

Future Veterinary Diagnostics

Bob Dahlhausen, DVM, MS

Abstract

The development of rapid, accurate, and sensitive diagnostic methods for detecting pathogens is the basis for treating, controlling, and eradicating infectious diseases of veterinary importance. Scientific and technological advancements have revolutionized the field of veterinary diagnostics. Genome sequencing has allowed efficient, sensitive, and specific diagnostic assays to be developed based on the detection of nucleic acids. The integration of advances in biochemistry, proteomics, engineering, and medicine offers enormous potential for the rapid and accurate diagnosis of viral, microbial, genetic, and metabolic disease. In the future, polymerase chain reaction assays, microarray testing, genomic analysis, and metabolic profiling will be accomplished in a rapid, portable, sensitive, and cost-efficient manner. Copyright 2010 Elsevier Inc. All rights reserved.

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Since the discovery in 1953 by James D. Watson and Francis Crick that DNA and RNA provide the code to life, there has been a global effort to understand how the genome sequences of numerous organisms are related to health and disease.

The rapidly advancing field of diagnostics, tests used to detect a medical condition or causative pathogen responsible for an infection, will drastically change the practice of veterinary medicine in the near future. The ability to detect nucleic acids has had and still has a major impact on diagnostic processes in clinical medicine.¹

Classical pathogen detection and identification are based on culture methodology, biochemical tests, and microscopy. The culture method is still a core technology in many clinical laboratories because it can provide important information about the viability of the pathogen and its susceptibility to therapeutic agents. Likewise, the historic gold standard for diagnosing viral infection has been selective cultivation followed by electron microscopy. Recovery of the live virus remains a very slow, technically demanding, and expensive diagnostic tool.

The development of rapid, accurate, and sensitive diagnostic methods for detecting disease pathogens is fundamental for treating, controlling, and eradicating infectious disease. Rapid scientific and technological developments have revolutionized the possibilities of diagnosing and treating disease. Researchers now have tools for observation at the level of the atom, to sequence entire genomes, and to understand the molecular basis of disease.² Recent biotechnological developments in microtechnologies and nanotechnologies have led to the proliferation of new, rapid diagnostic tests that hold promise for the improved management and control of infectious disease in animals. Emerging technologies are rapidly being adapted to improve our ability to de-

From Veterinary Molecular Diagnostics, Inc., Milford, OH USA.

Address correspondence to: Bob Dahlhausen, DVM, MS, Veterinary Molecular Diagnostics, Inc., 5989 Meijer Dr, Ste 5, Milford, OH 45150. E-mail: DrBob@one.net.

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tect new etiological agents, to provide rapid, accurate diagnostic tests for previously difficult infections, and to develop new response-based paradigms for diagnostic testing. Molecular tools in particular are advancing toward laboratory-on-chip and fully integrated technologies that are capable of same-day detection and pathotyping of agents in clinical specimens. Future diagnostic technologies that are rapid, portable, low cost, and of greater sensitivity/specificity will aid the veterinarian in the early disease detection in a point-of-care setting.

Nucleic Amplification Tests

Scientific advancements over the previous 2 decades have enabled the sensitive detection and characterization of bacterial and viral nucleic acids. Amplification technologies (e.g., polymerase chain reaction [PCR], nucleic acid sequence–based amplification [NASBA]) allow for specific detection of genetic targets. Furthermore, technological improvements in sequence determination systems allow for organisms to be fully characterized as to genotype, subtype, variant, mutants, and resistance patterns.

For the veterinary clinical laboratory, commercially standardized assays are not readily available. Most laboratories offering nucleic amplification tests (NAT)–based diagnostics use methods developed in-house or those adapted from research publications. There is no standardization among these in-house assays or an official regulatory body to monitor quality assurance. Differences in laboratory experience and technique, primer and probe combinations, methods of nucleic acid isolation, and control standards contribute to the reproducibility and variability observed among laboratories.¹

Assays must be precise and reproducible and have positive and negative controls in place to prevent invalid test results. It is also recommended that these assays have an internal control to validate nucleic acid isolation and to test for amplification inhibitors.

In a blind study, veterinary laboratories offering NAT diagnostics for psittacine circovirus were evaluated.³ The findings of this investigation revealed that accuracy was 100% for 2 laboratories, 95% for 2 laboratories, and 71% for 1 laboratory, whereas 1 laboratory had a test specificity of 20%.³ The authors concluded that although results from most laboratories were accurate, both false-positive and false-negative results were reported by at least 2 laboratories. They also concluded that 1 laboratory may be generating large numbers of falsely reported results. Veterinarians must realize that the accuracy of re-

sults depends completely on the accuracy of the operating standards of the individual laboratory. Laboratories should always strive for the highest standards of assay precision. Differences in assay precision make it difficult to compare data from different laboratories without defined standards.

Another consideration in NAT-based diagnostics concerns sample quality. Preservation of sample integrity is essential for accurate test results, especially if detection or quantification of RNA is desired. Unlike DNA, RNA is unstable and is readily degraded by naturally occurring endonucleases (RNases) that are present in the environment and tissues. Samples for RNA testing should be handled with gloves to prevent contamination with RNases present on the skin. Samples for RNA testing should be promptly submitted to the laboratory or stored in a freezer at -80°C until forwarded for testing. Samples may also be treated with buffers designed to minimize RNA degradation, with several commercial additives being available for this purpose, but their use varies among laboratories. It is best to consult the specific laboratory for their protocol for sample submission. Although DNA tends to be more stable than RNA, naturally occurring endonucleases that degrade DNA do exist. Test samples should be aseptically collected and refrigerated or frozen until submission. The ability of PCR to detect extremely low levels of DNA in a submitted patient sample results in the very real potential of false-positive test results due to DNA contamination.

Polymerase Chain Reaction

PCR is a technique that started as a research tool and was eventually integrated into medical diagnostic applications. In 1971, Kleppe demonstrated that DNA could be artificially replicated in vitro with a DNA polymerase enzyme.⁴ Combined with methods allowing for the synthesis of DNA oligonucleotides, Kary Mullis developed the technique of PCR in 1983 and won the Nobel Prize in Chemistry for his work.⁵

PCR has become one of the most widely used technologies in veterinary research and molecular diagnostics of infectious disease. The wide range of applications include the detection of DNA or RNA derived from infectious bacterial, viral, mycoplasmal, parasitic, protozoal, or fungal organisms; evaluation of specific genetic defects; and quantization of cytokines and cellular growth factors. PCR testing can be used on a wide range of samples, including tissue from biopsies or necropsies, blood, feces, tissue, soil, cerebrospinal fluid, and swabs of mucosal surfaces.



Figure 1. Bio-Rad iCycler thermocycler unit (Bio-Rad, Hercules, CA USA) with real-time computer interface (Courtesy Veterinary Molecular Diagnostics, Inc.).

PCR uses the highly specific molecular recognition ability of Watson-Crick base pairing to provide the selectivity needed for a nucleic acid probe to bind to a targeted DNA sequence and allow for its exponential amplification. Specific fragments of DNA are replicated by cyclical heating and cooling through repeated temperature stages in a thermal cycler unit (Fig 1). Each cycle can double the amount of existing target DNA. Repeatedly amplifying the target sequence over 30 to 40 cycles theoretically allows for millions (2^{30}) of DNA copies to be produced. PCR assays are thus extremely sensitive. They are capable of detecting femtograms (10^{-15} g) of target DNA sequence in a large mixture, or up to a microgram (10^{-6} g) of nontarget DNA. The DNA obtained at the end of the reaction can be used for a variety of applications, including sequencing, cloning, or as a probe for in situ hybridization.

Nested PCR

The nested PCR consists of 2 sequential PCR amplifications. The product from the first-round PCR is used as the template for a second PCR amplification. A second set of primers, internal to the first set, amplify a smaller fragment of DNA within the first-round product. In regular PCR, primers may bind to incorrect regions of the DNA, giving unexpected amplification products. The benefit of nested PCR is that increased specificity is achieved through confirmation of the correct primer binding during the first PCR amplification process. The nested PCR is more sensitive than conventional single-round qualitative PCR.

Real-Time PCR

The implementation of nucleic acid technology into routine diagnostics has greatly advanced with the

introduction of real-time PCR.¹ Real-time PCR amplification uses the detection and quantification of a fluorescent reporter molecule whose signal increases in proportion to the amount of target amplification product generated. Basic methods involve the detection of a DNA-binding dye, such as SYBR Green (Molecular Probes, Eugene, OR USA). The dye intercalates between double-stranded DNA formed in the PCR reaction and, when exposed to an excitation source, fluoresces. A laser scanner detects the fluorescence emitted by dye-impregnated DNA strands formed through the PCR amplification. Software traces this fluorescence as a running graph as the amplification reaction in real time (Fig 2).

A more specific strategy for real-time assays includes the use of a confirmatory probe sequence that matches part of the amplification product. These real-time amplicon detection technologies include TaqMan (Applied Biosystems, Inc., Foster City, CA USA) or hydrolysis probes,⁶ molecular beacon technology,⁷ and hybridization probes.⁸ Fluorescence is only detected if the probe is bound to the amplified target, thereby enhancing specificity to an already highly specific assay. These probes do not react with primer-dimers or other nonspecific products that may be generated during the PCR reaction as may occur with SYBR Green reactions. However, a well-designed and optimized SYBR Green amplification is a very effective testing technology. Unlike TaqMan probes, the SYBR Green reporter remains intact so a melt curve analysis can be performed on the amplification product. Melt curve analysis measures the dissociation characteristics of double-stranded DNA during heating. Melt curve analysis is sensitive enough to detect single-nucleotide polymorphisms (SNP) and can distinguish between homozygous and heterozygous gene alleles by the dissociation patterns produced (Fig 3). The advancement of this technology to produce high-resolution melt analysis will provide higher sensitivity for SNP detection within entire dye-stained amplicons for genomic analysis.

The main advantage of real-time detection is that it accurately quantifies the number of RNA or DNA copies present in the test sample. Real-time technologies can measure DNA presence over a large dynamic range, providing important information regarding the magnitude of viral involvement within a test sample. This information is helpful in distinguishing clinically active infections from transient environmental exposures. Quantification also aids in the treatment of patients with chronic infectious disease and monitoring disease progression and resolution.⁹

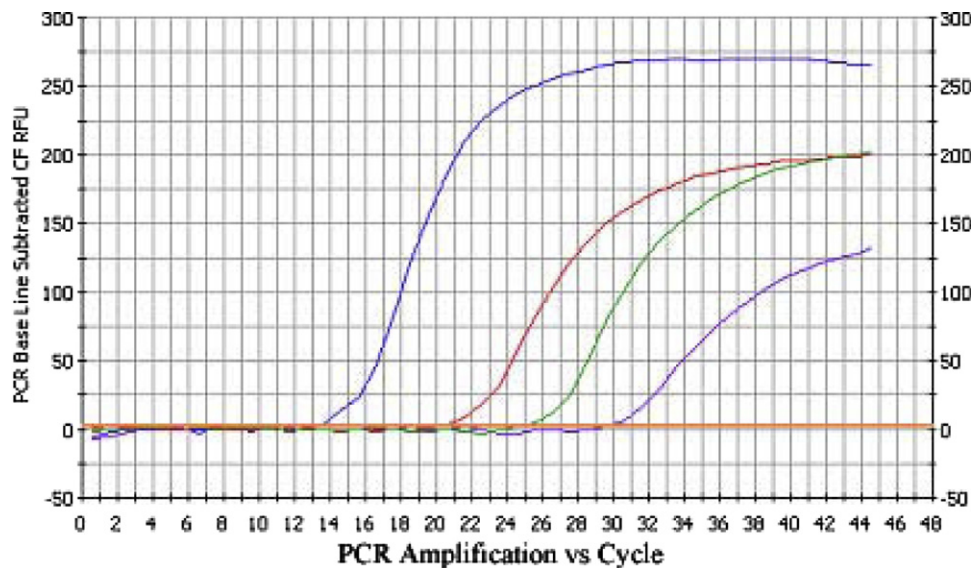


Figure 2. Real-time PCR amplification graph. Every 3 unit cycles represent an approximate 10-fold difference in amplicon concentration (Courtesy Veterinary Molecular Diagnostics, Inc.).

Reverse-transcription PCR

Standard PCR techniques cannot be used to detect the presence of RNA-based viruses in clinical samples. The direct amplification of RNA in PCR systems is not possible because of the polymerase enzyme's inability to synthesize DNA from an RNA template. The inability to synthesize DNA from an RNA template is overcome by the use of a reverse-transcriptase (RT) enzyme before the PCR reaction. In RT-PCR, RNA is converted into a complementary DNA (cDNA) strand by an RNA-dependent DNA polymer-

ase (reverse transcriptase). The cDNA produced is then used as a template in a conventional PCR reaction. It is important that all genomic DNA within the test sample be destroyed before the RT-PCR to prevent masking the presence of cDNA derived from the RT step.

In addition to diagnosing RNA viral infections in an individual patient, RT-PCR has been applied to monitoring disease in flocks and large groups of animals. Environmental air-sampling, referred to as "sniffing," was paired with real-time RT-PCR to detect exotic Newcastle disease virus in commercial

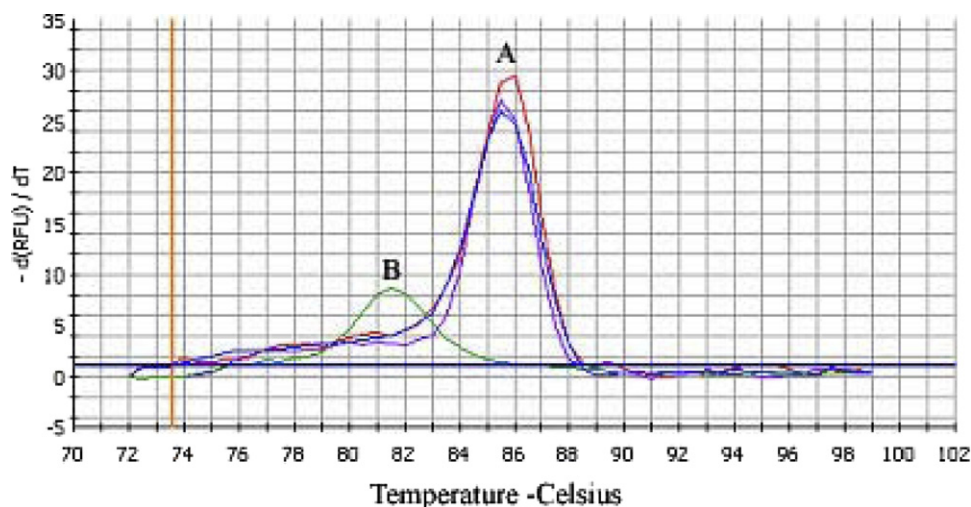


Figure 3. DNA melt curve dissociation graph. PCR products in group A are identical; product in group B is different. Melt curve analysis of this nature can be used to differentiate single nucleotide polymorphisms in gene amplification studies (Courtesy Veterinary Molecular Diagnostics, Inc.).

poultry operations in 2003.¹⁰ The same approach was successfully used to detect H6N2 influenza in commercial quail. This methodology offers the advantage of detecting virus in contaminated environments by collecting airborne particulates, including virus comingled with dust and debris, without having to handle individual birds.

Nucleic Acid Sequence-based Amplification

PCR currently is still the main target amplification technique used in both research and diagnostic laboratories. A second target amplification technique, known as NASBA, was specifically developed for the quantitative amplification of RNA targets such as retroviruses and other RNA viruses.^{11,12} NASBA's main advantage is that it works at isothermic conditions and can give quicker and more sensitive results than PCR. It has been used to develop rapid diagnostic tests for several pathogenic viruses with single-stranded RNA genomes, including influenza A,¹³ foot-and-mouth disease virus,¹⁴ and severe acute respiratory syndrome (SARS)-associated coronavirus.¹⁵

Loop-mediated Isothermal PCR

Loop-mediated isothermal PCR (LAMP) is a 1-step amplification reaction that amplifies a target DNA sequence with high sensitivity and specificity under isothermal conditions. LAMP uses a DNA polymerase with inner and outer primers that recognizes 6 separate regions within a target DNA sequence. The assay has high specificity because the amplification reaction only occurs when all 6 regions within the target DNA are correctly recognized by the primers.⁹ LAMP exhibits less sensitivity to inhibitory substances present in biological samples than regular PCR.

LAMP has been applied to pathogens that cause food-borne diseases. Testing kits that use this technology are commercially available for detecting *Salmonella* spp., *Legionella* spp., *Listeria* spp., verotoxin-producing *Escherichia coli*, and *Campylobacter* spp.

Many amplification methods, including PCR, ligase chain reaction, NASBA, and strand displacement amplification, are used in commercially available diagnostic test kits. These diagnostic test kits are promoted as providing a rapid, sensitive, and specific detection of important pathogens, especially those that are not readily identified by more conventional methods. Conventional detection and identification of noroviruses are performed by electron micros-

copy, which is less sensitive and more time consuming than NAT diagnostics.

Multiplex PCR

Molecular-based testing for multiple pathogens in a large number of animals is expensive. The development of new variations of PCR have allowed for simultaneously performing multiple tests on a single diagnostic sample, thereby affording some savings on a per-test basis. In multiplex PCR, multiple primer pairs are used to amplify more than one gene or pathogen in a single PCR reaction. Multiplex assays must be carefully designed for specific primer selection. Primers selected must be to regions that are uniquely different and characterized by low affinity for primer-to-primer interactions.

Nucleic amplification technologies will continue to be a benchmark for pathogen detection in veterinary diagnostics. Currently, there are 3542 reference sequences for 2416 viral genomes and 40 reference sequences for viroids in GenBank, an open-access public database of nucleotide reference sequences (<http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html>).

The exponential replication of DNA afforded by the PCR process allows for excellent diagnostic sensitivity and specificity. However, the instability and variability inherent in molecular enzymatic processes limit the application of this technology outside an institutional setting to a research facility or dedicated molecular laboratory. New technologies such as the lab-on-a-chip have been developed and have potential in veterinary point-of-care diagnostics. Defined as a miniaturized analytical laboratory constructed on a small chip, this technology has promising applications when combined with NAT diagnostic processes. Both PCR and LAMP assays have been used to facilitate the development of lab-on-a-chip-based NAT devices.¹⁶ Such devices can offer molecular-based testing in a rapid, effective, low-cost, and disposable automatic format as a point-of-care diagnostic tool.

Microarrays

The concept of the DNA or oligonucleotide array began in the mid-1980s as a derivative of the Southern blot technique in which DNA fragments are linked to a substrate and probed with a known gene or target DNA sequence. The "probe" refers to the DNA that is immobilized on a solid support such as a glass slide or a silicon wafer, whereas "target" refers

to the pool of nucleic acids whose identity or abundance is being detected.

Both high-density microarrays for high-throughput screening applications and lower-density microarrays have been developed for diagnostic applications.¹⁷ Microarray technology represents an important tool for the investigation of various areas of biology, mainly because of its ability to simultaneously detect and analyze thousands of different genes in a single trial.

DNA Microarray

A DNA microarray is a miniaturized form of dot blot in a predefined, ordered fashion at a high density.¹⁷ It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, each containing picomoles (10^{-12} moles) of a specific DNA sequence. The DNA spots (gene chips) are attached in an equidistant, ordered arrangement to a solid surface, such as a glass, plastic, or silicon chip. Alternative microarray platforms include polyacrylamide gel pads, microsphere beads, 3-dimensional films, and solid-state electronics.¹⁸ Microarrays may have from a hundred to thousands of test sites that can range in size from 10 to 500 μm . DNA arrays are different from other types of microarrays only because they either measure DNA or use DNA as part of the detection system.

Hybridization is performed with corresponding probes attached to the solid support. These can be cDNAs, oligonucleotides of varying length, or genomic sequences that are either radioactively or fluorescently labeled. Artificial single-stranded DNA or RNA ligands, short oligonucleotides or peptide molecules that can be generated against amino acids, drugs, proteins, and other molecules are called aptamers.¹⁹ Aptamers bind to a specific target molecule with high affinity and selectivity ranging from the micromolar to the nanomolar level, and they can discriminate between closely related targets.^{16,20} A high-density array can be generated by applying aptamers to the substrate with robotic pins or inkjet technology, or by an in situ photolithographic synthesis process. These techniques allow for the production of a high-density chip containing thousands of short probes targeted at different loci within a single gene or multiple genomes. High-density microarrays may have up to 106 test sites in a $1 \times 2 \text{ cm}^2$ area.¹⁷

The core principle of DNA microarrays is based on hybridization probing, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between nucleotide base pairs. Hybridization occurs between

2 cDNA sequences such as the nucleic acid probes immobilized on the solid surface of a chip and the mobile DNA, cDNA, or messenger RNA (mRNA) sample. A high number of complementary base pairs in a nucleotide sequence allows for tighter noncovalent bonding between the 2 strands to occur. Probe-target hybridization is usually quantified through the detection of a fluorophore, silver, or chemiluminescence-labeled target. After the hybridization step is complete, the microarray is scanned. The fluorescent tags are excited by a laser, read by a microscopic camera, and computer integrated into a digital image of the array. Software analyzes each microarray spot calculating the red-to-green fluorescence ratio and subtracting out background data. The nature and relative abundance of nucleic acid sequences in the target are determined. DNA microarrays have been used for the detection of infectious and genetic disease, cancer diagnostics, and forensic and genetic identification purposes. Microarray systems are very definitive and highly scalable because hundreds to tens of thousands of possible DNA elements can be interrogated in a single experiment.

Infectious Disease

Examination of animal and zoonotic pathogens typically focuses on 3 objectives: (1) determining the presence or absence of a pathogen; (2) determining pathogen viability; and (3) enumerating the pathogenic agents of a specific disease. DNA microarrays are well suited for determining the presence or absence of specific pathogens. DNA microarrays also permit relatively rapid interrogation of a clinical sample against thousands of genetic targets, allowing for simultaneous detection and discrimination among hundreds of pathogenic agents of veterinary interest.¹⁸ However, DNA microarrays are not very useful in inferring pathogen viability.

The first important step in microarray-based pathogen detection is probe selection and design. Many issues associated with probe design for DNA microarrays can impact the overall fidelity of the assay and particularly the levels of specificity and sensitivity. One of the first applications of a bacterial high-density DNA probe array was for the diagnostic capability of identifying *Mycobacteria* spp.²¹ Using RT-PCR and a DNA microarray based on the mycobacterial heat shock protein, this technology was used for the rapid identification of different mycobacterial species in mixed infections²² and array-based genotyping of mycobacterial organisms.²³ Other applications to bacterial infections have included the diagnosis of *Salmonella* spp., *Escherichia coli* O157:H7,

Mycoplasma hyopneumoniae, gene profiling of *Pasteurella multocida*, *Mycobacterium avium* subsp, *Paratuberculosis* spp., *Rhodococcus equi*, *Borrelia burgdorferi*, *Staphylococcus aureus* enterotoxin genes and *Clostridium perfringens* toxin genes, *Bacillus anthracis*, *C. perfringens*, *Brucella* spp., *Bordetella pertussis* and *B. paraper-tussis*, and *Actinobacillus pleuropneumoniae*.²⁴

Microarray technology has also been used to generate a “lipopolysaccharide array” against the outer membrane of Gram-negative bacteria. Monoclonal antibodies specific for *Escherichia coli* O111, *E. coli* O157, *Francisella tularensis*, and *Salmonella enteric* serovar Typhimurium O antigens were used to evaluate the assay. The detection limit of antibodies was reported to be 10 ng/mL, which is 100-fold more sensitive than conventional immunofluorescence assays.²⁴

Viral Disease

The diagnosis of viral diseases requires the identification of the viral pathogen in the clinical sample and a subsequent correlation between the identified virus and the clinical disease. Conventional methods of viral detection include isolation and in vitro culture, and immunological methods such as direct fluorescence antibody or enzyme immunoassay. With some viral disease agents, identification is readily accomplished. Other viruses (e.g., norovirus), however, are refractory to in vitro culture. Immunological methods, dependent on the quality and availability of antibodies, may also be ineffective for identifying viruses with constantly evolving serotypes. Although PCR has revolutionized viral diagnostics by increasing detection sensitivity and specificity, the maximum number of viruses detectable in a single assay is relatively small. In diseases where different viruses are present or where no etiologic agent has been identified, the value of these conventional methods may be limited.

Unlike PCR technology where the target agent must be known to use specific test primers, microarrays can allow for the rapid diagnosis of multiple pathogenic agents in disease outbreaks and epidemics of unknown etiology. One of the first studies in multiple pathogen detection used a microarray chip to discern 2 viral pathogens, porcine reproductive and respiratory syndrome virus and foot and mouth disease virus.²⁵ The microarray detection device demonstrated rapid and accurate detection of these 2 specific viruses. In another study, a low-density microarray, using 15 capture-oligonucleotides targeting the conserved influenza matrix gene, accu-

rately subtyped H1N1, H3N2, and H5N1 influenza A viruses in less than 12 hours.^{26,27}

Researchers led by Joseph DeRisi of the University of California at San Francisco have combined genome databases of sequenced viruses with DNA microarray technology.²⁸ Using available sequence data from viral genomes, they designed a long oligonucleotide viral microarray. The microarray was capable of simultaneously detecting hundreds of viruses in a single clinical sample. The approach used randomized primers in an RT-PCR to amplify any viral RNA that was present in the clinical sample. The PCR product was then hybridized to the microarray comprising 1600 probes, representing nearly 140 respiratory virus genomes. Viruses that were represented on the microarray were readily detected and identified by specific hybridization to the appropriate oligonucleotides. This methodology enabled the simultaneous detection of hundreds of viruses, including essentially all respiratory tract viruses. This microarray system greatly expanded the spectrum of detectable viruses in a single assay while simultaneously providing the capability to discriminate among viral subtypes. Similarly, Sengupta and colleagues developed microarrays with 476 probes to distinguish among various influenza viruses.²⁹

Although initial efforts were focused on only a few hundred viruses, DeRisi and colleagues devised a microarray representing all known human, animal, aquatic, and plant viruses called the ViroChip. The most highly conserved genomic sequences from within viral families were chosen for representation on the microarray. Viruses represented included double- and single-stranded DNA viruses, retroviruses, and both positive- and negative-stranded RNA viruses. DNA fragments of virtually every virus ever discovered, about 22,000 different viral sequences, were represented on the ViroChip.

The ViroChip greatly accelerates the ability of researchers to diagnose viral infections and identify new viral epidemics within a very short time. This technology can help to rapidly identify known viruses and classify new ones based on their genetic makeup. The ViroChip has been credited with the rapid recognition of a novel coronavirus in 2003, the etiologic agent responsible for SARS.³⁰

Although the ViroChip represents sequences from known referenced viruses, equally important is its potential to facilitate viral discovery in diseases of unknown etiology. As new viruses evolve, they still maintain certain characteristic-conserved regions in the viral genome. By using highly conserved sequences from all known viral taxonomy, the ViroChip can identify similar sequences in previously

unknown agents and newly evolved viruses within recognized families.

Proventricular dilation disease (PDD) has been diagnosed in psittacine birds for the past 40 years, but the underlying etiologic cause has not been identified. Although the disease exhibits characteristics of a viral infection and numerous viral etiologies have been proposed, confirmation of a definitive agent remained elusive. In 2008, Kistler and coworkers used the ViroChip to interrogate samples from 2 PDD case/control series collected on 2 different continents.³¹ Tissues from birds with confirmed PDD displayed a novel bornavirus signature in 62.5% of the PDD cases and none of the control tissues. The bornavirus-positive samples were further confirmed by virus-specific PCR testing and the complete genome sequenced. Currently, 7 genotypes of the avian bornavirus have been identified.

Microarray-based viral detection offers a powerful alternative for determination of viral subtypes. Classic serotyping of viruses is tedious and limited by availability of antisera. Conserved array elements are capable of broadly detecting many, if not all, viral serotypes. Unique hybridization patterns are often observed for different viral serotypes, which enables rapid identification.

Because of the high resolution of microarray hybridization, it will see future application in the study of viral pathogenesis and diagnosis of veterinary diseases. Microarray hybridization is a viable approach for detecting unsequenced or uncharacterized viruses and novel virus discovery. It can be used to differentiate among viral subtypes and provide important information on how a new virus relates to those that have been classified. Application of this type of information can provide clues about viral origin and possible treatment strategies. This comprehensive and unbiased analysis of viral prevalence in a given biological sample also increases the feasibility of introducing molecular testing for viruses that are easily detected using classical diagnostic testing methods.¹

Coupling PCR with Microarrays for Pathogen Detection

When target nucleic acids are abundant, then direct microarray interrogation of pathogen targets is achievable in the absence of amplification.¹⁸ Direct hybridization strategies involve extracting RNA or DNA from the sample and applying the material directly to a microarray without additional amplification. Direct hybridization provides the least bias in gene detection, but also has the lowest level of ana-

lytical sensitivity. Another strategy bypasses these limitations and yields an extremely broad-reaching and unbiased detection strategy. It uses microarrays composed of carefully selected viral sequences, coupled to a random PCR amplification. One approach is to amplify one or more universal genes (e.g., 16S rRNA, 18S rRNA, 23S rRNA genes) and to screen for pathogen-specific polymorphisms. Greisen and coworkers were one of the first groups to amplify fragments of the 16S rRNA gene using group-specific PCR primers and, with Southern blots that had pathogen-specific probes, identified pathogenic bacteria in cerebrospinal fluid.³² Microarrays can be used in a similar fashion to interrogate PCR products to distinguish between multiple pathogens. A second PCR strategy is to use multiplex PCR to amplify a number of discreet, pathogen-specific genetic markers that are subsequently detected on a DNA microarray.

Simultaneous detection of multiple pathogens should be viewed in the context of an entire analytical process that spans sample collection to final testing.¹⁸ Physical differences between target organisms often require very different strategies to extract nucleic acids for analysis after which the DNA microarray can then serve as the actual detection device.

The reader is referred to the review by Wang for an encompassing review of microarray technology.³³ In the area of microarrays for microbiological applications, several general reviews are available. Specific reviews are available on DNA microarray analyses of host-pathogen interactions³⁴ and the use of microarrays for the molecular diagnosis of *Mycobacteria* spp.³⁵ Generalized reviews in the medical-related areas include microarrays for disease gene discovery,³⁶ microarrays in medicine,³⁷ and microarrays for molecular pathology.³⁸

Genomics

Early methods of DNA sequencing, determining the nucleotide order of a DNA segment, gene, or genome, were costly and slow. Although the first sequence of a human gene locus was published in 1990,³⁹ it took 10 years to report the sequence of the entire human genome in 2000.⁴⁰ The recent development of cost-effective, high-throughput sequencing now allows for efficient, full sequencing of an organism's genome. As DNA sequence data have become more available, the field of comparative genomics has rapidly progressed. Attention has turned to generating whole genome assemblies and genomics resources for veterinary species. In July 2005, a publicly accessible annotated genome assem-

bly of the domestic dog,⁴¹ and subsequently a draft sequence of the domestic cat,^{42,43} were released. With complete genome sequences now available for the dog, cat, and horse,³⁹ the tools are now available to research very specific questions about animal genomes and cancer.

DNA microarrays have technologically advanced genomics research.⁴⁴ DNA microarrays are used to detect DNA or RNA (most commonly as cDNA after RT) and are referred to as gene expression analysis or expression profiling.²⁶ Gene expression analysis is based on the assumption that cells react to changes in their environment by increasing or decreasing transcription of appropriate genes. These changes in expression levels can be detected or quantified by using specific scientific methodology such as real-time RT-PCR and microarray technology.

The use of a distinct DNA array for gene expression profiling was first described in 1987 to identify genes whose expression was modulated by interferon.⁴⁵ Determining the level, or volume, at which a certain gene is expressed is called microarray expression analysis. Miniaturized microarrays were first applied to expression profiling in 1995.⁴⁶ In veterinary medicine, genome libraries have allowed for the development of new, high-density microarrays to facilitate whole genome and gene-targeted profiling at high resolution and throughput.⁴⁷ New, high-density microarrays have rapidly advanced our knowledge and understanding of pet and food animal genomes and have fostered the development of a rapidly growing class of molecular diagnostics focused on diagnostic testing for genetic traits encoded by genomic DNA.

Scientists can use a microarray to examine the expression of hundreds or thousands of genes within a single sample, in a single assay. Through computer analysis, the amount of mRNA bound to each site on the array is precisely measured, generating a profile of global gene expression within the cell. Once researchers have characterized the expression patterns of various genes involved in many diseases, the expression pattern of cDNA from an individual can be hybridized to determine if it matches the expression pattern of a known disease. If a gene is over expressed in a certain disease state, then more sample cDNA, compared with control cDNA, is detected. In microarray comparative genomic hybridization, the increased or decreased expression of a particular gene(s) is determined. A change in the number of copies of a particular gene may correlate to a certain disease state. Microarrays may also be used in veterinary diagnostics by comparing gene expression of infected cells or tissues with that of uninfected cells

or tissues. Expression profiling can also identify genes whose expression is changed in response to disease-causing agents.⁴⁸ Revealing how multiple genes work together to produce physical and chemical responses and analysis of patterns of coordinated gene expression can help identify genes involved in the development of various diseases.

A canine-specific microarray using mRNA and expressed sequence tag sequences has been developed.⁴⁹ The elucidation of these biological networks (e.g., disease, development, nutrition) and identification of associated molecular mechanisms will allow for the development of more precise diagnostics for these processes.⁴⁹

Single Nucleotide Polymorphisms

DNA microarrays can be designed specifically for genotyping, thereby enabling them to measure single base pair changes at many thousands of points throughout a genome. DNA microarrays are useful for genotyping point mutations, single nucleotide polymorphisms (SNPs), and short tandem repeats (STRs) among alleles within or between populations.⁵⁰ SNP technology has been diversely applied in areas such as analyzing forensics, measuring disease predisposition, evaluating germ line mutations in individuals and somatic mutations in neoplasias, diagnosing infectious and genetic disease, and diagnosing cancer. Once an SNP pattern is associated with a particular disease, SNP microarrays can be used to test an individual for that disease expression pattern. A determination can be made if the individual is susceptible or at risk of developing that specific disease condition.

In sheep, diagnostic microarrays are used to determine specific SNPs that predispose animals to scrapie, a prion protein disease. Individuals with these SNPs accumulate the prion protein disease cellular protein within their central nervous system.⁵¹

Oncology

Initial microarray platforms available for veterinary oncology were genomic and cDNA microarrays generated by binding DNA fragments onto a glass or silicon surface. More recently, high-density oligonucleotide arrays, where short DNA sequences are synthesized directly on the surface of a “chip,” have allowed for thousands of features to be represented. DNA copy number variation, gene expression levels, and genetic alterations can be determined at a substantially increased resolution.

High-density microarrays represent a powerful tool for comparative genomic studies. Microarray-based comparative genomic hybridization merges molecular diagnostics with traditional chromosome analysis.^{52,53} Applications for high-density microarrays have been extended to many areas of medicine and medical genetics.⁵⁴ New syndromes have been discovered, and the phenotypes of existing disease conditions have been expanded.

A comparative study of canine chronic myelogenous leukemia showed that dogs diagnosed with chronic myelogenous leukemia also present with a functionally active genetic translocation previously identified in humans.⁵⁵ This study resulted in the first molecular cytogenetic test for the presence of a clinically significant genomic alteration in a veterinary cancer.

Several tests are now available for canine patients to identify oncogenes and clonal lymphocyte populations.⁵⁶ These tests can be placed into 3 broad categories: (1) detection of individual mutations in oncogenes, (2) detection of chromosomal translocations, deletions, and duplications, and (3) detection of clonality in lymphoma and leukemia through unique antigen receptor genes of B- and T-cells. Each of these testing methods has been used in veterinary medicine on an experimental basis and is now being offered by several laboratories.⁵⁶

Future Microarray Technologies

The original emphasis on microarrays was on DNA technologies. Researchers have exploited the diversity of this technology to further extend applications to the development of protein, antibody, and carbohydrate microarrays. Macbeath and Schreiber⁵⁷ developed the first small-molecule microarray in 1999 and the first protein microarray in 2000. Soon afterward, other array types were developed, such as cell arrays, carbohydrate arrays, and proteome arrays.

Newer microarray formats that developed at the turn of the 21st century provide a host of other biomolecules that can be presented on a chip, including proteins (e.g., whole proteomes, enzymes, antibodies),^{58,59} small molecules (e.g., drug-like molecules, peptides, carbohydrates),^{60,61} and even whole cells and tissues for simultaneous, multiplexed experimentation.⁶²

Protein Microarrays

Within the last decade, protein microarrays have entered the field of proteomic research.^{63,64} Pro-

teomics is the term used to describe the study of proteins expressed by a genome. Technologies that had previously been established for DNA microarrays were adapted to the generation of protein arrays with glass slides being initially used as a solid support in planar microarrays. Bead arrays or liquid array systems rely on the use of different bead types that can be distinguished either by color, size, or shape. Currently, protein microarrays are highly miniaturized and parallelized solid-phase assay systems that use a large number of different capture molecules immobilized in microspots. Protein microarray technology has enormous potential for in vitro diagnostics and disease monitoring.

Protein microarray technology has enormous potential for application within the veterinary diagnostic testing arena. Antibodies in an immunoassay format have been widely used and are well established as highly sensitive tools for disease detection. With monoclonal antibody production, it is possible to produce pure and highly specific antibodies against almost any type of antigen. As a next-generation tool, protein arrays in their role as miniaturized, multiplexed immunoassays are perfectly suited for generating a maximum of diagnostically relevant information from very minute samples.⁶⁵ Protein microarrays offer unparalleled throughput, minimal reagent consumption, and sensitive simultaneous detection of multiple targets.⁵⁸ Currently, there is rapid advancement of antibody microarrays that have been developed for clinical, biothreat, and point-of-care applications. Early applications included the development of a protein microarray with 35 antibodies, which was used to subtype the 20 most common *Salmonella* serovars,⁶⁶ and a similar microarray was developed for *E. coli*.⁶⁷ Lipopolysaccharide, carbohydrate-based, and whole-cell microarrays have also been used for antibody-based detection of pathogens such as *Francisella tularensis*.^{68,69}

Liquid array technology has been adapted to the detection of avian influenza (AI) virus antibody in commercial poultry.⁷⁰ The assay uses recombinant AI virus nucleoprotein conjugated to microspheres to detect the influenza-specific antibody. The AI virus liquid array has a 99.3% sensitivity and a 94.7% specificity. This liquid array format can theoretically detect 100 different analytes in a single assay. Therefore, this technology has the potential to simultaneously detect and subtype all influenza hemagglutinin and neuraminidase proteins, as well as antibodies specific to the other relevant avian diseases.

Protein microarrays with high pathogen proteome content offer a valuable platform for high-throughput serology. These antigen microarrays

have been used to identify seropositive individuals by using the presence of serum antibodies to detect exposure to a specific pathogen. Zhu and coworkers monitored the antibody profiles of patients with SARS using protein microarrays containing 82 purified coronavirus proteins.⁷¹ Immunoreactivity against the coronavirus nucleocapsid proteins remained high for 120 to 320 days postinfection, which provided a means to check for exposure long after infection has occurred. The integration of DNA and protein-based microarray methods may extend the range of rapid clinical diagnostic testing.

In protein expression analysis, proteins or biomarkers from complex biological samples are identified and correlated with diagnostic patterns that are unique to specific cancer and disease conditions. The main challenge in the development of biomarker diagnostics is the biological variability among patient samples as well as the large dynamic range of biomarker concentrations. Both disease and cancer biomarker discovery (oncoproteomics) has been studied extensively using proteomics. Biomarkers have been identified that correlate with bladder cancer, breast cancer, colorectal cancer, esophageal cancer, gastrointestinal stromal tumors, glioma, hepatocellular carcinoma, leukemia, lung cancer, lymphoma, nasopharyngeal carcinoma, ovarian cancer, pancreatic cancer, prostate cancer, and urothelial carcinoma.⁷² Protein microarrays designed with hundreds of copies of protein-coding oligonucleotides or “bio-bar-codes” can serve to amplify the protein target and are capable of detecting proteins down to attomolar concentrations.⁷³

Besides protein expression analysis, protein microarrays can also be used to determine the functional analysis of proteins, including protein interaction involving immobilized proteins or peptides, low molecular weight compounds, DNA, oligosaccharides, tissues, or cells. Protein microarrays have been developed for the detection of allergen-specific immunoglobulin E reactivity and detection of specific auto antibodies associated with autoimmune diseases. In the field of autoimmune disease diagnostics, multiplexed assays are currently entering the diagnostic market,⁷⁴ and in the future, sets of tumor marker panels may also be applied to monitor treatment therapy for specific disease processes. Immunoassay panels have been developed for fertility, cardiac disease, tumors, cytokines and growth factors, cell adhesion molecules, thyroid function, and drug residues. Virtually every biological component from diverse small molecules, macromolecules (e.g., DNA, proteins), and entire living cells have been placed on microarrays.

Biomarkers

A biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic intervention.”⁷⁵ Research regarding biomarkers associated with disease has greatly advanced in both the human and veterinary medical fields. The challenge of this information is to determine which marker or combination of markers is optimal for the condition to which they are applied. Evidence from research studies suggests that using multiple markers may be superior to using single markers alone. Multiple marker combinations provide information of greater diagnostic and prognostic value than any single marker alone.

The most promising use of veterinary biomarkers are those used to evaluate cardiovascular disease in dogs and cats.⁷⁶⁻⁷⁸ Currently, circulating natriuretic peptides are regarded as the most promising markers used in the evaluation of such patients. Other markers include those of myocyte injury typified by troponins, markers of myocyte stress (adrenomedullin), matrix metalloproteinases, markers of endothelial function, and markers of inflammation (C-reactive protein).⁷⁹ Concentrations of some of these substances are known to change in response to heart failure and the treatment of cardiac disease in veterinary patients.^{76,77}

Clinical application of biomarkers in the future includes the detection of subclinical diseases, diagnosis of acute or chronic syndromes, risk determination, therapy selection, and monitoring of disease progression or response to treatment.⁷⁸ Presently, most biomarker testing takes place at dedicated laboratories, thereby increasing the expense of the test and the time needed to perform the test. The development of biosensor technology will enable biomarker testing to be accomplished in a smaller, faster, cheaper, and portable format.

Biosensors and Immunosensors

A biosensor can be generally defined as a device that consists of a biological recognition system and a transducer. A biosensor is a compact analytical device that integrates a biological element on a solid-state surface, enabling a reversible biospecific interaction with an analyte.⁸⁰ The interaction of the analyte with the bioreceptor produces an effect measured by the transducer, a device used to convert the information into a measurable signal. The biological component of a biosensor can include any molecule used for biomolecular recognition. Biosensors have been de-

signed using specific binding proteins, lectins, nucleic acids, membranes, whole cells, antibodies, or antibody fragments. Biosensors that detect the immunological interaction between a specific antibody and antigen are referred to as immunoreaction-based biosensors or immunosensors.⁸⁰ Some biosensors use aptamers, single-stranded DNA or RNA oligonucleotide sequences that recognize various target molecules with high affinity and specificity. These ligand-binding oligonucleotides mimic properties of antibodies in a variety of diagnostic formats.

There are 2 main types of biosensors: biocatalytic and bioaffinity-based biosensors, and they are primarily categorized on the detection principle applied. Biocatalytic sensors use enzymes to catalyze a signaling biochemical reaction, and bioaffinity-based sensors monitor the binding event itself. Transducers convert the biological interaction to a detectable signal and can be based on electrochemical (e.g., potentiometric, amperometric, conductometric), optical, microgravimetric, or thermometric (calorimetric) principles.

Biosensors have been designed using carbon nanotubes and nanoparticles for signal amplification. Combining the molecular specificity of biological recognition with an operationally simple transducer, extend these small units' diagnostic capability toward portability and ease of use. Recent developments in immunosensors have produced solid state systems that allow for rapid and continuous monitoring of antibody-antigen reactions in real time.

The specificity of the biosensor system is determined by the immobilized detection molecule.²⁰ Innovative strategies using biomarkers represent alternative strategies for reliable cancer testing and for detecting clinical markers predictive of cardiovascular and other diseases. Biosensors using antibodies or DNA/RNA strands can detect infectious disease agents and be used for hormone and drug assays.^{20,81}

Currently, most biomarker testing takes place at a dedicated, centralized laboratory, but in the future this technology will provide a means of rapid, convenient, and economical testing at the "point of patient care." In cancer, there is a strong connection between early detection and positive patient outcome, thereby improving patient survival and disease prognosis in many cases. In addition to changes within the host genome, complex molecular alterations such as over or under expression of a protein can occur during the course of tumorigenesis.⁸² Biosensors can detect these changes and can analyze molecular biomarkers for tumor diagnosis and classification, monitor response to treatment, and detect recurrence of the disease. Cancer biosensors have

been developed in a panel format for the simultaneous detection of different cancer biomarkers.^{83,84}

Because most cancer biomarkers are not specific to a particular tumor, the use of an array to detect multiple markers increases the diagnostic value of their definitive diagnosis.⁸⁵ Recently, biosensors have been developed to detect breast cancer, ovarian cancer, gastrointestinal tract carcinoma, chronic myelogenous leukemia, and prostate cancer.²⁰ Prostate-specific antigen is the most reliable tumor marker to detect prostate cancer in its early stages. The recently developed biosensors for prostate-specific antigen detection are sensitive to a limit of 4 pg/mL.⁸⁶

The importance of inflammatory markers in the early detection of cardiovascular disease has been demonstrated.⁸¹ Biosensors are being developed to detect C-reactive protein, cardiac troponin, myoglobin, and natriuretic peptide in a sensitive and efficient manner. Based on a competitive immunoassay in a portable biosensor, good correlation between salivary cortisol and levels of "free" cortisol in serum has been demonstrated.⁸⁷

Nucleic Acid-based Biosensors

A nucleic acid biosensor is an analytical device that integrates an oligonucleotide with a signal transducer.⁸⁸ The nucleic acid probe is immobilized on the transducer and acts as the biorecognition molecule to detect DNA/RNA fragments. Several DNA-based biosensors have recently been developed for the detection of virus-related sequences and other infectious agents.¹⁹ DNA or RNA ligands can also be synthesized to be used to detect amino acids, drugs, proteins, and other molecules. DNA or RNA ligands can bind to their targets with affinity ranging from the micromolar to the nanomolar level and can discriminate between closely related targets.²⁰ There is a growing need for small, fast, efficient and portable biosensors.

Metabolomics

The analysis of thousands of small molecules (metabolites) (e.g., sugars, organic acids, amino acids, nucleotides) by means of a global approach is referred to as metabolomics. Combined with pattern recognition processes, metabolomics defines a metabolic phenotype (metabotype) in the study species.⁸⁹ This approach combines high-throughput sample analysis with computer-assisted pattern recognition techniques whereby the full metabolite profile (metabolome) of a cell, tissue, or organism is

determined. Metabolomics is a powerful investigative tool for studying the biochemical effects of disease and screening for potential pharmacologic agents. Alterations in the metabolic profile often present much earlier in the course of disease than induced histopathological changes. Metabolomics can be a sensitive, early indicator of a disease process, and because metabolites are conserved across species lines, screening patterns can be applied to a wide range of species.⁸⁹

The application of metabolomics in veterinary medicine is in its infancy. Whitfield and colleagues used metabolomics to distinguish canine congenital portosystemic vascular anomalies from acquired hepatopathies.⁹⁰ Other early applications include the analysis of cadmium toxicity in rodents,⁹¹ assessment of sublethal stress in aquatic organisms,⁹² and neurotransmitter deficits in cerebral tissue from a mouse model of human batten disease.⁹³

Although current applications to the field of veterinary medicine are rare, metabolomics use in early preclinical safety assessment make it a rapidly developing tool in the field of drug investigations. Metabolomics enables a noninvasive systems assessment of a broad spectrum of biologic responses by an individual to the effects induced by therapeutic compounds, which could impact all stages of veterinary drug research and development.

Molecular Theranostics

Molecular theranostics is an emerging area in which molecular diagnostic tools are used to provide rapid (less than 1 hour), accurate, and informative diagnostic microbiology assays. Conventional microbial culture and sensitivity testing methods require at least 2 days' time because of the reliance on growth and isolation of microorganisms. Veterinarians must frequently treat patients empirically with broad-spectrum antimicrobial agents, which may not be needed or may not be effective. The common use of broad-spectrum antimicrobials is associated with an increasing rate of microorganism resistance, which may complicate patient treatment. Early diagnosis and treatment of diseases reduce the risk of the patient developing long-term disease complications. For some diseases, a prompt treatment will also reduce further transmission of the disease to other animals or humans.

Assays based on the detection of nucleic acids offer enormous potential for the rapid and accurate diagnosis of microbial infections. This assay-based testing can be extended to include the detection and

characterization of genes or mutations associated with antimicrobial resistance and virulence.⁹⁴

Advances in genomics and proteomics will provide the essential nucleotide or amino-acid sequence data required to design accurate assays. Efficiency will also depend on the development of rapid, simple, and efficient methods for microbial nucleic acid or protein extraction from a variety of clinical samples. Finally, rapid and specific assays, and the ability to detect all or most targeted microorganisms in multiplex target amplification systems, or signal amplification technologies, are needed. This will help future technologies reach a level of analytical sensitivity appropriate for testing directly from clinical samples without previous enrichment, thereby leading to more judicious antimicrobial usage and a concurrent reduction in antimicrobial-resistant organisms.⁹⁴ To be useful, diagnostic methods must be accurate, simple, and affordable.

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