 16S rRNA gene target and the high sensitivity of PCR can be a limitation due to false positive test results when analyzing clinical samples. Even small amounts of bacterial DNA contamination either within the clinical sample, or introduced during some part of the molecular procedure may result in a false positive broad-range PCR result, or a result that is difficult to interpret because of the amplification of one or more common commensal organism(s). Investigators have outlined several sources of bacterial DNA contamination that may occur during the sample collection, nucleic acid extraction or PCR analysis steps (Millar et al., 2002, 2007; Vandecasteele et al., 2002; Fenollar et al., 2006, 2008b). Despite clinical laboratories having standard operating procedures for the collection of all types of clinical samples, contamination by commensal organisms remains problematic with many specimen types (Blake and Doherty, 2006; Dhillon et al., 2009; Marlowe et al., 2010; Patton and Schmitt, 2010; Ishikawa et al., 2011). DNase or restriction enzymes may also be used to de-contaminate the PCR reaction mixture (Millar et al., 2002; Vandecasteele et al., 2002). Broad-range PCR assays should only be performed using good molecular laboratory practices as previously outlined (Mitchell et al., 2011). In addition, it is critical that a human DNA amplification control as well as a positive and negative control (Tanner et al., 1998; Millar et al., 2002; Bosshard et al., 2003b; Greub et al., 2005; Fenollar et al., 2008b) be included during sample extraction and carried throughout the procedure. Negative controls should consist of a negative sample control (i.e., an aliquot of a known negative clinical sample such as blood from a person without bacteraemia), and a negative reaction mixture control (i.e., contains all reagents but no clinical sample extract) in order to detect reagent contamination. Commercial reagents may also be the source of bacterial contamination and may need to be sterilized using filtration of other methods prior to use (Muhl et al., 2010; Chang et al., 2011).

Currently there is no universal DNA extraction protocol that is effective for both Gram-negative and Gram-positive bacteria and the extraction method impacts the analytical sensitivity of the assay. Prior studies of broad-spectrum PCR have used enzyme treatment (lysozyme, proteinase K), freezing and thawing or boiling,

mechanical disruption, a commercial method (i.e., QiAmp Blood Kit, Qiagen or automated instrument) or a combination of these methods for DNA extraction (Schuurman et al., 2004; Zucol et al., 2006; Rosey et al., 2007; Fenollar et al., 2008b). Zucol and colleagues determined the analytical sensitivities of broad-range RT-PCR based assays employing one to three different genomic DNA extraction protocols in combination with one or three different primer pairs targeting the 16S rRNA gene to detect a panel of 22 bacterial species (Zucol et al., 2006). A DNA extraction protocol using lysozyme, lysostaphin and proteinase K followed by PCR amplification using primers Bak11W/Bak2 (796 bp product), resulted in the best overall sensitivity; 82% of the strains were detected at a low concentration (Zucol et al., 2006). The selection of primers and probes for broad-range PCR assays also effects analytical sensitivity. Primers targeting the 16S rRNA gene are used in most of these assays, while some have also targeted the 23S rRNA gene (Greisen et al., 1994). As outlined by Baker et al. (2003), it is not possible to develop a 16S rRNA gene primer set that will be entirely universal. Therefore, different conserved regions within the 16S rRNA gene and various primer sequences for been employed for analysis of human clinical specimens (Greisen et al., 1994; Baker et al., 2003). The target loci and the primer sequences impact taxonomic assignments. In addition, analysis of existing primer sets demonstrates that environmental organisms within the Archaea and Eukarya can also be detected. Another limitation of this approach is that a definitive identification is not always possible due to the lack and quality of data in public databases as well as insufficient sequence divergence within the 16S rRNA gene sequence between certain taxonomic groupings (Petti, 2007; Petti et al., 2008a; Simmon et al., 2006).

Despite these limitations, the diagnostic utility of broad range 16S rDNA PCR has been evaluated in a variety of clinical conditions including endocarditis, meningitis, bone and joint infections and endophthalmitis (Fenollar and Raoult, 2004; Sontakke et al., 2009). This method has also been used to determine the cause of infection in pediatric patients with fever and neutropenia (Jordan et al., 2005; Ammann et al., 2007; Reier-Nilsen et al., 2009) or culture-negative intracerebral abscesses (Petti et al., 2008b) as well as to determine a cause of death in patients with unexplained mortality and critical illness (Nikkari et al., 2002). The implementation of 16S broad-range PCR to detect and differentiate bacteria in heart valve specimens substantially improves the diagnosis of culture-negative endocarditis (Millar et al., 2001; Marin et al., 2007). Although heart valve culture remains part of the Duke classification scheme for the diagnosis of infectious endocarditis, its poor sensitivity (7.8–25.4%) and specificity (71.6–88.6%) has led several investigators to recommend that culture testing be replaced by broad-range PCR as a major diagnostic criterion because of its much higher sensitivity (range = 41.2–96%) and specificity (95–100%). Several studies have documented the reliability of broad-range PCR for the detection of bacteria in excised heart valves and this assay is being increasingly used in conjunction with blood cultures to determine the microbial aetiology in both blood culture-positive and -negative endocarditis cases (Millar et al., 2001; Moore et al., 2001; Bosshard et al., 2003b; Lepidi et al., 2003, 2004; Breitkopf et al., 2005; Greub et al., 2005; Houpikian and Raoult, 2005; Raoult et al., 2005; Rice and Madico, 2005; Rovery et al., 2005; Marin et al., 2007; Dreier et al., 2008; Munoz et al., 2008; Voldstedlund et al., 2008; Tang, 2009; Fournier et al., 2010; Caram et al., 2008). Broad-range 16S rDNA gene PCR on heart valve tissue allows diagnosis of unusual causative agents of culture-negative endocarditis including *Cardiobacterium hominis* (Mueller et al., 1999), *Bartonella quintana* (Houpikian and Raoult, 2005; Dreier et al., 2008), *Tropheryma whipplei* (Lepidi et al., 2004; Thuny et al., 2010), *Mycoplasma* spp. (Fenollar et al., 2004) and *Coxiella burnetii* (Issartel et al., 2002; Thuny et al., 2010).

Interpretation of all broad-range PCR results should be correlated with the clinical picture due to the potential for DNA contamination from other sources. A 'positive' broad-range 16S PCR result should be correlated with the patient's clinical history and examination findings along with the results of other pertinent diagnostic test information to determine the likelihood of a particular disease. For example, in the case of a cerebrospinal fluid (CSF) sample testing positive by 16S broad-range PCR for a bacterial meningitis pathogen, the patient's clinical presentation and other CSF test results (i.e., CSF glucose, protein, cell count and culture) should be consistent with a diagnosis of acute meningitis. Further strength is also added to a 16S broad-range result of detection of a rare or unusual pathogen when the same micro-organism is detected in longitudinal samples, multiple samples of the same kind from several patients or several different specimen types. Interpretation of the presence of common specimen/culture contaminants by broad-range 16S PCR may be particularly difficult when the isolate may also be a potential cause of infection (i.e., *Streptococcus viridans* sp. or coagulase negative staphylococci).

The clinical relevance of bacterial DNA detection may also not indicate active infection in patients who have received prior antibiotic treatment since the broad-range PCR assay will also detect nucleic acid from nonviable cells. Broad-range PCR studies for the detection of bacterial DNA in heart valve tissue have clearly demonstrated that bacteria are slowly cleared from tissue despite antibiotic therapy, so that molecular assay results should be interpreted with caution because the bacteria may no longer be viable. Rovery and colleagues showed that bacterial DNA was found on heart valves more often in patients who underwent heart valve replacement while still on antibiotic therapy (60%) versus those having surgery after completion of their antibiotic treatment (37%, $p = 0.02$) (Rovery et al., 2005). The presence of bacterial DNA was however highly correlated with the presence of histological lesions ($p = 0.0001$) and with the presence of bacteria in the tissue Gram stain ($p < 0.0001$).

3.2. Broad-range PCR with electrospray ionization mass spectrometry detection

A new commercial high-throughput bioidentification system (PLEX-ID, Abbott Molecular) has recently become available, that combines broad-range PCR using several gene targets (Table 1) with product detection and analysis by electrospray ionization mass spectrometry (PCR/ESI-MS) (Hofstadler et al., 2005; Baldwin et al., 2009). It shows great promise for identifying a broad range of pathogens without prior knowledge of the pathogen's nucleic acid sequence (http://us.plex-id.com/stystemoverview) (Ecker et al., 2008; Baldwin et al., 2009). PCR/ESI-MS can also provide results that include information about an organism's genotype, resistance, or virulence profile. Although currently restricted to epidemiological genomic studies and pathogen discovery, this technology may have a major diagnostic advantage in being able to provide detection, identification and characterization of a broad range of pathogens directly from clinical specimens. This instrument also has the potential to process up to 300 samples in a 24 h period.

PCR/ESI-MS has so far had limited clinical evaluation but several recently published studies demonstrate the potential range of human diagnostic applications in rapid detection of bacteraemia, and the identification and typing of a wide range of pathogens (i.e., *S. aureus*, *Candida* spp., *Mycobacterium* spp., *Erlichia* spp., Influenza A and other respiratory viruses, and pan-orthopoxviruses) (Eshoo et al., 2009, 2010; Hall et al., 2009; Wolk et al., 2009; Deyde et al., 2010; Metzgar et al., 2010; Chen et al., 2011a,b; Gu et al., 2011; Kaleta et al., 2011; Massire et al., 2011; Wang et al., 2011). However, further validation of PCR/ESI-MS in the clinical laboratory will need to be performed against standard procedures in

Table 1
Broad-range primers for bacteria used for PCR/ESI-MS.

Gene target	Bacterial detection	Primer pairs**
16S rDNA	Universal	346,347,348,361
23S rDNA	Universal	349,360
*rpo*C	Bacteroidetes, fusobacteria, spirochaetes, bacilli, proteobacteria (α/β/γ)	354
*rpo*C	Proteobacteria (α/β)	363
*val*S	Some γ-proteobacteria: *Erwinia*	358
*rpo*B	*Pantoea*, *Pectobacterium*	359
*rpo*B	Proteobacteria (α/β)	362
*tuf*B	Some β-proteobacteria: *Eikenella*, *Neisseria*, *Achromobacter*, *Bordetella*, *Burkholderia*, *Ralstonia*	367
*rpl*B	Clostridia, fusobacteria, bacilli, and ε-proteobacteria (*Campylobacter*, *Helicobacter*, *Wolinella*)	356, 449
*inf*B	Bacilli	352
*ssp*E	*Bacillus cereus*	355

** Adapted from Baldwin et al. (2009). For specific primer sequences see Hofstadler et al. (2005).

order to demonstrate the role of this system in potentially replacing other identification methods that are currently faster or as fast while being much less expensive to perform.

3.3. Microarrays

Microarrays are used as a high-throughput large-scale screening systems that allow for simultaneous identification and characterization of a very large number (up to several hundred thousand) of alleles of interest. Microarrays are therefore a very powerful tool with greater capacity (100–1000X) compared to other molecular methods (i.e., real-time PCR) that can only analyze a small number of targets. Microarray-based genotyping can potentially identify new genes by including sequences derived from related bacteria or identify polymorphisms that are detectable by oligonucleotide hybridization (Miller and Tang, 2009). DNA microarrays are also being used to diagnose infection by the identification of pathogens and simultaneous detection of drug-resistance and virulence-associated traits in these isolates (Bager et al., 1999).

Technical details of various microarray configurations including probe length and synthesis, number of possible features (i.e., density of the microarray), and the solid-surface used have been previously outlined in detail (Bager et al., 1999; Miller and Tang, 2009). Briefly, all microarrays consist of millions of pre-designed and developed oligonucleotide probes (i.e., consisting of either dsDNA, PCR amplicons 200–800 bp from a known genetic sequence, shotgun library clones or cDNA) that are specifically bound and immobilized on the solid-surface support of the array. The target nucleic acid extracted from a pathogen or clinical specimen of interest is fluorescently labeled and subsequently hybridized to the probe matrix of the microarray. A successful hybridization event between the labeled target molecule and an immobilized probe oligonucleotide creates increased fluorescence intensity above the background level, which can then be measured using either a fluorescent scanner or a flow cytometer. The large amount of complex data generated from a microarray assay can then be analyzed by using a variety of methods with sophisticated bioinformatics algorithms (Zhang et al., 2009; Kaewpongsri et al., 2010; Loewe and Nelson, 2011).

Manufacturing and genomic advances that have allowed expanded development of several commercial microarray formats include solid-support, high-density bead–bead, electronic and liquid suspension bead-based arrays that can be performed and analyzed using bioinformatics software on an automated system (Miller and Tang, 2009). Traditionally, the probes were printed onto glass slides, but commercial systems rely on several other techniques for microarray production and spatial matrix arrangement including: (i) *in situ* synthesis of the oligonucleotides onto the surface of a quartz wafer (GeneChips® Affymetrix), (ii) high-density bead arrays (SAM, Sentrix BeadChip, Illumina, San Diego, CA), (iii) low-density electronic arrays that use a microelectronic device (NanoCHIP® 400, Nanogen, San Diego, CA), and (iv) liquid-based suspension bead-based arrays that use colored microspheres for the solid support of applied probes (Luminex Corp., Austin, TX).

Microarrays theoretically appear to be an ideal technology for clinical microbiology application because pathogens could be not only detected and identified, but also genotyped and further profiled to determine antimicrobial resistance and virulence traits using a single test. Although microarrays are used for complex human genetics applications they have not been as widely used by clinical microbiology laboratories but are increasingly being implemented for simultaneous detection, identification and genotyping of important viral infections. The major factors limiting implementation of printed and commercial microarray assay formats in the clinical microbiology setting are outlined in Table 2 (Miller and Tang, 2009). Although several commercial suppliers have developed infectious diseases microarrays for research purposes or will manufacture customer-specified assays, commercial assays that have been used diagnostically detect a large panel of respiratory viruses (Petti and Hillyard, 2007; Nolte et al., 2007; Pabbataju et al., 2008; Merante et al., 2007) including the only FDA-approved xTAG Respiratory Viral Panel FAST assay (i.e., eight different respiratory viruses including strains of Influenza A, B), which has a liquid suspension bead-based format (Luminex) (Krunic et al., 2007; Merante et al., 2007; Pabbaraju et al., 2008) (Luminex Molecular Diagnostics, Toronto, Ont. Although other commercial NanoChip® Nanogen) microarray panel tests were previously developed for infection testing (human papilloma virus, transplantation, bacterial pneumonia, infection control, tuberculosis and a gastrointestinal panel), the company discontinued its microarray business in 2007. However, a multitude of clinical applications have been or are in development for liquid suspension bead-based microarrays (Luminex), and this method will most likely be successfully developed commercially for other select diagnostic multiplex panel tests (e.g., viral encephalitis panel, antibiotic resistance panel etc.)

Low or middle-density microarray assays are also increasingly being used for diagnostic testing or epidemiological investigations. To date, microarray techniques have been most widely applied for the detection, identification and genotyping of human papilloma viruses (HPV) known to causing cervical dysplasia, and cancer of the cervix and other sites (Hwang et al., 2003; Oh et al., 2004; Albrecht et al., 2006; Gheit et al., 2006; Klug et al., 2008; Ermel et al., 2010; Eklund et al., 2010; Schopp et al., 2010; Cho et al., 2011). Currently there are about 100 identified genotypes of HPV, of which about 40 are genital HPV types that have been assigned to either oncogenic or 'high-risk' or non-oncogenic or 'low-risk' categories based on their detection in cervical cancers (Munoz et al., 2003). Worldwide, HPV types 16 and 18 have been shown to cause the majority of cervical cancers, approximately a half of high grade precancerous lesions and a quarter of low grade lesions, whereas low-risk HPV types 6 and 11 cause most genital warts and some low grade cervical lesions (Munoz et al., 2003; Li et al., 2011). Epidemiologic surveillance of high-risk HPV genotypes is therefore important for not only clinical care but also vaccine development. One of the earliest studies of the clinical efficacy of an HPV oligonucleotide microarray for detections of 15 types of high-risk HPVs (16/18/31/33/35/39/45/51/52/54/58/59/66/68/69), and seven types of low-risk HPVS (6/11/34/40/42/43/44) from various histological cervical lesions showed that this was a highly comparable method to PCR-RFLP analysis; HPV DNAs were detected in 158 and 174 of the 234 cervical samples by PCR-RFLP compared to

Table 2
Major advantage and disadvantages of various microarray formats for diagnostic purposes[**].

Format	Supplier	Advantages	Disadvantages
Printed arrays	In-house manufacture by a core facility NimbleGen (Roche) Agilent	• Inexpensive • Simple to manufacture • Flexibility to change spot probes as required • Assay steps manual	• Need reliable access to a core facility(dust, humidity and temperature controlled) • Little supervision over quality control of manufacture • Requires extensive in-house probe design and manufacture • Requires expensive assay validation • Requires access to in-house bioinformatics to analyze and assess quality of the data • Can be inflexibility to change assay probes as required • Can be non-conducive to user-defined development (exception: Agilent)
In-situ synthesized oligonucleotide arrays	GeneChip®(Affymetrix)	• Custom-assay production • Automated	• Most expensive • High-density so (15,000 to >10^6) spot probes per assay • Complex to manufacture – probes chemically synthesized directly on quartz wafers
Electronic	NanoChip® (Nanogen)	• Electric fields promote active hybridization to nucleic acids onto a microelectronic device • Low-density (400 maximum) but adequate for diagnostic panel assays • Less expensive than high-density format • Allows flexible testing of multiple targets in a single sample or multiple samples on the same microarray cartridge	• Commercially available products discontinued (2007)
Liquid-based suspension bead-based arrays	Luminex Molecular Diagnostics	• Probes or universal sequence tags are attached to spectrally unique microspheres; bead hybridization with fluorescently labeled target DNA measured by flow cytometry • Low-density (100 maximum) but adequate for diagnostic panel assays • Less expensive than high-density format • Only FDA-approved commercial assay (xTAG Respiratory Panel) • Most flexible, practical format for clinical use • Multitude of clinical applications	• Requires careful validation of the positive fluorescent threshold for each analyte in a user-defined multiplex-bead-based assay

[**] Adapted from Miller and Tang (2009).

HPV microarray method, respectively (Hwang et al., 2003). Subsequent studies have compared several commercial HPV detection and genotyping assays, and highlighted the differences in their performance with regards to type-specific detection rates of various HPV genotypes (Klug et al., 2008; Ermel et al., 2010; Eklund et al., 2010; Schopp et al., 2010; Cho et al., 2011). Eklund and colleagues (2010) for the WHO HPV Laboratory Network recently performed a global proficiency study of HPV genotyping used in HPV surveillance and vaccinology by distributing 43 coded samples composed of titration series of purified plasmids of 16 HPV types (6/1/16/18/31/33/35/39/45/51/52/56/58/59/66/68) whereby detection of at least 50 IU of HPV 16 or HPV 18 DNA and of 500 genome equivalents of the other 14 less prevalent HPV types was considered proficient. Participating laboratories used more than 21 HPV-genotyping assays including the Linear Array (Roche), Lineblots (in-house, PGMY), InnoLiPa (Innogenetics), DNA chip (Biocore), as well as type-specific real-time PCR(GenoID), PCR-RFLP, PCR-Luminex (Multimetrix), PCR sequencing and microarray assays (Papillocheck, Genetel, in-house). HPV 16 and 18 were detected in 98.7% and 92.2% of the data sets, respectively, while less common HPV types 56, 59 and 68 were detected in less than 80% of the data sets. Aside from non-detection of specific HPV types, false-positive HPV genotyping results occurred for two commercial tests and were also reported in twenty-eight data sets which also were considered nonproficient. Although the sensitivity of HPV tests for detection in cervical screening programs need not be as high for less commonly encountered oncogenic HPV types, clinical laboratories need to know the limitations of various microarray methods and follow recently published guidelines for evaluation of such tests before implementation (Meijer et al., 2009).

Several evaluations have also been done of microarrays that simultaneously assess a large number of microbial gene targets in order to detect and identify pathogens from clinical samples. However, sensitivity of microarray assay detection and identification of pathogens from clinical samples is highly dependent on target gene(s), and enhanced by specific microbial gene amplification before analysis by the array. Either a broad-range or a multiplex PCR is commonly used that targets conserved regions within the 16S bacterial and 28S fungal and intergenic transcribed spacers (ITSs) in rRNA genes prior to microarray analysis of downstream PCR products instead of relying on DNA target sequencing (Rood et al., 2008). An early application of this approach was the panel detection of 40 predominant human intestinal bacterial pathogens from clinical stool samples (Blyn et al., 2008; Ecker et al., 2008). Other clinical applications of user-defined microarrays include; rapid detection and characterization of methicillin-resistant *S. aureus* (MRSA) (Koessler et al., 2006), detection of antimicrobial resistance genes (Bager et al., 1999), and microbial typing of bacterial enterocolitis pathogens (Volokhov et al., 2003; Fitzgerald et al., 2007).

4. Microbial community analysis

Characterizing the properties of pathogenic microbial communities is a new frontier for clinical microbiology because studies of the human microbiome demonstrate that there are far more

unique species present at many body sites than are easily grown in the clinical laboratory (Turnbaugh et al., 2007). Several recent molecular studies of clinical samples from cystic fibrosis patients (Sibley et al., 2008a,b) as well as complex abscesses (Versalovic, 2009) have demonstrated the presence of a far more complex microbial flora than was previously found using phenotypic methods. Classic culture-based methods have most likely given clinical microbiologists a skewed view of the complex microbial community involved in some infections because only the cultivatable pathogens present in high numbers in a given clinical sample may be detected and reported (Petrosino et al., 2009). *Escherichia coli* is the prototypical gut micro-organism but this organism belongs to the γ-proteobacteria phylum, which in its entirety only represents <1% of the total microbial population in the human intestine (Hamady and Knight, 2009). *E. coli* has been thought of as a dominant resident in the human gut solely because it is readily cultivated *in vitro*.

Because of the far greater discriminatory sensitivity of culture-independent approaches, molecular methods are fundamental for characterizing complex human-associated microbial communities. Culture-independent molecular technologies have typically relied on either direct nucleic acid sequencing or electrophoresis in order to investigate community structure, community dynamics and community behavior (Rogers and Bruce, 2010). However, the revolution in high-throughput sequencing technologies in the last decade has allowed for unprecedented data output, orders of magnitude beyond traditional Sanger-based sequencing, at a fraction of the cost per nucleotide (Pallen et al., 2010). Several high throughput sequence-independent community-profiling approaches are also available and include denaturing gradient gel electrophoresis (Muyzer et al., 1993), automated ribosomal intergenic spacer analysis (Fisher and Triplett, 1999) and terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al., 1997). Here we will describe T-RFLP and deep 16S rRNA gene sequencing as tools for profiling microbial communities.

4.1. Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is an approach that has been extensively used to profile fungal, archeal and eubacterial communities and has comprehensively been reviewed in the context of other fingerprinting techniques in the past (Schutte et al., 2008). Traditionally, it has been used to profile environmental communities but it has also been exploited to investigate microbial communities associated with the oropharynx, gastrointestinal tract, lungs, vagina and other body sites (Rogers et al., 2003; Coolen et al., 2005; Sibley et al., 2008b; Yamamoto et al., 2009; Jakobsson et al., 2010). The technique utilizes one or both PCR primers labeled with a fluorescent dye (labeling both forward and reverse primers can improve resolving power) to amplify the universal 16S rRNA gene from a bacterial community of interest. The resulting amplicon is digested with one or multiple restriction enzymes (at four base pair recognition sequences), salts and primers are removed and the relative abundance of terminal restriction fragments (T-RFs) are resolved by polyacrylamide gel or capillary electrophoresis. The resulting data represents a semi-quantitative profile of the most dominant community members.

T-RF size is estimated by reference to internal size standards and the abundance is expressed as either peak height or peak area. T-RFs are assigned to operational taxonomic units or "bins". Accurate sizing of T-RFs can be challenging due to the fact that electrophoresis mobility can be influenced by sequence composition (Kaplan and Kitts, 2003) and fluorophores each have different electrophoresis properties (Tu et al., 1998). For this reason, comparisons between profiles should be done only between data generated with the same fluorophore. It is also known that discrepancies between true and experimentally determined T-RFs can exist (Kaplan and Kitts, 2003).

T-RFLP is inexpensive, high throughput, reproducible and can be a very effective tool for characterizing the dynamic changes that occur in human-associated microbial communities over time (Sibley et al., 2008b). Importantly, the technique is best suited for microbial communities with low to intermediate richness; in populations with greater than 50 distinct organisms the resolving power can be significantly reduced (Engebretson and Moyer, 2003). Like all PCR-based assays, the technique suffers from amplification bias. Appropriate primer selection is essential; however, it must be noted that a catchall, perfect universal primer set does not exist for community profiling (Marsh et al., 2000). Programs are available to optimize primer design and selection but can be limited by the incompleteness of current databases (Collins and Rocap, 2007).

The T-RFLP technique can suffer from the fact that a baseline to distinguish between signal and noise is not necessarily automated; both peak height and area can be used in the determination of this threshold. Several different strategies have been employed and include using a fixed detection threshold, a fixed percentage threshold (Sait et al., 2003), variable percentage thresholds (Osborne et al., 2006), use of a correction factor (Dunbar et al., 2001) and statistical methods (Abdo et al., 2006), each with their advantages and disadvantages (Schutte et al., 2008).

Web-based in silico prediction tools are available for microbial identification from T-RFLP data (Kent et al., 2003; Shyu et al., 2007) but these tools suffer from the inherent experimental issues associated with T-RFLP and the enormous size and unreliability of many of the sequences in current databases. T-RFLP is most robust when the in silico predictions are corroborated with parallel 16S rRNA sequencing (either clone-based or 454 pyrosequencing (Jakobsson et al., 2010). This is because microbial identification data based solely on in silico predictions are not often reliable and can be confusing because of the vast number of possible organisms predicted for each T-RF, many of which suffer from the "uncultured bacterium" phenomenon. Relevant relationships between microbial communities can be assessed by using various tools including principal component analysis, multi-dimensional scaling, self-organizing maps, cluster analysis and canonical correspondence analysis (Schutte et al., 2008).

Certainly, T-RFLP provides invaluable, inexpensive data that can be quite informative with respect to community dynamics particularly if the community members have been characterized using a more sophisticated sequencing-based approach or the identity of specific T-RFs have been determined experimentally. T-RFLP is an excellent tool for measuring the diversity that exists in an amplicon before proceeding to more involved community profiling experiments such as high throughput sequencing.

4.2. Deep sequencing

Nearly 20 different next-generation sequencing instruments are currently on the market (and more are on the way), each with their own advantages and disadvantages (Glenn, 2011). A detailed discussion regarding the technical aspects of all of these technologies is far beyond the scope of this article but excellent resources are available (Martinez and Nelson, 2010; Metzker, 2010). Sequencing experiments can range from 14 days with the Genome Analyzer IIx (Illumina) instrument to a mere two hours with the new Ion Torrent instrument (Life Technologies). Single experiments can generate hundreds of Gigabases of data (Glenn, 2011). Several of the platforms have been widely adopted and include the Roche 454 technology, the Solexa/Illumina platform and Life Technologies SOLiD platform (Metzker, 2010). The incredible amount of sequencing throughput provided by these new instruments has,

for the first time, allowed for a deep characterization of complex microbial communities.

The most common technology that is currently used for community profiling because of the robustness of the chemistry is the 454 FLX Titanium instrument that generates read length of approximately 400 bp with a totally of 500 Mb of data collected in a 10 h run. An upgrade to this instrument can increase the read length to >700 bp and generate 900 Mb of sequence per 18–20 h run. This sequencing by synthesis approach exploits the liberation of pyrophosphate to mediate by the ATP-dependent conversion of luciferin to oxyluciferin. Perhaps the most important advancement in high-throughput sequencing with respect to microbial community profiling has been the advent of barcoding pyrosequencing, which allows for hundreds of microbial communities to be sequenced to exceptional depth simultaneously (Hamady et al., 2008). Barcodes are effectively added to each sequence either by ligation or by amplification with a barcoded primer. There are several different barcoding strategies but it is generally recommended that error-correcting and error-detecting codes should be used (Hamady and Knight, 2009). Fortunately, it appears that different barcode sequences do not significantly alter the community analysis from replicate samples (Hamady et al., 2008; Turnbaugh et al., 2009).

Early versions of pyrosequencing platforms have been shown to be cost effective and improve diagnostic capability in the clinical laboratory particularly for organisms not amenable to biochemical testing (Luna et al., 2007). The ability to generate thousands of sequence reads from hundreds of samples, in a run that takes less than a day, is a very attractive technology for a clinical microbiology laboratory. In fact, significant advances have been made to adapt high-throughput pyrosequencing technology to the clinical laboratory setting and this method has been used to characterize the polymicrobial nature of various clinical entities including rhinosinusitis, surgical wounds and leg ulcers (Wolcott et al., 2009a,b, 2010a,b; Stephenson et al., 2010).

Nucleic acid extraction is perhaps the most important variable in any culture-independent approach to community profiling and high-throughput sequencing is no exception (Morgan et al., 2010). Sequencing data will not accurately correspond to the concentration of community members if the template DNA does not reflect the community composition. It appears that variability between commercially available DNA extraction kits and bead beating in hot phenol may not be tremendously important in terms of species presence/absence but concentration of each community member can vary significantly between extraction protocols (Wu et al., 2010). It is highly advised to rigorously evaluate and validate extraction methods to ensure data accurately reflects community composition (Morgan et al., 2010) before proceeding to costly sequencing efforts and caution must be made in comparing experimental data generated by using different extraction methods (Rogers and Bruce, 2010).

The number of sequence reads that are required from a clinical specimen is dependent on the goal of the study and the nature of the microbial community in the specimen. If broad brush strokes are sought, such as the relative proportion at the phyla-level, shallow sequencing at a level <100 sequence per sample would more than suffice (Ley et al., 2008). However, at a depth of at least 1000 sequences per sample one can confidently infer frequencies of organisms at >1% abundance. If the goal is a complete assessment of all the microbial constituents in a sample, much deeper sequencing may be required and often it is not even possible or practical to accomplish such a task. It has been estimated that if an entire 454 run (1 million reads) was used to for a single fecal sample (5×10^5 sequences) only five cells in every billion total cells would be sampled (Hamady and Knight, 2009). If the goal of a study were to detect rare pathogenic organisms a targeted PCR approach would obviously be more appropriate.

The 16S rRNA gene is approximately 1.5 Kb and thus is too large to be sequenced in a pyrosequencing reaction and surrogate hypervariable regions must be used as representatives. There are nine variable regions in the 16S rRNA gene (each vary with respect their utility for microbial identification) and although V2, V4 and V6 provide the lowest error rates when assigning taxonomy (Wang et al., 2007b; Liu et al., 2008), there is currently no standard for which regions to use as a proxy sequence. It has recently been suggested that a combination of V1–V3 and V7–9 might provide better representative characterization (Kumar et al., 2011). Regions V2 and V3 appear to be most effective for universal detection of pathogens (Chakravorty et al., 2007). As with any PCR application, primer selection is critical and primer bias always exists in any community analysis (Walters et al., 2011). However, appreciating what groups of organisms might be disproportionally represented in a given experimental design must be considered. If a metagenomic-based sequencing study was done in parallel to the community profiling, the organisms underrepresented because of PCR bias would not be overlooked (Hamady and Knight, 2009).

An important consideration with all next-generation sequencing technologies is error rate. Error rates are considerably better with the newer GS FLX instrument and can be <1%. The accuracy of next-generation pyrosequencing is comparable to Sanger sequencing (error rates of 0.03–0.07%) when assembly of multiple overlapping sequence reads can be used to correct for sequencing error. This is not possible for community analysis whereby every sequence read represents a distinct organism in the community. Strategies to remove sequences with errors in primer sequence or with read length abnormalities from the data set can significantly improve data quality (Huse et al., 2007). However, care must still be taken to avoid overestimating the diversity in the rare biosphere due to sequencing error (Quince et al., 2009; Reeder and Knight, 2009) and certainly distinguishing low frequency organisms from sequencing error will continue to be an area of intense investigation (Gilles et al., 2011). If the clinical lab is only interested in characterizing the dominant community members, the issues associated with over representing rare organisms would not be an issue because singleton or doubleton reads could be ignored (Petrosino et al., 2009). Chimeric 16S sequences can also be generated during PCR amplification. Software for detecting sequence hybrids exist (Huber et al., 2004; Edgar et al., 2011; Haas et al., 2011) but there has been no entirely effective solutions to deal with this issue in large pyrosequencing data sets.

With respect to 16S rRNA sequence, species, genus and strain-level operational taxonomic units are defined by 95%, 97% and 99% sequence identity, respectively. The most prominent reference databases for 16S rRNA sequence data include Ribosomal Database Project II (RCPII) (Cole et al., 2007), Greengenes (DeSantis et al., 2006) and ARB-SILVA (Pruesse et al., 2007). However, 16S sequencing is not often used in the clinical laboratory for strain-level comparisons because more accurate techniques exist. In a clinical laboratory setting, species level and in some cases strain level determinations are critical for many pathogens. This level of unequivocal taxonomic assignment can, in certain instances, be difficult with 400 bp of 16S rRNA sequence from pyrosequencing and often the most reliable perspective is at genera-level. Confirming specific pathogens by real-time PCR and characterizing them at the strain level by PFGE or MLST could still be required.

Current computational methods including UniFrac (Lozupone and Knight, 2005; Lozupone et al., 2011), SONS (Schloss and Handelsman, 2006), network-based comparisons (Ley et al., 2008) and QIIME (Caporaso et al., 2010) has significantly improved the capacity to analyze large sequence data sets. Hamady and Knight (2009) nicely outline the three major axes for ecological

analysis of microbial diversity from community profiling data: alpha vs. beta diversity, qualitative vs. quantitative analyses and phylogenic vs. taxon-based analyses (Hamady and Knight, 2009). The type of analysis employed depends entirely on the study design. In a clinical laboratory it is likely that a quantitative, taxon-based, alpha diversity metric would be most appropriate; meaning that a diagnostic platform would measure the concentration and number of taxa in each sample, with an approach that treats all taxa as phylogenetically equivalent entities.

4.3. Other clinical applications of deep sequencing

In addition to bacterial community analysis applications, high-throughput sequencing has been successfully applied to sequencing en masse of total DNA extracted from a microbial community, a technique referred to as metagenomics. The most impressive example of the power of metagenomics to date is the sequencing of nearly 3 million protein coding genes in the fecal metagenome by deep Illumina-based sequencing (Qin et al., 2010). Viral pathogens can also be detected directly using an unbiased metagenomics approach to clinical specimens (Nakamura et al., 2009). During the 2009 H1N1 outbreak there were no laboratory tests initially available to identify the virus and it has been suggested that the virus may have been circulating for months prior to its identification (Smith et al., 2009). Metagenomics was capable of detecting the influenza virus at titers near the limit of detection of RT-PCR (Greninger et al., 2010). This approach could play an important role in rapid detection of other novel pathogens during outbreaks of acute, unexplained illness (Tang and Chiu, 2010). It has already been applied to the diagnosis of lymphocytic choriomeningitis virus infection in transplant recipients (Palacios et al., 2008), in addition to defining the etiology of hemorrhagic fever outbreaks (Towner et al., 2008; Briese et al., 2009).

Bacterial genome sequencing, originally an extremely labor intensive and time consuming process, could realistically become a routine tool in diagnostics, epidemiology and infection control (Pallen et al., 2010). When no reference genome exists, 454-based pyrosequencing is best suited for de novo assembly due to the long read lengths; however, platforms that output shorter reads are suitable if a scaffold genome is available. The anthrax attacks of 2001 highlight the need for rapid bacterial genome sequencing. 454-based pyrosequencing technology was shown to be capable of generating a draft genome of *Bacillus anthracis* that could be used to assess relevant genetic variants in less than 24 h (Chen et al., 2010). More recently, the genome of the *E. coli* isolate implicated in the outbreak in Europe was completely sequenced in the matter of a few hours using the Ion Torrent platform. Sequencing a bacterial genome is no longer expensive and it has been estimated that a Streptococcal genome with greater than 150-fold coverage can cost as little as US$100 (Vernet et al., 2011). Whole genome sequencing has great value as an epidemiologic tool and was recently shown to be superior to the gold standard typing method, PFGE, during a recent Canadian *Listeria monocytogenes* outbreak (Gilmour et al., 2010). Similar observations have been made with multi-drug resistant *Acinetobacter baumannii* (Lewis et al., 2010). Genome wide SNP analysis is an extremely discriminating tool for studying transmission events and could be adopted by the clinical laboratory for routine and outbreak surveillance. Such approaches have generated an insightful perspective on the epidemiology of epidemics including the important community and hospital pathogen, methicillin-resistant *S. aureus* (MRSA) (Harris et al., 2010) and Group A *Streptococcus* (Beres et al., 2010). In addition to epidemiology applications, whole genome sequencing can also be effective for defining antibiotic resistance determinants of a particular pathogen, such as linezolid resistance in *Streptococcus pneumoniae* (Feng et al., 2009). Technology now exists for whole genome sequencing from single bacterial cells, which relies on multiple displacement amplification (Rodrigue et al., 2009; Woyke et al., 2010). It will be most interesting to see how such approaches are used to characterize rare, enigmatic members of the human microbiome.

There are several considerations as sequencing technologies continue to significantly improve output and begin to transition into the clinical laboratory. There is a real need to streamline the bioinformatics such that a technician in the clinical laboratory can manipulate deep sequencing data. The vast amounts of data that a clinical lab would produce are staggering and the computational infrastructure would require significant improvement, which would likely require taking advantage of distributed computing and robust server networks (Petrosino et al., 2009). For community profiling in clinical samples further data is required to indicate the precise clinically relevant biomarkers that predict the pathogenic potential of communities or are effective in disease risk management, before such approaches are commonplace. There are also several technical aspects that need to be resolved before a clear clinical application can be realized. For example, human-associated microbial communities contain both live and dead bacteria and PCR cannot distinguish between these two sources of nucleic acid. The protocol development in this regard has been exciting (Rogers et al., 2008; Nocker and Camper, 2009) but further validation in the high throughput sequencing pipeline is required (Rogers and Bruce, 2010).

5. Future directions

Although larger clinical microbiology laboratories are increasingly developing their own molecular assays and procedures in-house, several barriers exist in this process including rigorous validation for the population being tested, quality control, ensuring a high degree of automation, access to dedicated technologists who are trained and experienced in molecular techniques, access to capital to purchase the necessary equipment, and the expense of operating a molecular microbiology laboratory alongside the phenotypic culture-dependent testing. However, implementation of culture-independent testing methods in clinical microbiology will increase over the next decade not only due to the continued development of multiplexing methods (real-time PCR, liquid suspension bead-based arrays) but also due to improvements in future generations of sequencing platforms that increase capacity and read length while reducing cost (Martinez and Nelson, 2010). High-throughput sequencing technologies appear to be following Moore's law and increasingly the potential applications to clinical microbiology will become more apparent (Pallen et al., 2010). For example, in addition to the bacterial community analysis, deep sequencing has been applied to viral communities in the human body. Deep sequencing of intra-patient HIV viral genomes has revealed an intriguing perspective on viral resistance and ecology (Wang et al., 2007a; Eriksson et al., 2008; Le et al., 2009; Simen et al., 2009). One can envision the next generation of clinical microbiologists having the ability to perform complex analyses as diagnostic and epidemiological tools such as routine genome wide SNPs, high throughput community analysis and streamlined metagenomic testing for novel pathogen discovery within a reasonable clinical turnaround time. However, although the clinical microbiology diagnostic techniques 'tool-box' has the potential to significantly increase in the future, it is very unlikely that culture-independent approaches will entirely replace cultivation of microbes because of the importance of strain stocks and phenotypic information is not easily inferred from genotypic data.

As medical management of infectious diseases evolves so too will the need for alternate perspectives on complex chronic

infections. In future, molecular approaches may also be routinely used to simultaneously investigate host susceptibility for particular types of infection, as well as determine pathogen induced phenotypic changes in host gene expression during microbial infections that prognosticate clinical outcome.

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