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Pathogen Detection in the Genomic Era

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Introduction

In the 21st century, one of the greatest challenges to public health and clinical microbiologists is the rapid detection and identification of emerging and reemerging pathogens. Complex factors such as genetic variation in the host and pathogen, environmental changes, population pressures, and global travel can all influence the emergence of infectious diseases. The SARS epidemic of 2003 highlighted the potential of an emerging pathogen to spread globally in a very short time frame (Peruski and Peruski, 2003). The diagnostics of such infectious diseases has been greatly affected in the past 20 years. No longer is cultivation and microscopy the only means of detecting infectious agents. With the introduction of molecular diagnostics, the ability to detect minute amounts of microbial nucleic acids in clinical specimens has revolutionized clinical microbiology. In particular, the utility of PCR allows the detection and quantitation of specific agents in a matter of hours. PCR sequencing of specific segments of nucleic acid allows for the determination of specific drug resistance that now aids in guiding viral therapies.

In 1876, a German physician named Robert Koch was in a race to discover the causative agent of a disease that was destroying cattle and sheep in Europe. What Koch found was a rod-shaped bacteria in the blood of the dead cattle, which is known today as *Bacillus anthracis*. He was able to culture the bacteria on nutrients and then inject these cultured bacteria back into a healthy animal. The once healthy animal feel ill and died. Koch was able to again isolate the bacteria from the blood of the dead animal. By performing these series of experiments, Koch was able to demonstrate that an infectious disease, anthrax, was caused by a specific infectious agent (*B. anthracis*). This technique was again used to demonstrate that *Mycobacterium tuberculosis* was the causative agent of tuberculosis. Thus, Koch's series of experiments, known today as Koch's postulates, has provided the foundation for the study of the etiology of infectious diseases.

Although Koch's postulates have been very useful in determining the cause of many bacterial infections, there are times when these principles do not apply, for example, when an organism is uncultivable on artificial medium. There are also situations when a number of different pathogens can cause similar clinical symptoms or when a single pathogen can cause several pathologies. The application of molecular diagnostics can be very useful to overcoming these situations because these techniques allow for the rapid detection of fastidious, uncultivable, or unknown agents. Using universal primers or multiplex systems, a panel of organism targets can be used to screen for in any clinical specimen. Molecular methods are a widely used tool for epidemiological fingerprinting of isolates that are important to public health. Through the use of sequence-based identification, strain typing, and specific markers, comprehensive isolate fingerprinting can be used for the tracking and control of disease.

In 1995, scientists at the institute for genomic research (TIGR) unveiled the first two complete DNA sequences of the bacterial genomes *Haemophilus influenzae* and *Mycoplasma genitalium*. This was followed in 1996 by the first complete genome sequencing of an archaea, *Methanococcus jannaschii*. This scientific achievement was made possible through the use of automated sequencing equipment. Since 1995, TIGR and others have completed the genome sequence of many pathogens as well as a number of microorganisms of environmental relevance. Comparison of genomes can pinpoint differences between virulent and avirulent medically important pathogens. At the species level, genome comparisons can provide information about host or tissue specificity. On an evolutionary level, comparison of genomes can help to reveal the origins of microbial life (Doolittle, 2002).

Viral genomes are smaller and can mutate faster than bacterial genomes. Current technology is already tapping into the use of viral genomes for guiding drug therapies. Implementation of viral load testing and specific nucleic acid sequencing provides physicians with valuable information regarding the clinical response of patients on antiviral therapy and emergence of antiviral drug resistance (Smith et al., 2004).

The foundation for the study of biological processes at the protein level is being driven by the rapid progress in genomics. The identification and characterization of proteins expressed in cells (microbial and host) under different cellular states is a growing area of interest. Messenger RNA is often spliced in different ways to code for different proteins; simply knowing a gene sequence and its transcriptional expression is not enough to understand critical protein functions. The study of proteomics is the next frontier in understanding genomic functionality. The goal of proteomics is to define and characterize the complete set of proteins (the "proteome") in an organism, tissue, or cell and determine their spatial and temporal variation. It is being increasingly applied to the study of various microbial processes (e.g., host and pathogen interactions). Proteomics holds great promise for enhancing our knowledge of how a cell functions under various conditions, thus it may allow for breakthroughs in new generations of diagnostics, antimicrobial agents, and vaccine candidates.

Culture Confirmation and Tissue Pathogen Detection by Direct *In Situ* Hybridization

Though *in situ* hybridization (ISH) is typically performed in histology rather than clinical microbiology laboratories, it can provide extremely useful information to clinical microbiologists. Several pathogen targets have been used for direct hybridization to nucleic acid probes *in situ*. Bacterial targets include *Helicobacter pylori* (Makristathis et al., 2004), and *Legionella* spp. (Hayden et al., 2001b). Yeast forms of the dimorphic fungi (Hayden et al., 2001a), and molds such as *Aspergillus* spp., *Fusarium* spp., and *Pseudoallesheria* spp. (Hayden et al., 2002, 2003) have also proven to be useful as ISH targets. Typically, *in situ* hybridization is chosen when it is useful for the pathogen to be identified in association with intact cells or tissue, but branched DNA probes have been used to identify the presence of human papilloma virus (HPV) and the gene expression signal from HPV mRNA (Kenny et al., 2002). As a method for culture confirmation, PNA FISH (peptide nucleic acid fluorescent *in situ* hybridization) has been used to identify *Staphylococcus aureus* (Stender, 2003) and coagulase-negative staphylococci from positive blood cultures (AdvanDx, Woburn, MA, USA). Although not an *in situ* technique, a hybridization protection assay using an rRNA probe matrix has been used for rapid identification of bacteria and fungi from routine blood cultures (Marlowe et al., 2003). In this study, Enterobacteriaceae, *Pseudomonas aeruginosa*, other Gram-negative bacteria, *Staphylococcus aureus*, coagulase-negative staphylococci, streptococci, enterococci, other Gram-positive bacteria, anaerobes, and yeast were successfully identified using hybridization methods that proved to be both sensitive (100%) and specific (96%).

Specimen Automation: Nucleic Acid Extraction and Molecular Diagnostics

Efficient extraction and preparation of a specimen for nucleic acid analysis is critical to the quality of molecular testing results. During the past decade, improvements in novel and convenient extraction schemes have allowed the routine integration of molecular testing in many clinical diagnostic laboratories. More recently, the evolution of extraction systems into an automated format allows for the common practice of molecular testing. Several reviews serve as a reference for specific details of extraction chemistries and real-time instruments (Wolk et al., 2001; Wolk and Persing, 2002).

Specimen preparation automation can be divided into three categories: (i) nucleic acid extraction, (ii) specimen processing/PCR assay set-up, and (iii) general liquid handing systems. Some systems combine part or all of these categories into one system (Table 28.1). Many companies developed these automated systems so they can be integrated with their real-time PCR instruments for complete automation of extraction, sample processing, and amplification/detection.

TABLE 28.1. Automated specimen processing and real-time instruments.

Manufacturer	Instrument	Throughput	Web site	Extraction	Amplification preparation	General/liquid handing	Amplification/ detection
Abbott	m1000	48/run	www.abbottdiagnostics.com	×	×		
ABI	ABI Prism 6100 Nucleic acid PrepStation	96/run	www.appliedbiosystems.com	×			
	ABI Prism 6700 automated Nucleic acid workstation	192/run		×	×		
	ABI Prism 7000 Detection System	96/run					×
	ABI Prism 7300 Detection System	96/run					×
	ABI Prism 7500 Detection System	96/run					×
	ABI Prism 7900 HT Detection System	384/run					×
Beckman Coulter	Biomek FX Laboratory Automation Workstation		www.beckman.com	×	×	×	
	Biomek NX Laboratory Automation Workstation			×	×	×	
	Biomek 2000 Laboratory Automation Workstation			×	×	×	
	Biomek 3000 Laboratory Automation System			×	×	×	
Beckton Dickinson	BD Viper Sample Processor	550/8 h	www.bd.com	×	×		
Biomerieux	Nucleisense Extractor	10/run	www.biomerieux.com	×			
	EasyMAG	24/run					
	NucliSens miniMAG	12/run					×
	Easy Q	48/run					
Bio-Rad Laboratories	iCycler iQ	96/run	www.bio-rad.com				×
	iCycler iQ5	96/run					×
	MiniOpticon	48/run					×
	Opticon 2	96/run					×
	Chromo 4	96/run					×

Cephid	Smart Cycler 1600	16/run	www.cepheid.com					×
	Smart Cycler 3200	32/run						×
	Smart Cycler 4800	48/run						×
	Smart Cycler 6400	64/run						×
	Smart Cycler 8000	80/run						×
	Smart Cycler 9600	96/run						×
	GeneXpert	1/cartridge		×	×			×
Corbett Research	x-tractor gene	96/run	www.corbettresearch.com	×				
	CAS-1200 liquid handing system	96/run			×			
	Gradient Palm-Cycler	96-384/run						×
	Rotor-Gene 3000	36-72/run						×
Gen-Probe	Tigirs DTS	1000/12 h	www.gen-probe.com	×	×			×
Hamilton	MICROLAB STAR^{LET}		www.hamiltonrobotics.com	×	×	×		
Qiagen	Biorobot EZ1 workstation	6/run	www.qiagen.com	×				
	Biorobot M48 workstation	48/run		×				
	Biorobot M96 workstation	96/run		×				
	Biorobot MDx workstation	96/run		×	×			
	Biorobot 9604 workstation	192/run		×	×			
Roche	MagNA Lyser Instrument	16/run	www.roche-applied-science.com					
	MagNA Pure Compact System	8/run	www.roche-diagnostics.com	×				
	MagNA Pure LC System	32/run		×	×			
	Light Cycler	32/run						×
	Cobas Ampliprep	72/run		×	×			
	Cobas TaqMan 48	48/run						×
Stratagene	Mx3000P	96/run	www.mx3000p.com					×
	Mx4000	96/run	www.mx4000.com					×
Tecan	Freedom EVO clinical		www.tecan.com	×	×	×		

Automated Systems: Extraction Without Amplification

Qiagen (Qiagen, Valencia, CA, USA) has six different robotic systems for clinical research applications to extract nucleic acids from a variety of specimens. Other robotic systems are available for additional research applications. These automated extraction systems can be used with a variety of PCR and real-time PCR instruments for nucleic acid detection. The systems offered for clinical use are designed to accommodate laboratories depending on their specimen volume needs. The BioRobot EZ1 workstation can purify nucleic acids from 1 to 6 samples using prefilled EZ1 DNA and RNA kits that are available in cards. The BioRobot M48 and M96 can process up to 48 and 96 samples per run, respectively. These two systems use the MagAttract magnetic particle technology for DNA extraction and ultraviolet lights to decontaminate surfaces between runs. The BioRobot MDx workstation can process 8 to 96 samples and is equipped with vacuum processing to eliminate the need for centrifugation that was present with the earlier BioRobot 9604. The MDx system uses bar coding for specimen tracking. High-throughput testing is optimal with the BioRobot 9604, which is equipped to run QIAmp protocols for the extraction of DNA and RNA and has barcode specimen tracking. The Qiagen BioRobot has been evaluated for both DNA and RNA viruses and the extraction efficiency found to be equivalent to manual methods (Espy et al., 2001; Knepp et al., 2003; Forman and Valsamakis, 2004; Xu et al., 2004).

Automated Systems: Extraction with Amplification

Roche (Roche Molecular Biochemical, Indianapolis, IN, USA) has three automated instruments. The MagNA Lyser Instrument is an automated tissue homogenization unit that can be combined with their automated nucleic acid extractors. For nucleic acids, there is the MagNA Pure Compact and the MagNA Pure LC. The MagNA Pure Compact has a small footprint and can extract nucleic acids from 1 to 8 samples, using 2 different compact nucleic acid kits for small and large volumes. The MagNA Pure LC can process up to 32 samples in 1 to 3 h. The LC model automates both extraction and PCR set-up for the LightCycler capillaries, COBAS A-rings, and 96-well plates or tubes. The MagNA Pure LC has been evaluated with bacteria, parasites, fungi, DNA viruses, and RNA viruses; it was found to be equivalent to manual methods (Espy et al., 2001; Loeffler et al., 2002; Wolk et al., 2002; Germer et al., 2003; Holzl et al., 2003; Knepp et al., 2003; Leb et al., 2004; Lee et al., 2003; Cook et al., 2004; Dalesio et al., 2004; Muller et al., 2004; Tang et al., 2005). The COBAS Ampliprep is an automated system for the Cobas Amplicor analyzer and is not available in the United States except for research purposes. The COBAS Ampliprep has been reported in the literature to be suitable for routine testing with decreased hands-on time (Gartner et al., 2004; Stelzl et al., 2004). The next-generation COBAS, the COBAS TaqMan 48, is now available and has the capacity to run two different assays with individual PCR profiles. The LightCycler 2.0 system is also available, which allows 6-dye channel detection instead of the previous 4-dye channel detection.

Biomerieux (Biomerieux, Marcy l'Etoile, France) has three systems for nucleic acid extraction: the Nuclisens Extractor, which uses silica dioxide Boom extraction protocol (Boom et al., 1990), and the NucliSens easyMAG and the NucliSens miniMAG, which both use magnetic silica. The Nuclisens Extractor is an automated closed system. It has been compared to Qiagen manual extraction and found to be efficient for extraction of viral DNA and RNA (Gobbers et al., 2001). The easyMAG is an automated magnetic silica extraction system that will extract 24 samples in less than an hour. The miniMAG is a manual system that can extract 12 samples in about 35 min and offers a low-cost extraction system to a smaller molecular laboratory. Recent studies have suggested that the miniMAG consistently provides high yields of low titer of target (Tang et al., 2005). These systems are designed to extract both DNA and RNA for use with either Nucleic Acid Sequence-Based Identification (NASBA) or PCR amplification technologies. The NucliSens Easy Q can also be coupled with these extraction methods for detection with real-time NASBA. The miniMAG has been used with the EasyQ real-time NASBA HIV assay to accurately quantify HIV over a six-log range.

Applied Biosystems (ABI; Foster City, CA, USA) has two nucleic acid extraction systems, which use proprietary flow-through chemistry. The ABI PRISM 6100 nucleic acid prep station can program up to 300 defined extraction protocols. The ABI PRISM 6700 Automated Nucleic Acid Workstation is a fully automated system that extracts nucleic acid, prepares the PCR reactions, and initiates the reverse transcription step of RT-PCR for up to 96 samples. The ABI Prism 6700 was used to automate the large-scale surveillance of West Nile virus during outbreaks in the northeastern United States (Shi et al., 2001). This workstation can interface with the existing ABI PCR instruments, the ABI PRISM 7900HT, 7500, 7300, 7000, and GeneAmp 5700.

Corbett Research (distributed by Phenix Research Products, Hayward, CA, USA) offers two separate automated instruments, the X-Tractor Gene for RNA/DNA extraction and the CAS-1200 liquid handling system for set-up of both standard and real-time PCR reactions. The CAS-1200 can be configured to support 96-well plates or the Rotor-Gene well rotors for use with the Rotor-Gene 3000, a four-channel real-time PCR fluorescent detector.

Becton Dickinson (BD, Franklin Lakes, NJ, USA) has an automated sample processor for the BDProbeTec ET system. The BD Viper Sample Processor is designed to automate sample processing with high-volume testing of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (>500 samples/8-h shift). Gen-Probe (Gen-Probe Incorporated, San Diego, CA, USA) has launched the TIGRIS, which is a fully automated instrument for high-volume testing (1000 samples/12-h shift). The TIGRIS incorporates Gen-Probe's target capture technology for sample processing, Transcription-mediated Amplification (TMA) amplification, and Hybridization Protection Assay (HPA) detection into one instrument. Currently, the TIGRIS is limited to use with Gen-Probe's APTIMA *N. gonorrhoeae*/*C. trachomatis* assay and Procleix HIV/HCV blood screening assay. Gen-Probe also offers the DTS 800 and 1600 systems (manufactured by Tecan, Research Triangle Park, NC, USA), which automate the liquid handling of other various diagnostic kits. Abbott (Abbott

Laboratories, Abbott Park, IL, USA) is developing an automated sample preparation workstation. The m1000 is designed to process 48 samples in 2 h using a magnetic microparticle–based protocol and has been reported by Abbott to be integrated with the ABI PRISM 7000 detection systems.

Liquid Handling Systems

Beckman Coulter, Hamilton, and Tecan all offer robotic liquid handling systems, which offer flexibility in molecular diagnostic labs. Beckman Coulter (Fullerton, CA, USA) has available several liquid handling systems that can be used to extract nucleic acids, set up PCR and sequencing reactions, as well as immuno-detection assays. These systems include the Biomek NX, Biomek FX, Biomek 3000, and Biomek 2000. The Hamilton MICROLAB STAR^{let} (Hamilton Company, Reno, NV, USA) is a benchtop workstation for assay automation that can be used for nucleic acid extraction and PCR set-up. The vector software and various accessories allow the user to customize protocols to laboratory needs. Tecan supports the Freedom EVO PCR workstation, which also offers flexible software and accessories to allow the user to customize protocols to their needs.

Other Real-Time PCR Systems

Cepheid, Bio-Rad, and Stratagene all offer real-time PCR systems that could be combined with many automated systems. The Cepheid SmartCycler (Cepheid, Sunnyvale, CA, USA) offers flexible real-time PCR detection that allows multiple low sample number tests to be performed simultaneously or with up to 96 independently programmable reaction cells for higher throughput. Cepheid has announced plans to launch the GeneXpert system, which would be a real-time system with integrated sample preparation, amplification, and detection in a single-use cartridge that contains lyophilized reagents. Bio-Rad (Hercules, CA, USA) offers real-time detectors in the iCycler and now the iQ5 for a multiplex of up to five fluorophores. Additionally, they offer the MiniOpticon, a two-color 48-well system, the Opticon II, a two-color 96-well system, and the Chromo4, a four-color 96-well system (formerly MJ Research). The Stratagene (Stratagene, San Diego, CA, USA) systems include the Mx4000 and Mx3000P.

Extraction Summary

Although there have been many advances in the automation of nucleic acid extraction, sample processing, and amplification, there have been very few studies comparing the various automated systems. The limited studies available have demonstrated that the automated systems are comparable to each other as well as to manual methods (Espy et al., 2001; Knepp et al., 2003). Automation limits the risk of contamination, decreases hands-on time, and reduces repetitive-motion disorder among technicians. Ultimately, the decision to implement automation

into a molecular lab depends on cost, specimen-testing volume, and laboratory workflow.

Molecular Typing and Epidemiology

The use of molecular techniques in epidemiological studies is important for rapid, focused disease detection and for implementation of infection control measures. Molecular typing has become a valuable tool in defining disease source(s), determining the transmission modalities, and tracking outbreaks (Diekema et al., 2003; Soll et al., 2003). Pulse-field gel electrophoresis (PGFE) is considered the reference method for comparative purposes, although various methods are used (Tenover et al., 1995; Olive and Bean, 1999b; Goering, 2000b; Weller, 2000b). Although PGFE is considered a reliable, discriminatory, and reproducible method, it is expensive and time consuming, making this technique difficult for hospitals to use as a routine form of support for infection-control practitioners in outbreak investigations (Weller, 2000a; Shopsin and Kreiswirth, 2001; Stranden et al., 2003). Due to testing conditions, there is inherent gel-to-gel variability with PGFE, which makes it difficult to compare inter-institutional electrophoresis results. Public health laboratories have standardized PFGE for use in outbreak investigations, and digital imaging has provided some standardization and cross-gel comparative properties; still, the technique is relatively cumbersome and not practical in most hospital laboratories. With recent CDC and JCAHO recommendations to use molecular testing as the gold standard in investigation of hospital-acquired infections, hospitals are faced with difficult choices, referring specimens to reference laboratories or state public health laboratories for testing or investment in hospital-based infrastructure to support genotyping. Currently, there is a gap in our ability to provide results of molecular epidemiology in the hands of most clinical microbiologists and infection-control practitioners.

Though numerous bacterial genotyping methods have emerged for use in outbreak investigations and molecular epidemiology, few are completely standardized or practical. Among them, PCR-based amplification with subsequent typing (Rademaker and Savelkoul, 2004), genotyping via variable number tandem repeats (VNTR) (Mathema and Kreiswirth, 2004), multilocus sequence typing (MLST) (Hanage et al., 2004), and automated ribotyping (Pfaller and Hollis, 2004) provide options. Although all are useful tools,varying in their strengths and limitations (Tenover et al. 1997; Goering, 2000a), two methods appear to have promise for translation into a routine clinical laboratory practice: rep-PCR and ribotyping.

Repetitive-element sequence-based PCR (rep-PCR) is a DNA fingerprinting method that has been successfully used as a rapid molecular tool for outbreak investigation of oxacillin resistant *S. aureus* (ORSA) (Versalovic et al., 1991; Del Vecchio et al., 1995; van der Zee et al., 1999; Deplano et al., 2000) and has successfully demonstrated discriminatory typing for organisms such as *Salmonella typhi, Escherichia coli*, and *Bacillus* species (Olive and Bean, 1999a). The rep-PCR

method exploits the presence of repetitive elements dispersed throughout the chromosomes of ORSA and other organisms. Repetitive elements have different and specific positions within the genome among different strains of microorganisms. Primers, designed to amplify genetic regions between these repetitive elements, allow for generation of PCR amplification products of various sizes (Woods et al., 1993). Electrophoresis of the differently sized products, derived from different organisms strains, produces unique gel-banding pattern fingerprints, by which the strains can be compared and differentiated. Recent advancements and improvements to commercial rep-PCR fragment analysis using capillary electrophoresis (Bacterial Bar Codes, Inc., Atlanta, CA, USA) enables standardized and reproducible performance of rep-PCR and may provide a promising option for a rapid and cost-effective outbreak investigation in hospitals and communities.

Molecular Detection of Drug Resistance

Rapid and accurate determination of drug susceptibility of a clinical isolate can be useful for various aspects of patient therapy. The presence of resistance markers can also help distinguish ambiguous break points associated with susceptibility testing. Well-characterized resistant genes can be used to monitor their epidemiological spread in the community or hospital. Despite the fact that there still remains much to learn about these markers, the application of molecular diagnostic methods to detect drug resistance is evolving as a routine practice for some laboratories.

The use of molecular methods to detect resistance can be applied to bacteria, viruses, and fungi. The advantage of using molecular tests is that they do not rely on time-consuming incubations or media-dependent expression. Thus, educated choices for therapy can be initiated early in diagnosis to impact patient outcomes, particularly with slow-growing organisms such as *Mycobacterium* spp. (Inderleid and Pfyffer, 2003).

Antimicrobial resistance genes among bacteria include resistance for β-lactams, aminocylitols, aminoglycosides, chloramphenicol, fluoroquinolones, glycopeptides, isoniazids, macrolides, mupicurin, rifampin, sulfonamids, tetracyclines, and trimethaprim (Fluit et al., 2001; Rasheed and Tenover, 2003; Tenover and Rasheed, 2004). For a list of PCR primers used to target such resistance markers, see Tenover and Rasheed (2004).

Perhaps the most well-documented applied use of markers for bacteria are those of oxacillin-resistant *Staphylococcus aureus* (ORSA, formerly MRSA) and vancomycin-resistant enterococcus (VRE). The *mecA* gene mediates oxacillin resistance in most ORSA, and the *vanA* and *vanB* genes primarily mediate acquired vancomycin resistance in VRE. Commercially available tests for ORSA include latex agglutination tests for PBP2a (the product of *mecA)*, cycle probe technology, and PCR for the detection of *mecA* in *S. aureus*. PCR has been used to detect and track both ORSA and VRE (Clark et al.,1993; Gordts et al. 1995; Aarestrup et al., 1996; Satake et al., 1997; Hussain et al., 2000; Padiglione et al., 2000; Grisold et al., 2002; Jonas et al., 2002; Louie et al., 2002; Maes et al., 2002; Francois

et al., 2003; Strommenger et al., 2003; Sloan et al., 2004), A review by Diekema et al. (2004) highlights the fact that together, ORSA and VRE are the two most important resistant pathogens in U.S. hospitals, and their rapid detection remains a need. Antimicrobial resistance is continuing to increase worldwide. With active surveillance and proper isolation of infected patients, use of rapid PCR technology could play an important role in identifying carriers upon hospital admission and aide in the prevention and control efforts for ORSA and VRE. For routine use of these tests, not only would they have to be sensitive and specific, but they would also have to be cost effective with proven infection prevention studies.

The detection of viral mutations associated with drug resistance has been well documented. Examples include polymerase and protease inhibitors with human immunodeficiency virus (HIV), acyclovir and penciclovir resistance in herpes simplex virus, acyclovir resistance in varicella-zoster virus, ganciclovir resistance in cytomegalovirus, famciclovir and lamivudune resistance with hepatitis B virus, and amantidine resistance with influenza A (Shafer and Chou, 2003). Viral mutations are most commonly detected by direct sequencing of the specific viral reading frames, which encode the proteins that are targeted by currently available antiviral drugs. Genotypic resistance testing to mange HIV-1–infected patients is widely used by physicians.

There are currently two commercially available FDA-cleared sequencing assays that include reagent kits and software. These two kits are the Truegene HIV-1 genotyping kit and OpenGene DNA–sequencing system, (Bayer Corp, Tarrytown, NY, USA) and ViroSeq HIV-1 genotyping system (Applied Biosystems, Foster City, CA, USA). Genotype testing requires a skilled laboratory, which is proficient in sequencing, alignments, editing, mutation detection, and interpretation of sequences.

Commercially available line probe assays (LiPA), a reverse hybridization method, allow laboratories that are proficient in PCR and have limited sequencing capabilities to detect mutations without the need to sequence (Descamps et al., 1998; Schmit et al., 1998). Comparison studies of these HIV-1 viral genotyping methods have proved to be reliable and accurate. However, the LiPA is designed to identify known primary mutations associated with high-level drug resistance; direct sequencing can detect more and new mutations (Erali et al., 2001; Hanna and D'Aquila, 2001; Grant et al., 2003; Caliendo and Yen-Lieberman, 2004).

Rapid detection of antifungal resistance is useful, primarily due to the increase in fungal infections among immunocompromised patients. Current antifungal assays rely on fungal susceptibility testing, which is dependent on growth. The practical application of antifungal molecular testing is yet to be seen, as there is still much to learn about the genetic markers that mediate resistance. The genetic information needed to examine fungal resistance at the molecular level is much more complex than that of viruses, such as HIV-1, and could involve the evaluation of fungal gene expression. For a review of molecular mechanisms of antifungal resistance, see Edlind (2004).

The full potential of molecular diagnostics for drug-resistance testing in microbiology has not been reached, and its application is still in its infancy. As the

molecular mechanisms of antimicrobial resistance are described, newer technologies will enhance the utility of marker testing. Microarray technology has the promise to impact the rapid and accurate detection of multiple mutations associated with resistant bacteria, mycobacteria, viruses, and fungi. As with all molecular diagnostics, laboratories that perform molecular resistance testing need to ensure quality control of specimens. Currently, there is still a need to cultivate organisms for further testing of other antimicrobials or typing for epidemiological studies. Thus, it important to retain specimens or inoculate a culture until the laboratory can be sure a result is negative and the specimen can be discarded (Diekema et al., 2004). Until the full potential of drug-resistant markers is understood, rapid molecular antimicrobial testing must still be combined with traditional microbial cultivation.

Microbial Proteomics in Pathogen Detection

Though in its infancy, proteomic technology has the potential to play a key role in the future of clinical microbiology diagnostics as techniques become more rapid, affordable, and the list of applicable biomarkers expands. Mass spectroscopy (MS) and 2-D gel electrophoresis are the 2 common techniques in microbial proteomics (Douglas, 2004). In 2-D gel electrophoresis, proteins are first separated by their isoelectric point (pI) in glass tubes (Bjellqvist et al., 1982). Gels are then removed from glass and placed horizontally on top of polyacrylamide slab gels, and polyacrylamide gel electrophoresis (PAGE) further separates proteins with similar charges by their size (molecular weight). Gel electrophoresis is a simple method to catalogue microbial proteins grown under different conditions and disease states. A mass spectrometer can take proteins from PAGE and further separate them by producing charged particles (ions) (Shevchenko et al., 1996). The mass spectrometer differentially moves ionized molecules, separated by their mass-to-charge (m/z) ratio, through a vacuum by means of an electromagnetic field. For the sake of discussion, if one assumes that each component of the mixture has a different molecular weight, then the mass spectrum contains unique "peaks" for each compound that is present. For more information about the different types of mass spectroscopy, refer to Douglas (2004).

A few reports have begun to surface in the clinical microbiology literature and describe how proteomic methods may impact laboratories in the future. In one report, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) was used to rapidly identify fungal proteins that evoked a specific human immune response, which may prove to be linked to active infection and outcome (Pitarch et al., 2004). In another study, MALDI-TOF-MS, gelelectrophoresis, and tandem mass spectrometry were used to identify intra-amniotic proteins, which could lead to discovery of novel human biomarkers for human intra-amniotic infection (Gravett et al., 2004). Ultimately, these tools will help to elucidate the interaction of proteins with protein precursors, DNA, and mRNA to add to the understanding of pathogenesis and disease. Out of this understanding,

novel biomarkers for early detection of disease or disease outcomes are expected to occur.

One emerging technology, the Luminex xMap System, can identify multiple immune proteins, like serotype-specific antibodies, in a single well or tube multiplex format. It has been used to identify multiple immune proteins (Jones et al., 2002) and bacterial DNA (Dunbar et al., 2003), but routine applications in the clinical laboratory will require further translational.

Although mass spectrometry is typically used to identify proteins, high-performance mass spectrometry has recently been adapted and developed for use in conjunction with PCR for rapid identification and strain typing of emerging pathogens, such as *Bacillus anthracis* and coronavirus, among others (Van Ert et al., 2004; Ecker et al., 2005; Sampath et al., 2005)

Conclusion

Over the past 10 years, molecular methods have emerged as essential tools in the clinical microbiology workplace. Identification of bacterial, viral, fungal, and parasitic genomes drives the emergence of new technologies to identify pathogens more rapidly, but limitations in the single target approach still exist. Miniaturization of robotics and automation will allow even small health care facilities to implement molecular methods. New discoveries in the human genome, linking disease susceptibility with infection, or human mutations with antibiotic utility, will certainly continue to impact the clinical laboratory of the next 10 years, broadening the scope of clinical microbiology from that of detection to include prediction, via protein or nucleic acid targets. Finally, miniaturization of techniques such as mass spectroscopy of PCR amplicon could change the entire face of clinical microbiology, in that multiple pathogens could be amplified or simultaneously detected and genotyped to allow rapid detection of many emerging pathogens and other infections.

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