Molecular detection and genotyping of pathogens: more accurate and rapid answers

James Versalovic and James R. Lupski

Rapid molecular methods have enhanced the capabilities of laboratories to identify and characterize microbial pathogens in greater detail. Nucleic acid amplification strategies and advances in amplicon detection have been key aspects in the progress of molecular microbiology. Sophisticated new amplification–detection combinations are resulting in many applications in laboratory testing for infectious diseases. These applications include qualitative detection, sub-species-level DNA fingerprinting, molecular resistance testing and genotyping, and quantitative (viral load) testing. When applied selectively in the laboratory, these applications can enhance diagnostic approaches and clinical management and will probably evolve into standard laboratory and point-of-care testing protocols.

Microbial genomes are being sequenced at a staggering rate; there were 81 publicly available bacterial genome sequences as of June 1, 2002 (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html). Our understanding of sub-species-level or strain-level differences in bacterial and viral genomes has sharpened our appreciation of the differences between clones or quasi-species of microbial pathogens. Elevated mutation and rapid replication rates among RNA viruses have established the concept of viral quasi-species in clinical virology. The notion of genomic plasticity has been developed to explain substantial differences in genetic content between bacterial species and strains [1]. Approximately 10% of the genes of a species in a given genus are unique to each organism, and we are now beginning to appreciate the genetic diversity among bacterial strains of a given species.

This tremendous genetic diversity poses a formidable challenge in the speedy and accurate design of molecular assays. Conventional phenotypic methods for bacterial detection and identification have depended for decades on cultivation of microbial cultures in liquid or plated media. Various formulations have been developed to optimize recovery of different bacterial and fungal pathogens. Biochemical testing by manual and semi-automated methods has been a key element of bacterial identification for decades. The development of microbiological stains in the late nineteenth and early twentieth centuries enabled rapid microscopic smear analysis in the laboratory. Antigen and antibody detection (serological methods) made culture-independent pathogen detection possible. Antigen and antibody detection have relied on developments in direct (DFA) or indirect (IFA) immunofluorescence analysis and enzyme immunoassay (EIA)-based studies, but these methods are limited in sensitivity in comparison with *in vitro* nucleic acid amplification.

In the 1980s, molecular probes were applied in clinical microbiology for the first time. Researchers were quick to appreciate the conserved nature of the ribosomal RNA (rRNA) operon and Carl Woese began using these sequences to establish the field of bacterial phylogenetics [2]. Probes based on rRNA operon sequences were developed for molecular epidemiological strategies such as ribotyping [3]. In southern California, a fledgling enterprise later known as Gen-Probe (http://www.gen-probe.com) established the field of probe-based diagnostics by combining rRNA operon probes and DNA–RNA hybridization to identify specific bacterial, mycobacterial and fungal pathogens. Nucleic acid amplification strategies were subsequently developed and included molecular methods such as ligase chain reaction (LCR), PCR, strand-displacement amplification (SDA) and transcription-mediated amplification (TMA) (Table 1).

Amplification-based strategies enable the direct detection of microbial pathogens in clinical samples by molecular methods (Table 2). Amplified products, or amplicons, can be size-fractionated by agarose or polyacrylamide gel electrophoresis. PCR amplification can be combined with microplate-based hybridization or real-time detection to maximize sensitivity and specificity. Reverse hybridization includes sequence-specific oligonucleotide probes that are embedded in nitrocellulose strips and used to hybridize with PCR-generated target amplicons. Post-amplification dideoxy-DNA sequencing remains the gold standard for phylogenetic identification of bacteria and genotyping of bacterial and viral pathogens. Microarray-based hybridization has been introduced for human immunodeficiency

James Versalovic*
Depts of Pathology, and Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX 77030, USA, and Dept of Pathology, Texas Children's Hospital, 6621 Fannin Street, MC 1-2261 Houston, TX 77030 USA.
*e-mail: jamesv@bcm.tmc.edu

James R. Lupski
Depts of Molecular and Human Genetics, and Pediatrics, Baylor College of Medicine, and Dept of Pediatrics, Texas Children's Hospital.

1471-4914/02/$ – see front matter PII: S0966-842X(02)02438-1

Table 1. Common nucleic acid amplification methods used in clinical laboratories

Acronym (target)	Method	Brief description
bDNA (DNA or RNA)	Branched DNA	Serial hybridization-based signal amplification using complex branched DNA probes
Hybrid capture (DNA)	Hybrid capture®	Signal amplification based on a combination of hybridization with target-sequence-specific RNA probes and antibodies recognizing RNA–DNA hybrids
LCR (DNA)	Ligase chain reaction	Ligation-dependent amplification using probe pairs that bind to adjacent target sequences on each strand of DNA
PCR (DNA)	Polymerase chain reaction	Polymerization-dependent amplification using primers to initiate DNA synthesis on opposite DNA strands
NASBA (DNA or RNA)	Nucleic acid sequence-based amplification	Transcription-dependent amplification based on promoter primers that recognize specific target sequences and enable RNA amplicon synthesis
RT-PCR (RNA)	Reverse transcription-polymerase chain reaction	PCR following reverse transcription of RNA target sequence to duplex DNA
SDA (DNA)	Strand-displacement amplification	Target amplification based on restriction enzyme cleavage and strand displacement by DNA synthesis
TMA (DNA or RNA)	Transcription-mediated amplification	Transcription-dependent amplification based on promoter primers that recognize specific target sequences and enable RNA amplicon synthesis

virus type 1 (HIV-1) genotyping but remains in the developmental stages for clinical laboratory applications.

This review focuses on qualitative molecular detection and genotyping of bacterial and viral pathogens (Table 2). We address only qualitative testing and genotyping, and refer the reader to other references [4] for discussions of viral load (quantitative) testing. Our goal is to stress the most important diagnostic issues with emerging and rapid molecular methods in clinical microbiology and infectious diseases. This review does not contain details about methods but rather highlights the key issues pertaining to clinical applications of molecular methods for pathogen detection and genotyping.

Bacterial pathogens

Bacterial detection and identification

Bacteriological culture and biochemical identification remain the predominant approaches in the clinical laboratory for the detection and identification of bacteria. Cultivation of bacteria in liquid or plated media, coupled with microscopic smear analysis and biochemical testing, enables microbiologists to identify many important pathogens. However, phenotypic approaches can be limited in their ability to detect specific organisms because of unique growth requirements or biochemical inertness.

Bordetella pertussis and *Mycobacterium tuberculosis* are important and fastidious respiratory bacterial pathogens. Bacteriological culture of *B. pertussis* requires specialized media not available in most clinical laboratories. Real-time PCR detection of *B. pertussis* and *Bordetella parapertussis* has been demonstrated in respiratory specimens for the diagnosis of pertussis or whooping cough [5,6]. With real-time PCR systems (e.g. LightCycler; Roche Diagnostics, http://www.roche.com), amplification and detection are performed in closed tubes in small-footprint homogeneous systems, and turnaround times for diagnosis can be reduced to several hours.

Diagnosis of *M. tuberculosis* infection can require 10–14 days of culture followed by probe-based diagnostics. Nucleic acid amplification can be performed by two Food and Drug Administration (FDA)-approved methods: PCR (Amplicor; Roche Diagnostics) or TMA [Amplified Mycobacterium Tuberculosis Direct (AMTD); Gen-Probe] [7,8]. Nucleic acid amplification enables the laboratory to detect the presence of *M. tuberculosis* DNA in respiratory specimens, usually following acid-fast bacillus (AFB) smear analysis, which indicates the presence of mycobacteria. The clinical use of either PCR (Amplicor or 'home brew' methods) or transcription-mediated amplification (AMTD) for the rapid diagnosis of tuberculosis with smear-negative respiratory or extrapulmonary specimens is being reconsidered. Emerging data with modified versions of these assays support the use of amplification for the rapid diagnosis of tuberculosis with the smear-negative specimens [9,10].

Obligate intracellular pathogens such as *Chlamydia* and *Mycoplasma* species represent fertile opportunities for molecular diagnostics. The laboratory requirement for mammalian cell culture makes it difficult to obtain timely results and requires specialized laboratory expertise.

Table 2. US FDA-approved tests for qualitative pathogen detection[a]

Test	Method	Vendor
CMV detection	Hybrid capture®	Digene (http://www.digene.com/)
CMV typing, screening	Hybridization	Digene
CMV pp67 mRNA	NASBA	Biomerieux (http://www.biomerieux.com/)
C. trachomatis DNA Test	Hybrid capture®	Digene
C. trachomatis and *N. gonorrhoeae* screening and/or detection	Hybridization and amplification	Gen-Probe (http://www.gen-probe.com/)
C. trachomatis detection	LCR	Abbott Laboratories (http://abbott.com/)
C. trachomatis detection	PCR	Roche Diagnostics (http://www.roche.com/)
C. trachomatis and *N. gonorrhoeae* detection	SDA (BD Probetec ET)	Becton-Dickinson (http://www.bd.com/)
C. trachomatis detection	TMA	Gen-Probe
Culture confirmation for *Mycobacteria spp.*; different fungi and bacteria*	Hybridization	Gen-Probe
Gardnerella and *Trichomonas vaginalis* and *Candida spp.*	Hybridization	Becton-Dickinson
HCV detection	PCR	Roche Diagnostics
HCV/HIV-1 detection (blood donor centers)	TMA	Gen-Probe and Bayer (http://www.bayer.com/)
HPV DNA Test	Hybrid capture®	Digene
HPV typing/screening	Hybridization	Digene
M. tuberculosis detection	PCR	Roche Diagnostics
M. tuberculosis detection	TMA	Gen-Probe
N. gonorrhoeae detection	LCR	Abbott Laboratories
N. gonorrhoeae DNA Test	Hybrid capture®	Digene
Streptococcus pyogenes (Group A streptococcus)	Hybridization	Gen-Probe

[a]Abbreviations: *C. trachomatis, Chamydia trachomatis; M. tuberculosis, Mycobacterium tuberculosis; N. gonorrhoeae, Neisseria gonorrhoeae*; NASBA, nucleic acid sequence-based amplification. See Table 1 for additional abbreviations.

Alternative strategies such as evaluation of cold agglutinins for *Mycoplasma pneumoniae* infection lack the requisite sensitivity and specificity. Nucleic acid amplification techniques have become the gold standard for the detection of *Chlamydia trachomatis* and have significantly improved the detection of other *Chlamydia* and of *Mycoplasma* species. Chromosomal DNA targets of the respiratory pathogens *Chlamydia pneumoniae* [11] and *M. pneumoniae* can be detected by real-time PCR and used for the diagnosis of respiratory infections, including atypical pneumonia. PCR targets in *M. pneumoniae* include ATPase operon genes, which permit species-specific detection and correlation with infection. Multiple commercial amplification methods have been developed for the combined detection of the genitourinary tract pathogens *C. trachomatis* and *Neisseria gonorrhoeae* [12]. Commercial approaches include multiple FDA-approved methods such as LCR, PCR, SDA and TMA as amplification methods and genus- and species-specific probes for hybridization-based detection (Table 2). Commercial systems have been developed for sequence-based identification of bacterial and fungal pathogens (e.g. MicroSeq; Applied Biosystems, http://www.appliedbiosystems.com) based on conserved rRNA gene targets.

Bacterial strain typing

Clinical correlations of molecular epidemiological studies are important aspects of these investigations. Biochemical methods are not helpful for distinguishing individual clones or strains, and antibiograms (antibiotic susceptibility profiles) are of limited use in this setting. Most clinical microbiology laboratories in the USA use pulsed-field gel electrophoresis (PFGE) as the primary method, alone or in combination with other non-PCR or PCR-based typing methods [13]. Alternative DNA fingerprinting or typing strategies include hybridization-based approaches such as ribotyping [2] or bacterial restriction endonuclease analysis (BRENDA) [13]. PCR-based strategies include randomly amplified polymorphic DNA (RAPD) analysis [13] and repetitive element-based PCR (rep-PCR) [13,14] and facilitate the detection of low-copy-number DNA targets. Post-amplification DNA sequencing of

polymorphic genes is being used for molecular epidemiological analyses, primarily in the research setting. Rapid DNA fingerprinting approaches depend on the combination of rapid nucleic acid amplification and detection methods to facilitate real-time analysis.

The presence of conserved, interspersed repetitive DNA elements in bacterial chromosomes creates opportunities for rapid PCR-based DNA fingerprinting (rep-PCR). PCR amplification of unique-sequence chromosomal DNA residing between high-copy-number interspersed repetitive DNA elements of bacterial pathogens is the basis for a rational PCR-based fingerprinting strategy. Optimization of PCR reactions with standardized reagents, including primers, has culminated in the development of commercial kits with improved accuracy and reproducibility. Advances in amplicon detection strategies include the combination of microfluidic-based fragment separation and fluorescence detection with the compilation of electronic DNA profile libraries for strain identification (DiversiMap; Bacterial Barcodes, http://www.bacbarcodes.com). Such refined molecular strategies will enable sophisticated molecular epidemiological studies and possibly assist in identification of bacterial pathogens. Moreover, the strategy of designing primers to endogenous repetitive sequence elements can be generalized to the analysis of fungal and other pathogens.

An infection control unit or microbiology laboratory will usually initiate a request for molecular epidemiology when unusual organisms or patterns of infection are observed. Application of molecular epidemiological methods have proved cost effective for both the surveillance and study of nosocomial outbreaks [15]. Nosocomial organisms that are examined routinely by DNA fingerprinting include methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Less commonly studied by molecular methods are organisms such as methicillin-resistant *Staphylococcus epidermidis* (MRSE), extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae*, and *Clostridium difficile*. The persistence and spread of organisms resistant to antimicrobial agents has justified the application of molecular methods in epidemiological studies and has led to insights into patterns and modes of transmission. For example, methicillin resistance can be first detected by oxacillin disc testing, but the resistance profile is insufficient to study clonal relationships among bacteria. DNA-typing methods that have been used successfully with MRSA, include PFGE, chromosomal RFLP, RAPD and rep-PCR [16,17]. Post-amplification DNA sequencing with multilocus and protein A gene-based strategies have also been useful in characterizing individual clones of MRSA [18,19] and represent alternative approaches in reference or research laboratories. If strains are distinguishable by one method, one can assume that they are different strains. By contrast, strains initially assigned identical DNA profiles by one method might be distinguishable by different molecular methods, depending on the resolution of the respective techniques.

Bacterial resistance testing

Molecular resistance testing supplements conventional antimicrobial susceptibility testing for the determination of differential susceptibility of microbial pathogens to multiple antibacterial agents. Mechanisms of antimicrobial resistance include alterations in: (1) protein pumps on the cell surface; (2) drug-modifying enzymes; or (3) drug targets by mutations in chromosomal or plasmid genes. Rapid molecular testing has been applied to the detection of particular genetic determinants conferring resistance and might gradually evolve into more standardized approaches in the clinical laboratory and point-of-care setting. Approaches include the detection of a resistance gene (e.g. *mecA* in staphylococci) and of a mutation (e.g. macrolide resistance in *Helicobacter pylori*).

The *mecA* gene in staphylococci encodes penicillin-binding protein 2′ (PBP 2′ or PBP2a), a protein with low affinity for β-lactam antimicrobial agents. Because of this low affinity, PBP 2′ is thought to function and confer methicillin resistance in the presence of β-lactams. Virtually all methicillin-resistant staphylococci contain the *mecA* gene, which and it is now considered the gold standard test for methicillin resistance in staphylococci [20,21]. Thus, the PCR assay detects the presence (resistant) or absence (susceptible) of the *mecA* gene in staphylococcal isolates. In the USA, revised National Committee for Clinical Laboratory Standards (NCCLS) breakpoints [22] have improved conventional antimicrobial susceptibility testing based on disk diffusion with oxacillin. However, molecular methods enable confirmation if results are indeterminate or a rapid assessment is required. Molecular methods enable the direct detection of resistance determinants without the delays of sequential culture-based identification and susceptibility testing. Rapid molecular approaches of *mecA* detection include cycling probe technology (Velogene; ID Biomedical, http://www.idbiomedical.com) and real-time detection of PCR products using fluorescence resonance energy transfer (FRET) in the LightCycler (Roche Diagnostics) [23]. User-developed methods are based on published PCR-based strategies that enable amplification of the 3′ segment of the *mecA* gene in coagulase-negative and coagulase-positive staphylococci. Additionally, rapid MecA antigen detection methods have been developed based on latex agglutination.

Macrolides are an important class of antimicrobial agent; they have bacteriostatic effects based on the ability to block protein translation. Macrolides bind 23S rRNA and interfere with the peptidyltransferase reaction. Macrolide resistance is caused by methylation or mutational alteration of conserved region V in 23S rRNA of the large ribosomal subunit [24]. Point mutations in the 23S rRNA gene have been identified in *Helicobacter pylori*; these confer resistance to clarithromycin [25]. Two point mutations account for more than 90% of macrolide resistance in this organism and PCR-based mutation detection approaches have been developed, including PCR-restriction fragment length polymorphism (PCR-RFLP), reverse hybridization with linear probe arrays (line probe-based reverse hybridization) and real-time detection using FRET probes.

Mutations in the *rpoB* gene encoding the β subunit of RNA polymerase confer resistance to rifampin and might serve as a surrogate marker for multidrug resistance in *M. tuberculosis* [26]. The fastidious nature of *M. tuberculosis* and the lengthy periods required for antimicrobial susceptibility testing mean that molecular methods present the distinct advantage of timely recognition of resistance determinants. Post-amplification PCR-RFLP, reverse hybridization and dideoxy-DNA sequencing have been used for the rapid characterization of sequences within a conserved and functionally important region of *rpoB* [26].

Viral pathogens

Viral detection

Viral detection by molecular methods has included several important advances in the diagnosis of infection by DNA and RNA viral pathogens. Direct detection of viral pathogens by nucleic acid amplification methods has eliminated the absolute requirement for time-consuming viral culture and has enabled the rapid diagnosis of viral infections. In addition to quantitative (viral load) testing and viral genotyping, qualitative detection by molecular methods has become a standard diagnostic approach in clinical virology.

Detection of herpes simplex virus (HSV) in the cerebrospinal fluid by DNA amplification is the test of choice for the diagnosis of HSV encephalitis and meningitis [27]. Although routinely culturable in most clinical specimens, HSV is difficult to culture from paediatric or adult cerebrospinal fluid specimens. Therefore, PCR-based detection gained rapid acceptance, unlike methods such as electroencephalography and brain biopsy sampling. Real-time detection of HSV DNA with FRET probes has facilitated ultrasensitive detection with improved specificity and serotype analysis by post-PCR melting curve studies [28].

Multiple DNA and RNA detection methods are being applied to the molecular diagnosis of cytomegalovirus (CMV) infection (for an excellent review, see [29]). Serological testing has a limited role with CMV because individuals are often infected latently. CMV commonly causes disease upon reactivation as a result of immunosuppression and diagnostic methods that provide an accurate prediction of the onset of reactivation disease are a primary goal of molecular CMV detection and quantitation. CMV DNA detection by nucleic acid amplification of human plasma is particularly useful in neutropenic patients, an important issue with stem cell transplantation and leucocyte-depleted patients. Molecular methods provide alternative approaches with greater sensitivity than CMV antigen (pp65) detection in peripheral blood leucocytes [30], although pp65 antigen detection appears to be superior for guiding the initiation of antiviral therapy [31]. Nucleic acid amplification of CMV DNA in peripheral blood leucocytes requires fewer cells than the pp65 antigenemia assay and is more sensitive than CMV DNA detection in plasma.

Signal or target nucleic acid amplification methods are currently used for the detection of hepatitis C virus (HCV) RNA in serum or plasma [32,33]. Patients with chronic, untreated HCV infection have stable serum RNA levels, and HCV RNA loads can therefore be useful for patient management [34]. Recent developments in antiviral therapy, including high-dose interferon and multidrug regimens with ribavirin, have increased the potential use of serum RNA load determinations in treatment follow-up and in the definition of patients requiring therapy. Qualitative RNA testing can detect low levels of HCV RNA in plasma or serum (e.g. qualitative Amplicor, 60 IU ml^{-1} versus Amplicor Monitor, 600 IU ml^{-1}), and qualitative nucleic acid amplification-based testing can therefore be used to report the presence or absence of HCV RNA in patients who are negative by viral-load testing. In addition to its use in the determination of treatment endpoints, qualitative HCV RNA testing can be helpful in supporting a diagnosis of HCV infection in patients with equivocal results by serological testing.

Human papillomaviruses (HPV) are the primary aetiological agents of cervical neoplasia [35]. Cervical cancers, including squamous cell carcinomas and adenocarcinomas, and their pathological precursors, are firmly linked with HPV infection. Cervical Papanicolaou (Pap) smears continue to be the primary strategy for cervical cancer screening. However, direct detection of HPV DNA in cervical cytological specimens has emerged as a widely recognized adjunctive test, especially for patients with atypical squamous cells of undetermined significance (ASCUS) or atypical glandular cells of undetermined significance (AGUS)

[36]. Signal and target amplification approaches and *in situ* hybridization methods have been used to detect HPV DNA in clinical specimens. The Hybrid Capture® signal amplification system (Digene, http://www.digene.com) [37] is the only FDA-approved strategy for the diagnosis of HPV infection in cervical specimens and is now used by the majority of testing laboratories.

Application of HPV DNA testing in conjunction with Pap cervical cytology is an attractive strategy for triaging patients during cervical cancer screening. Women would continue to undergo routine Pap cervical cytology and individuals with a cytological diagnosis of ASCUS or AGUS would be referred for HPV DNA testing [36,38]. Specimens collected by thin-preparation cytology could be submitted for HPV DNA detection without resampling. Detection of HPV DNA – usually HPV of high-risk genotypes – in cervical specimens would support referral for colposcopy. The majority of women are negative for HPV DNA and could avoid colposcopy, returning months later for routine Pap testing. Combination strategies of liquid-based cervical cytology and reflex HPV DNA testing were more cost effective than patient evaluation and management strategies based solely on conventional cytology and colposcopy [38].

Viral genotyping

The emergence of drug-resistant HIV-1 has been associated with treatment failure in infected patients [39,40]. As with most viral pathogens, the susceptibility of HIV-1 to antiviral agents is difficult to assess using culture-based methods. Identification of specific mutations in genes encoding antiviral target proteins such as HIV-1 reverse transcriptase (RT) or protease (P) enable the determination of the presence and nature of antiviral resistance [40]. HIV-1 genotyping has improved the virological responses and management of patients treated with antiretroviral agents [41]. Most clinical laboratories perform genotyping by DNA sequencing. The predominant commercial platform, the Trugene system (Visible Genetics, http://www.visgen.com), was cleared in September 2001 by the FDA for HIV-1 genotyping.

Multiple strategies that are being developed for HIV-1 genotyping include post-amplification slab gel and capillary nucleic acid sequencing, microarray-based sequencing, and line probe-based reverse hybridization of HIV-1 amplicons [42,43]. Commercially available kits and user-developed sequencing protocols are being used. Methods vary with respect to the detection of specific mutations in mixed viral populations and inter-method comparisons have been performed [42]. Such strategies require considerable expertise in molecular diagnostics and highlight the emerging importance of sequencing and mutation identification in the diagnostic molecular microbiology laboratory.

HCV genotyping is commonly performed to assist physicians in the management of HCV-infected individuals [44]. At least six genotypes have been officially recognized and affect the duration of antiviral regimens when combined with viral load and qualitative molecular testing [44]. Although HCV genotype 1 accounts for the majority of HCV infections in the USA, genotypes 2 and 3 comprise up to one-third of HCV infections in North America. The most important distinction for laboratories is to stratify genotypes as type 1 or non-type-1. The presence of HCV genotype 1 indicates that 48 weeks of therapy is probably required, whereas the detection of HCV genotypes 2 and 3 permit cessation of therapy at 24 weeks, if the patient responds adequately to therapy (as determined by viral load testing).

The 5′-untranslated region (5′UTR) is the most highly conserved region of HCV and has been used as the preferred target for HCV detection by nucleic acid amplification. Reverse transcription-polymerase chain reaction (RT-PCR)-based amplification of HCV 5′UTR target sequences can be combined effectively with post-PCR reverse hybridization or sequencing. Reverse hybridization of the 5′UTR target region using linear probe arrays (line probes) [45] (INNO-LiPa; Innogenetics, http://www.innogenetics.com) is the most commonly used approach for HCV genotyping based on proficiency testing data from the College of American Pathologists (2002 HCVN survey; http://www.cap.org). A minority of laboratories in the USA use other approaches, such as DNA sequencing or type-specific PCR. Reverse hybridization (non-sequencing approach) is highly effective at distinguishing the HCV genotype 1 from other genotypes. Subtype determination is less reliable with reverse hybridization and is addressed most effectively with post-amplification sequencing of the NS5B coding region. For example, the line probe assay might have difficulty distinguishing HCV subtypes 1a from 1b and 2a from 2c. The clinical relevance of HCV subtyping remains unclear.

Concluding remarks

Multiple applications based on molecular methods have contributed significantly to our ability to study microbial pathogens in the clinical laboratory. Selective applications, including detection of pathogens that are especially difficult to assess by conventional culture-based methods, have significantly enhanced efforts to improve timely diagnosis and patient management. Future developments will include integration of amplification and signal detection technologies in single instruments, and miniaturization of technologies including applications of

microarrays and microfluidics. Future point-of-care testing could include bedside or in-clinic use of DNA chips for the rapid assessment of selected microbial pathogens.

Acknowledgments

We would like to thank M. Healy for critical review and V.J. Gotting for assistance with manuscript preparation. The authors disclose that they are cofounders of, and scientific advisors to, Bacterial Barcodes, and co-inventors on patents (US5523215; US5691136; European 0610396) describing the rep-PCR DNA fingerprinting technology.

References

1 Dobrindt, U. and Hacker, J. (2001) Whole genome plasticity in pathogenic bacteria. *Curr. Opin. Microbiol.* 4, 550–557
2 Woese, C.R. *et al.* (1985) A phylogenetic definition of the major eubacterial taxa. *Syst. Appl. Microbiol.* 6, 143–151
3 Grimont, F. and Grimont, P.A.D. (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann. Inst. Pasteur* 137B, 165–175
4 Berger, A. and Preiser, W. (2002) Viral genome quantification as a tool for improving patient management: the example of HIV, HBV, HCV and CMV. *J. Antimicrob. Chemother.* 49, 713–721
5 Sloan, L.M. *et al.* (2002) Multiplex lightcycler PCR for detection and discrimination of *Bordetella pertussis* and *Bordetella parapertussis*. *J. Clin. Microbiol.* 40, 96–100
6 Kosters, K. *et al.* (2002) Real-time LightCycler PCR for detection and discrimination of *Bordetella pertussis* and *Bordetella parapertussis*. *J. Clin. Microbiol.* 40, 1719–1722
7 Vuorinen, P. *et al.* (1995) Direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens by Gen-Probe Amplified *Mycobacterium tuberculosis* direct test and Roche Amplicor *Mycobacterium tuberculosis* test. *J. Clin. Microbiol.* 33, 1856–1859
8 Ichiyama, S. *et al.* (1996) Evaluation of Gen-Probe Amplified *Mycobacterium tuberculosis* direct test and Roche PCR-microwell plate hybridization method (Amplicor Mycobacterium) for direct detection of mycobacteria. *J. Clin. Microbiol.* 34, 130–133
9 Woods, G.L. (1999) Molecular methods in the detection and identification of mycobacterial infections. *Arch. Pathol. Lab. Med.* 123, 1002–1006
10 Kaul, K.L. (2001) Molecular detection of *Mycobacterium tuberculosis*: impact on patient care. 47, 1553–1558
11 Kuoppa, Y. *et al.* (2002) Quantitative detection of respiratory *Chlamydia pneumoniae* infection by real-time PCR. *J. Clin. Microbiol.* 40, 2273–2274
12 Van Dyck, E. *et al.* (2001) Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by enzyme immunoassay, culture, and three nucleic acid amplification tests. *J. Clin. Microbiol.* 39, 1751–1756
13 Versalovic, J. *et al.* (1993) DNA-based identification and epidemiologic typing of bacterial pathogens. *Arch. Pathol. Lab. Med.* 117, 1088–1098
14 Versalovic, J. *et al.* (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell. Biol.* 5, 25–40
15 Hacek, D.M. *et al.* (1999) Medical and economic benefit of a comprehensive infection control program that includes routine determination of microbial clonality. *Am. J. Clin. Pathol.* 111, 647–654
16 Pfaller, M.A. *et al.* (2001) Integration of molecular characterization of microorganisms in a global antimicrobial surveillance program. *Clin. Infect Dis.* 32, S156–S167
17 DePlano, A. *et al.* (2000) Multicenter evaluation of epidemiological typing of methicillin-resistant *Staphylococcus aureus* strains by repetitive element PCR analysis. *J. Clin. Microbiol.* 38, 3527–3533
18 Oliveira, D.C. *et al.* (2001) Comparison of DNA sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin resistant *Staphylococcus aureus*. *J. Clin. Microbiol.* 39, 574–580
19 Shopsin, B. and Kreiswirth, B.N. (2001) Molecular epidemiology of methicillin-resistant *Staphylococcus aureus*. *Emerg. Infect. Dis.* 7, 323–326
20 Sakoulas, G. *et al.* (2001) Methicillin-resistant *Staphylococcus aureus*: comparison of susceptibility testing methods and analysis of *mecA*-positive susceptible strains. *J. Clin. Microbiol.* 39, 3946–3951
21 Del Vecchio, V.G. *et al* (1995) Molecular genotyping of methicillin-resistant *Staphylococcus aureus* via fluorophore-enhanced repetitive sequence PCR. *J. Clin. Microbiol.* 33, 2141–2144
22 National Committee for Clinical Laboratory Standards (2002) *Performance standards for antimicrobial susceptibility testing: twelfth informational supplement*. NCCLS document M100-S12. National Committee for Clinical Laboratory Standards. Wayne, PA, USA
23 Reischl, U. (2000) Rapid identification of methicillin-resistant *Staphylococcus aureus* and simultaneous species confirmation using real-time fluorescence PCR. *J. Clin. Microbiol.* 38, 2429–2433
24 Owen, R.J. (2002) Molecular testing for antibiotic resistance in *Helicobacter pylori*. *Gut* 50, 285–289
25 Versalovic, J. *et al.* (1996) Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrob. Agents Chemother.* 40, 477–480
26 Soini, H. and Musser, J.M. (2001) Molecular diagnosis of mycobacteria. *Clin. Chem.* 47, 809–814
27 Lakeman, F.D. *et al.* (1995) Diagnosis of herpes simplex encephalitis: application of polymerase chain reaction to cerebrospinal fluid from brain-biopsied patients and correlation with disease. *J. Infect. Dis.* 171, 857–863
28 Espy, M.J. *et al.* (2001) Detection of herpes simplex virus DNA in genital and dermal specimens by LightCycler PCR after extraction using the IsoQuick, MagNA Pure, and BioRobot 9604 methods. *J. Clin. Microbiol.* 39, 2233–2236
29 Yen-Lieberman, B. (2000) Diagnosis of human cytomegalovirus disease. *Clin. Microbiol. Newsletter* 22, 105–109
30 Boeckh, M. *et al.* (1997) Plasma polymerase chain reaction for cytomegalovirus DNA after allogeneic marrow transplantation: comparison with polymerase chain reaction using peripheral blood leucocytes, pp65 antigenemia, and viral culture. *Transplantation* 64, 108–113
31 Solano, C. *et al.* (2001) Qualitative plasma PCR assay (AMPLICOR CMV test) versus pp65 antigenemia assay for monitoring cytomegalovirus viremia and guiding preemptive ganciclovir therapy in allogeneic stem cell transplantation. *J. Clin. Microbiol.* 39, 3938–3941
32 Gretch, D. (1997) Diagnostic tests for hepatitis C. *Hepatology* 26(S), 43S–47S
33 Urdea, M.S. *et al.* (1997) Hepatitis C – diagnosis and monitoring. *Clin. Chem.* 43, 1507–1511
34 Liang, T.J. (1998) Combination therapy for hepatitis C infection. *New Engl. J. Med.* 339, 1549–1550
35 Stoler, M.H. (2000) Human papillomaviruses and cervical neoplasia: a model for carcinogenesis. *Int. J. Gynecol. Pathol.* 19, 16–28
36 Manos, M.M. *et al.* (1999) Identifying women with cervical neoplasia: using human papillomavirus DNA testing for equivocal Papanicolaou results. *JAMA.* 281, 1605–1610
37 Clavel, C. *et al.* (1999) Hybrid capture II-based human papillomavirus detection, a sensitive test to detect in routine high-grade cervical lesions: a preliminary study on 1518 women. *Br. J. Cancer* 80, 1306–1311
38 Kim, J.J. *et al.* (2002) Cost-effectiveness of alternative triage strategies for atypical squamous cells of undetermined significance. *JAMA* 287, 2382–2390
39 Carpenter, C.C. *et al.* (2000) Antiretroviral therapy in adults: updated recommendations of the International AIDS Society-USA Panel. *JAMA* 283, 381–390
40 Hirsch, M.S. *et al.* (1998) Antiretroviral drug resistance testing in adults with HIV infection: implications for clinical management. International AIDS Society – USA panel. *JAMA* 279, 1984–1991
41 Durant, J. *et al.* (1999) Drug-resistance genotyping in HIV-1 therapy: the VIRADAPT randomised controlled trial. *Lancet* 353, 2195–2199
42 Wilson, J.W. *et al.* (2000) Comparative evaluation of three human immunodeficiency virus genotyping systems: the HIV-GenotypR method, the HIV PRT GeneChip assay and the HIV-1 RT line probe assay. *J. Clin. Microbiol.* 38, 3022–3028
43 Erali, M. *et al.* (2001) Human immunodeficiency virus type 1 drug resistance testing: a comparison of three sequence-based methods. *J. Clin. Microbiol.* 39, 2157–2165
44 Zein, N. (2000) Clinical significance of hepatitis C virus genotypes. *Clin. Microbiol. Rev.* 13, 223–235
45 Stuyver, L. *et al.* (1996) Second-generation line probe assay for hepatitis C virus genotyping. *J. Clin. Microbiol.* 34, 2259–2266