Chapter 16
Rapid Detection of Bioterrorism Pathogens

David Perlin

16.1 Introduction

Pathogen identification is a crucial first defense against bioterrorism. A major emphasis of our national biodefense strategy is to establish fast, accurate and sensitive assays for diagnosis of infectious disease agents likely to be used in a bioterrorist event. The Centers for Disease Control and Prevention and National Institutes of Health's National Institute of Allergy and Infectious Diseases have identified three priority classes or "select agents" of pathogens and toxins. These are designated A, B, and C, and are likely candidates since they can be easily disseminated, usually in aerosol form, require small numbers of organisms or molecules to cause disease, and result in rapid morbidity and mortality.

The challenge for most infectious diseases specialists is that these agents are rarely encountered in normal practice, many are seen in remote outbreaks in distant countries, and one (smallpox) has been eradicated with the last case observed several decades ago. As our first line of defense, the vast majority of physicians may not be able to distinguish the early events of a bioterrorist event from other atypical pneumonias or cutaneous infections. The challenge of rapid pathogen or toxin recognition is to aid physicians in the diagnostic process and to help identify at an early stage a potential deliberate outbreak [1]. Such assays will ensure early and appropriate treatment of infected patients, and will alert public health authorities and law enforcement to help contain an outbreak.

A major lesson of the October 2001 anthrax outbreak was that aggressive therapeutic intervention saves lives, since highly virulent organisms like anthrax can respond well to antimicrobial therapy when diagnosed in the early stages [2, 3]. Unfortunately, the early signs and symptoms of many of these diseases are nonspecific so it is critical that highly sensitive and reliable tools are available to identify infected individuals. The first step toward effective patient and

D. Perlin
Public Health Research Institute, UMDNJ-New Jersey Medical School, 225 Warren Street, Newark, New Jersey, USA
e-mail: perlinds@umdnj.edu

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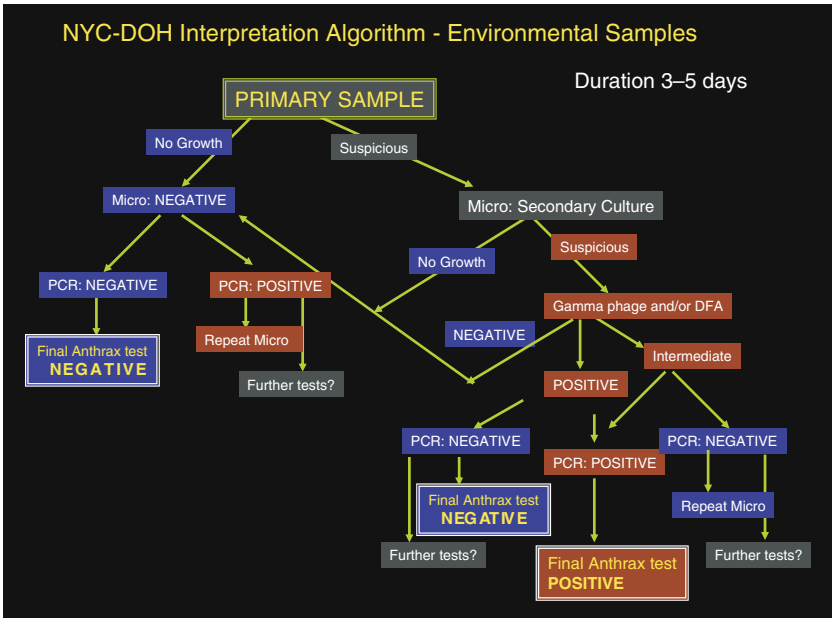
public health control is to identify rapidly the infecting pathogen and its source. Some of these select agents are highly transmissible in the early stages of disease and it is critical to identify infected patients to limit the risk to the remainder of the population. In some cases, such as hemorrhagic fever with Ebola virus, it is hypothesized that patients become infected through contact with an infected animal. Yet, the natural reservoir of the virus is unknown, as is the manner in which the virus first appears in a human at the start of an outbreak [4]. Whether the initial source can be elucidated or not, rapid diagnostic procedures are critical to support infection control measures that monitor and limit the spread of infectious diseases agents [5, 6]. Finally, accurately defining the scope and progression of an infectious disease outbreak helps mobilize resources more efficiently and eases public anxiety that can lead to panic [7].

16.2 Limitation of Conventional Diagnostics

Rapid clinical diagnosis and aggressive preemptive therapy can limit the fatalities associated with a biological agent of mass destruction [8, 9]. Most clinical laboratories, however, still rely upon culture-based technology with phenotypic endpoints, approved by FDA and/or CDC. These assays can take several days for definitive results. In addition to time delays, these techniques often lack adequate sensitivity and specificity and some organisms are difficult to isolate in culture. Delays often translate into the initiation of empiric therapy in the absence of positive pathogen identification. The problem is not limited to a bioterrorism outbreak as hospital and public health laboratories, confounded by inadequate and slow methodology for pathogen detection, often have difficulty identifying pathogens. When occurring with very ill and/or immunocompromised patients, these delays can increase morbidity and mortality.

In a bioterrorist event, like many naturally occurring disease outbreaks, there is a need to obtain rapid pathogen identification from clinical specimens but also from environmental specimens to minimize fomite-based transmission. Time delays measured in days can prevent adequate public health measures from being instituted to contain an outbreak. Such delays also subject exposed individuals to needless stress and anxiety. The inadequacy of phenotypic-based diagnostic assays is illustrated graphically by the "gold standard" public health laboratory-testing algorithm that was in place for positive identification of *Bacillus anthracis* from environmental samples during the October 2001 anthrax outbreak (Fig. 16.1a). A complicated matrix of phenotypic and biochemical assays required 3–5 days for a positive endpoint. What was needed was a streamlined approach that could progress from primary specimen to a positive or negative outcome in a matter of hours or less (Fig. 16.1b). Rapid diagnostics have finally come of age and offer exceptional promise in this regard for accurate pathogen identification.



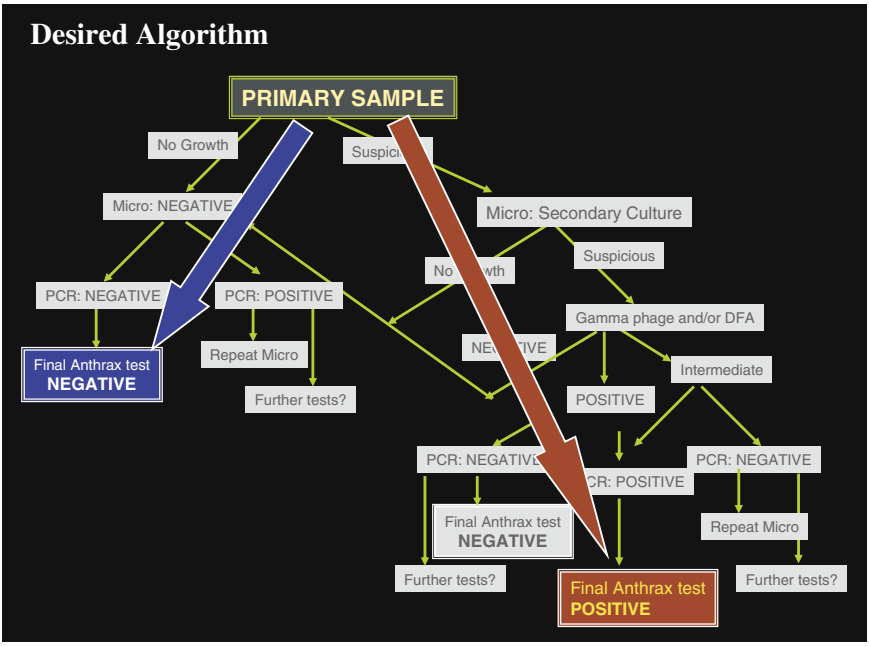


Fig. 16.1 (**a**) Schemes for identification of *Bacillus anthracis*. New York City Department of Health interpretation algorithm for identification of *Bacillus anthracis* from environmental samples based on CDC guidelines. (**b**) Desired pathway for identification of *Bacillus anthracis*

16.3 Rapid Identification Methods

Rapid identification assays can be loosely divided into tests that either utilize antigen-antibody or other antibody-specific binding to identify a specific pathogen or genetic tests that identify pathogen-specific DNA or RNA sequences.

16.3.1 Serologies: Antigen–Antibody Interactions

Serological techniques have been invaluable for detecting active infections with organisms that are difficult to culture and for documenting previous infection/immunity. While conventional serodiagnosis has a limited ability to detect acute infections with select agents, it is invaluable for monitoring disease kinetics and dispersion of these agents within exposed or high-risk populations, especially where asymptomatic infections are frequent. Such information is crucial to therapeutic intervention and prophylaxis, as well as to isolation protocols in the context of a biological attack. Serodiagnosis is particularly useful for mass screening of infectious diseases because such techniques are generally simple to perform, inexpensive and amenable to high throughput technologies. The assay takes advantage of the exquisite sensitivity and specificity of antigen-antibody interactions. Antigen capture assays represent a somewhat more recent addition to serological techniques. These methods have been useful for rapidly diagnosing acute infections where antigen levels are relatively high, especially in the urine where they may be concentrated.

The Enzyme Linked Immunosorbent Assay (ELISA) has become the workhorse of most clinical laboratories. It relies on specific antigen-antibody interactions to identify a pathogen [10]. Typically, a preliminary test can be performed quickly, usually within 3–4 h. A positive ELISA test can be confirmed by performing a Western Blot with target-specific antibodies or by an immunofluorescent antibody (IFA). Unfortunately, pathogen antigen production or a host antibody response is not always robust enough to permit reliable testing, especially in the early stages of an infection. In addition, a false positive result with an ELISA test can occur due to interference from other antibodies. Although the ELISA test is highly specific, an antibody response may not be detected in either the early or late stages of a disease. Improved sensitivity may be obtained by expanding the class of antibodies detected to IgG, IgA and/or IgM classes of antibodies [11].

ELISA platforms are especially well suited for toxin detection and, when combined with procedures such as time-resolved fluorometry, sensitivities of 4–20 pg/mL can be obtained in a typical 2 h assay for molecules such as botulinum type A or B neurotoxin and *Staphylococcus aureus* enterotoxin B [12]. A newer format for antigen-antibody detection where the detector antibody is labeled by chemiluminescence facilitates an even greater sensitivity [13]. In one approach, target antigen is first complexed to paramagnetic beads

coated with capture antibody and then identified using a detector antibody. An electrochemical flow cell with photon detector is used to detect the target-antibody interactions with detection limits approaching 200 fmol/L and dynamic ranges of six orders of magnitude [10]. More recently, a novel type of biosensor for rapid pathogen identification has been described using B cells as sensing elements. This system know as CANARY (cellular analysis and notification of antigen risks and yields) utilizes B lymphocytes genetically engineered to express both cytosolic aquorin, a calcium-sensitive bioluminescent protein, and membrane bound antibodies specific for a given pathogen or toxin [14]. Interactions of antigen with antibody elevate intracellular calcium levels resulting in light emission by the cytosolic aquorin molecules. The system responds in a fashion that is more rapid, sensitive, and specific than most antigen detection systems, and has been shown to detect *Yersinia pestis* in less than 3 min at levels of 50 colony forming units [14].

16.3.2 Antigen–Non-Antibody Target Interactions

In recent years, the recognition that pathogens can evoke specific T-cell responses has been used to develop assays such those used in the enzyme-linked immunospot assay (ELISPOT) T-SPOT TB® and Quantiferon-TB for the diagnosis of latent tuberculosis infection. The exquisite sensitivity of these assays in which signature TB peptides elicit specific interferon gamma release makes them applicable to immunosuppressed individuals, while the specificity of the assay overcomes problems such as prior vaccination with Bacille Calmette-Guérin (BCG). These assays cannot distinguish between drug sensitive and resistant forms of TB, but are particularly valuable in following diseases transmission in settings where multidrug resistant (MDR) or extremely drug resistant (XDR) strains are prevalent.

Enzymatic activity has been used for some time in the detection of microorganisms. For example, the presence of significant levels of *Helicobacter pylori* in gastric secretions has been made using the detection of urease activity of the bacillus. Although bacterial urease production is not unique to *H. pylori*, the other urea producers are not found in the human stomach, the site of replication of *H. pylori*.

An example of a bioterrorism antigen detection system utilizing a specific enzymatic interaction with a substrate rather than binding to an antibody is the micromechanosensor reported by Liu, et al. [15]. The functional nature of a toxin is detected utilizing microfabricated cantilevers [16]. In this proof of concept model, botulinum neurotoxin B is detected by its activity as a endopeptidase, cleaving its neurotarget synaptobrevin 2 (also referred to as VAMP 2). The reporting system detects the large change in resonance frequency of the vibrations of the cantilever following release of the agarose bead, which is bound to the cantilever by synaptobrevin. The cantalever system is most

sensitive in gaseous or vacuum milieus and the fluid medium damps the vibration to some degree. It can detect botulinum toxin at 8 nM concentration within 15 min and is applicable to on-chip electronic technology that greatly increases sensitivity.

16.3.3 Genetics: Exploiting Genomic Differences

Genomic differences between microbes offer an alternative to culturing for detection and identification of pathogens by providing species-specific DNA targets that can be accurately resolved by molecular methodology. Nucleic acid-based molecular approaches for pathogen identification overcome many of the deficiencies associated with conventional methods by exploiting both large- and small-scale genomic differences between organisms. Polymerase chain reaction (PCR)-based amplification of highly conserved ribosomal RNA (rRNA) genes, intergenic sequences, and specific toxin genes is currently the most reliable approach for identification of bacterial, fungal and many viral pathogenic agents. When combined with microarray or fluorescence-based oligonucleotide detection systems, these molecular approaches provide quantitative, high fidelity analysis [8, 17, 18]. Most importantly, these genetic probing systems offer rapid turn around time (1–6 h) and are suitable for high throughput, automated multiplex operations critical for use in clinical diagnostic laboratories.

The need for rapid diagnostics was never more apparent than during the severe acute respiratory syndrome (SARS) epidemic of the spring of 2003. In the early stages of the epidemic, physicians and public health officials were relatively helpless to contain a fast moving, globally spreading epidemic of severe atypical pneumonia caused by an unknown respiratory agent. Once the viral agent was identified, genomic sequences of the SARS coronavirus (CoV) were used to develop a diagnostic assay within days that rapidly (within 1–2 h) and reliably identified the SARS CoV in a range of clinical and environmental specimens [19]. Armed with molecular tools, physicians and public health officials in China and Canada, hit hardest by the disease, could confirm the cause of rapidly spreading atypical pneumonias, monitor virus levels in patients and explore potential sources for the outbreak [20–23]. This single event highlighted the importance of rapid molecular diagnostics in outbreak control and disease management, signaling the arrival of a new era in diagnostics.

16.3.3.1 Attributes of a Comprehensive Diagnostic Test

An effective diagnostic assay must be rapid, sensitive and specific as well as being simple and robust to facilitate use in clinical and public health laboratories. Assays should detect a wide variety of pathogens and, where possible, have capacity to detect “designer” organisms (heterologously expressed toxin or

virulence genes) created through recombinant technologies. Genetic-based molecular assays are currently in development that not only include all category A through C pathogen and toxin genes but also include a wide range of common bacteria, viruses and fungi. With this approach, it is possible to recognize in clinical (respiratory secretions, blood, urine, tissue), environmental (including letters, nasal swabs and hair) and food or water samples the presence of a pathogenic organism that is present as a homogeneous population or is cloaked by dispersion within a large population of nonpathogenic organisms. It is also possible to detect unnatural events such as the expression of a lethal toxin gene (such as a botulinum toxin genes) in a recipient nonpathogenic organism such as *Escherichia coli* or *Bacillus subtilis*. Similarly, a mixed powder containing anthrax-laden spores in a background of 99.9% *B. subtilis* spores would be perceived as harmless, until the 0.1% *B. anthracis* component began to cause disease. Such complex mixtures can be easily resolved by molecular diagnostics.

Specificity and sensitivity are also key elements of the diagnostic assay. When specific probes are used, they must be shown to interact only with their designated targets to avoid false positive responses. The advent of real-time self-reporting probes capable of allele-specific sequence discrimination at the level of a single nucleotide allows these probes to react with exceptional fidelity [24, 25]. Sensitivity is a major requirement especially because in early stages of a disease few pathogens may be present for detection. Some pathogens like *Francisella tularensis*, the etiological agent of tularemia, need only a few organisms to cause lethal disease [26].

16.3.3.2 DNA Microarrays

Microarrays of nucleic acids were developed to utilize the enormous amount of information provided by genome projects but have clear potential in mass screening and diagnostics [27, 28]. A microarray allows thousands of targets to be analyzed simultaneously, being particularly useful for novel virus identification and characterization. Microarrays consist of gene and genome-specific nucleic acid fragments, either cloned gene segments or long (70–80 mers) oligonucleotides, which are fixed to a glass slide or other solid matrix such as those used for computer chips [29]. One such product is the GeneChip®, a high density, oligonucleotide-based DNA array developed at Affymetrix. Target DNA or RNA is labeled and hybridized to complementary DNA sequences on the microarray. Scanning lasers are used to detect high affinity interactions and each addressable position corresponds to a known target.

The application of this maturing technology may be best illustrated during the outbreak of SARS during early 2003. To assist in trying to identity the pathogen, the CDC referred specimens containing the unknown agent to many laboratories, including that of Dr. Joseph DeRisi. DeRisi had developed a microarray chip called Virochip containing nucleic acids specific for numerous viruses known to cause human disease. Hybridization of the unknown virus genome segments to the chip revealed the presence of a previously

uncharacterized coronavirus. Subsequent molecular characterization and phylogenetic sequence comparisons confirmed that the virus was a new member of this family [30, 31].

Microarray technology is powerful but also expensive, technically demanding and labor intensive. Amplifying the sample above background is critical to achieve dependable results. A highly purified nucleic acid sample is best for the assay as interfering substances may limit hybridization. False positive interactions can usually be minimized through careful microarray development with suitable redundancy of targets. It is best, however, to verify independently "positive hits" with a different technique. Like any hybridization-based assay, target specificity is critical, although absolute fidelity can be fine tuned. Allele-specificity at the level of single nucleotide changes can be desirable for subtyping of species [32]. The ability to detect imperfect matches within a family is equally desirable for the discovery of new disease agents and variants of old ones (SARS CoV). Of course, genetic microarrays like other genetic approaches can detect the presence of toxin genes, but they cannot be used to directly detect toxins.

A trend toward fully automated microfluidic applications involving chip-based capillary electrophoresis that can perform in-line reagent dispensing, hybridization, and detection significantly reduces sample sizes and improves accuracy [33]. The combination of array hybridization followed by direct viral sequence recovery provides a general strategy for the rapid identification and characterization of novel viruses and emerging infectious diseases. The ability of DNA microarrays to identify either multiple gene targets from single or multiple pathogens in a single sample has the capacity to transform detection of emerging pathogens. It is particularly useful to evaluate rapidly changing disease agents such as influenza [34]. New platforms such as the GreeneChipPm, which is a panmicrobial microarray comprising 29,455 sixty-mer oligonucleotides, is suitable for comprehensive detection of a wide range of vertebrate viruses, bacteria, fungi, and parasites [35]. This technology has the potential to transform blood testing by providing an integrated platform for comprehensive testing that replaces multiple individual assays [35, 36].

16.3.3.3 Real-Time Probes

The simplest PCR form involves the use of specific primers to amplify a known target fragment of DNA or RNA and detection the product with an intercalating dye. This approach relies upon the specificity of linear DNA-DNA or DNA-RNA hybridization probes in the amplification process. Probe-target hybridization is highly temperature dependent, however, and, depending on the nucleotide composition of the probe, random annealing can pose a problem. Sequences with high G + C contents are especially vulnerable since the temperature profile for annealing is shifted and false priming may occur.

In general, standard PCR-based amplification is insufficient for identification purposes due to relatively high levels of false positive results. A solution to

this problem is the use of fluorescent probes such as the 5′ endonuclease, adjacent linear and hairpin oligoprobes and the self-fluorescing amplicons that require high fidelity binding to target sequences for detection [18, 37–39]. Such high fidelity probes, especially self-reporting probes, have additional advantages in that both PCR amplification and detection can be done in a sealed tube, greatly reducing the possibility of contamination. They can also be used in real-time assays in which product formation is continuously monitored and validated. This is an important consideration for a clinical microbiology lab because PCR amplification has the potential to amplify small amounts of target DNA from contaminating organisms or even human DNA. Real-time PCR does not require post-PCR sample handling, preventing potential PCR product carry-over contamination and facilitating high throughput assays. Real-time PCR assays using high fidelity probes are also rapid (0.5–2 h), quantitative and have a large dynamic range exceeding 1 million-fold of starting target.

Probing systems including LightCycler™ [40, 41], TaqMan™ [42] and Molecular Beacons [24, 43] are widely used to identify pathogens in real-time assays. The LightCycler system measures the fluorescence resonance energy transfer (FRET) between two linear oligonucleotide probes labeled with different fluorophores in a glass capillary tube format. The probes are hybridized to the target in a head-to-tail motif during the annealing stage of the PCR which bring the fluorophores in a close proximity, causing a transfer of energy resulting in an emission of a detectable fluorescent light. TaqMan probes are linear oligonucleotides that contain a 5′ reporter dye and 3′ acceptor with overlapping emission-absorption spectra. The reporter dye remains quenched by the 3′ acceptor, while hybridized to its target. Cleavage of the 5′ reporter by 5′ nuclease activity of Taq DNA polymerase results in strong fluorescence signals. TaqMan can be utilized in a 96 well format, amenable to high throughput screening. One limitation of FRET-based systems is that multiplexing is more limited since several quenchers in the same reaction are required and spectral overlap can be a problem. Molecular Beacons are small, single stranded nucleic acid hairpin probes that brightly fluoresce when bound to their targets [43]. The probes possess a stem and loop structure in which the loop contains a complementary target sequence. The stem forms by the annealing of short complementary nucleotide sequence arms adjacent to the target sequence. A fluorophore is covalently linked to one end of the stem sequence with a quencher covalently linked to the other end. In free solution, Molecular Beacons do not fluoresce because the stem structure keeps the fluorophore close to the quencher and fluorescence energy is absorbed and released as heat. In the presence of target DNA, however, the loop sequence anneals to the target and a probe-target hybrid is formed forcing the stems containing the fluorophore and quencher apart, and fluorescence occurs. Molecular Beacons are better suited than most linear probes to monitor authentic amplicons in PCR reactions because a single nucleotide mismatch can prevent a Molecular Beacon from binding to its target and lighting up [24]. Both TaqMan and Molecular Beacons can detect single nucleotide changes and are highly suitable for allelic discrimination [24, 44].

16.3.3.4 Multiplex Assays

A single multiplex reaction assay that combines numerous probes and is capable of identifying multiple pathogens is a more efficient and cost-effective approach for a clinical microbiology lab, greatly expanding the capacity of pathogens being surveyed. Multiplex assays require that probes representing different targets can be reliably resolved in the same reaction tube or well. Typically, different probes are labeled with a range of fluorophores that have unique emission spectra that can be discerned with discrete optics or dispersed onto an array for detection. When dealing with fluorophores, the spectral properties of the probe-target hybrid must be significantly different from the unbound probes to permit unambiguous probe identification. LightCycler™, TaqMan™ and Molecular Beacon probes are all suitable for multiplex assays. The ability to multiplex PCR by probe color and melting temperature (T(m)) greatly expands the power of real-time analysis. Novel labeling techniques are evolving quickly that will allow more than 50 targets to be simultaneously evaluated in a single reaction. For example, MassTag PCR, which has been used detect viral hemorrhagic fever, is a multiplex assay in which microbial gene targets are coded by a library of 64 distinct mass tags. Nucleic acids are amplified by multiplex PCR using up to 64 primers, each labeled by a photocleavable link with a different molecular weight tag. After separation of the amplification products from unincorporated primers and release of the mass tags from the amplicons by UV irradiation, tag identity is analyzed by mass spectrometry [45].

16.3.3.5 Target Selection

A number of target sequences have been proposed for the identification of pathogens likely to be used in a bioterrorist event. Some targets are specific to a single species, subspecies, toxin or virulence factor (Table 16.1). Other targets can be used more generally such as ribosomal RNA (rRNA) genes or heat shock genes. These genes contain highly conserved DNA sequences (usually required for function) interspaced with variable regions that have been widely utilized in species-specific genetic assays. Ribosomal genes in fungi and bacteria have conserved sequences that are ideal for universal primer targeting, contain variable sequence regions that are species-specific, and are present in high copy-number tandem repeats [46]. The gene for the small-subunit ribosomal RNA (16 S-like) has been especially useful in evolutionary studies of distant phylogenetic relationships, remaining quite stable during evolution of all organisms [47]. The 16 S-like ribosomal genes can be amplified from total DNA isolated from essentially any organism using a single set of primers recognizing the conserved regions of the gene.

DNA or RNA from a wide variety of organisms can be amplified using a single set of “universal” PCR primers that bind to conserved regions of these genes. Species determination may then be performed by analyzing the

Table 16.1 Targets for real-time detection of toxins and secretion systems

Name	Organism	Gene(s)
Ricin	*R. communis*	*RTA & RTB*
Staphylococcus Enterotoxin B	*S. aureus*	*entB*
Botulinum A	*C. botulism*	*BoNT/A*
Botulinum B	*C. botulism*	*BoNT/B*
Botulinum C	*C. botulism*	*BN/C1*
Botulinum D	*C. botulism*	*BoNT/D*
Botulinum E	*C. botulism*	*BotE*
Botulinum F	*C. botulism*	*BotF*
Botulinum G	*C. botulism*	*BoNT/G*
Difficile A	*C. difficile*	*ToxA*
Difficile B	*C. difficile*	*ToxB*
Perfrigens Type A	*C. perfrigens*	*cpE*
Epsilon toxin	*C. perfrigens*	*extD*
Shiga toxin 1 & 2	*E. coli*	*STX1 & 2*
Listeriolysin	*L. monocytogenes*	*hly1*
Diptheria	*C. diphtheriae*	*Tox*
Yersinia translocon proteins	*Yersinia spp.*	*YorB, YorD, LcrV*
Pseudomonas translocon proteins	*P. aeruginosa*	*PopB, PopD, PcrV*
Shigella translocon proteins	*S. flexneri*	*IpaB, IpaC*
EPEC translocon proteins	*E. coli*	*EspB, EspD*
Salmonella translocon proteins	*Salmonella spp.*	*AF056246*
Xanthamonas translocon protein	*X. campestris*	*HrpF*

species-specific sequences contained in regions within the resulting amplicons [48]. There are advantages to both types of targets. Specific targets can be detected in samples that are heavily contaminated with nonpathogenic bacteria. They also make it possible to detect virulence factors inserted into normally innocuous bacteria. "Universal" target amplification approaches have the advantage that they can be more easily multiplexed. A single set of primers serves to amplify multiple species, permitting the development of more general diagnostic assays. The same two approaches can be used to develop targets for viral detection assays, although "universal primers" are generally more restricted to bacteria, fungi and specific families of viruses.

16.3.3.6 Sample Processing

Sample processing development is an integral component of a successful diagnostic program. During a bioterrorist event, depending on its size and duration, a public health lab may need to process tens of thousands of specimens. Rapid nucleic acid extraction from clinical and environmental samples will be critical for downstream molecular evaluation. Extraction of genetic materials must be automated, ultrasensitive and have high throughput capability to process and organize large sample populations. The detection of nucleic acids from infecting microorganisms or viruses in whole blood, tissues, respiratory secretions, urine,

and other body fluids can be influenced by numerous factors. Sample preparation depends on the type of biological material that vary in consistency and viscosity. Highly viscous samples (such as mucous) can be difficult to handle and process. Bacterial and fungal spores are encased in a heat and largely chemical resistant shell, making the isolation of nucleic acids troublesome. New cell and spore disruption techniques involving mechanical, chemical, enzymatic and thermal treatments have improved extraction efficiencies markedly [48–51].

All of these procedures are suitable for robotic high throughput processing. When possible, liquid biological samples should be centrifuged to concentrate bacteria, spores and fungi prior to nucleic acid extraction, increasing purity and efficiency. Anticoagulants such as EDTA, heparin or citrate can limit product formation by interfering with the PCR as can large excesses of free genomic DNA. Differential surface-based binding procedures have been developed to purify and concentrate target DNA away from genomic DNA and host-associated inhibitors, improving sensitivity. Magnetic bead technology is an ideal choice for nucleic acid isolation and purification because of its greater affinity for nucleic acids than other conventional methods. It reduces the risk of sample cross contamination found in other extraction methods by eliminating centrifugation and other manual steps during the extraction and purification process. Magnetic bead based nucleic acid extraction can be performed from micro ($<100\,\mu L$) volume samples, is completely automated for high throughput and can rapidly isolate purified nucleic acid in about 1 h. Commercial systems such as MagNA Pure LC™, KingFisher™ and NucliSens easyMAG™ are readily available in clinical laboratories for extraction of both DNA and RNA from a wide range of pathogenic bacteria, viruses and fungi present in clinical samples. These standardized products allow for highly efficient target capture and are scalable over a wide range of nucleic acid levels. The GeneXpert® System is a bench-top sized fully self-contained microfluidic system for sample extraction and nucleic acid detection.

The Cepheid MIDAS II (microfluidic DNA analysis system) provides rapid, on-site testing for bioterror pathogens from environmental samples. It automatically processes biological samples, extracts the nucleic acid, and prepares it for testing. The system then transfers the extracted nucleic acid and PCR reagents to a real-time thermal cycler with eight independently programmable reaction sites. All of the critical processes of the analysis are performed in a closed microfluidic system, including post-analysis clean-up and decontamination. This technique allows for continuous, automated operation over an extended time with an assay time is less than 30 min for pathogen detection.

16.3.4 Validation of Diagnostic Assays

One of the primary outcomes of rapid diagnostics development is to facilitate therapeutic intervention by detecting infectious agents early in infection. This

goal requires that a new molecular detection technique be optimized for both sensitivity and specificity, and be validated. An important consideration is to be able to quantitatively compare culture and antigen-based detection in individuals with molecular probes. Once molecular diagnostic approaches are validated, the new diagnostic tools can be refined and used to assess earlier predictors of disease such as elevated temperature or measurable immunological responses. Ideally, validation of a new diagnostic should occur by statistically demonstrating its equivalence or superiority to conventional detection methodology on clinical samples.

Typically, such validations are best determined from clinical specimens obtained from patients in endemic areas of disease. For most select agents, human infections are rare and occur in remote regions outside the USA, making validation on human populations impractical. A partial solution is to use well-developed animal infection models to both optimize and provide initial validation for new diagnostic tools. The primary advantage of an animal infection model is that infection and progression of disease can be more precisely defined. The goal of optimization studies in animals is to achieve the highest possible level of detection while maintaining fidelity of identification in the absence of false positives.

16.4 In Place and on the Horizon

Rapid advances in the genomic sequencing of bacteria and viruses over the past few years have made it possible to consider sequencing the genomes of all pathogens affecting humans as well as the crops and livestock upon which our lives depend. The Chem-Bio Non-Proliferation program of the US Department of Energy began a large-scale effort of pathogen detection in early 2000 in an effort to provide biosecurity at the 2002 Winter Olympic Games in Salt Lake City, Utah [52, 53]. Molecular assays were developed at the Lawrence Livermore National Lab for likely bioterrorist agents by utilizing whole genome comparison methods to recognize unique regions of pathogen genomes suitable for identification. Genetic-based rapid assays were developed for all major threat list agents for which adequate genomic sequence is available, as well as for other pathogens requested by various government agencies. The assays were validated by CDC and were used at the 2002 Winter Olympics [52, 53]. The program continues to add new pathogens to expand the diversity of the detection platform.

The Olympic air monitoring utilized 15–20 monitor stations over an area centered on Salt Lake City. Filters were removed on a 4 h basis and tested for genome fragments of bioterrorism pathogens. During the screening period, a sample collected at the city airport was positive by initial screening. The airport was alerted regarding the potential for evacuation, but confirmatory tests were negative [53]; the cause of the false positive test result was not specified.

In 2003, the US Department of Homeland Security expanded this program into BioWatch, a multicity (initially 20) program. From this "early warning" system, there has been one report of a positive assay. The incident originated in Houston, Texas where air filters detected genomic evidence of *F. tularensis*, the cause of tularemia, on air monitoring filters between October 4 and 6, 2003 [54]; subsequent assays were negative. The source of the positive test was not clear but tularemia is endemic in the state. The $60 million/year system was expanded to 31 cities in late 2003. Yet, it has been criticized for being unable to detect small releases of pathogens [55].

In July 2003, the United States Postal Service employed at mail processing centers a high throughput Bio-agent Detection System (BDS) developed by Northrop Grumman Company. The automated system samples air from critical points around the mail sorting machines. The air is drawn through a spinning membrane of chemically enhanced water that removes contaminants. DNA is then sampled by PCR probing methodology developed by Cepheid, Inc. The system is fully automated with run completion in 30 min. If a biological agent is detected, a system alert is generated that that shuts down operations. BDS was developed initially for anthrax, but it is being expanded to include other pathogens and will be adapted for toxins, as well.

The Science Applications International Corporation is developing a biosensor that combines advanced genomic and signal processing techniques to identify all known, newly emergent, and bioengineered pathogens (including all viruses, bacteria, fungi and protozoa). Known as TIGER (triangulation identification for genetic evaluation of risks), the biosensor uses mass spectrometry to determine the mass of core genetic material selectively extracted from a pathogen. TIGER uses specialized algorithms to read a pathogen's genetic signature. The sensor then checks the pathogen's mass against the masses of known pathogens in its database. This system differs from most antibody-based biosensors that cannot detect unknown or bioengineered pathogens.

An antibody-based microarray, which can be fabricated with a wide range of pathogen or toxin-specific antibodies, is a rapidly emerging approach that hold great promise for disease detection proteomics [56]. Similarly, mass spectrometry-based proteomics is emerging as an important tool, which can be used to study protein-protein interactions on a small and proteome-wide scale and generate quantitative protein profiles from diverse species [57, 58]. The ability of mass spectrometry to identify accurately thousands of proteins from complex samples will continue to improve and impact biology and medicine [57].

Direct nucleic acid detection methods may not be sufficiently sensitive to detect pathogens that are either present at low levels in body fluids or tissues. A promising approach to assess host-pathogen interactions at a very early stage of infection is to develop a signature for the host's immunological response. Both PCR and antigen capture assays require the presence of the causative agent. A number of recent studies suggest that there is a pathogen-specific difference in the innate host immune response and that these differences are

detectible by transcriptional profiling with DNA microarrays [59]. In this approach, a molecular immunological signature or bar code-like response would be generated that corresponds to a given pathogen.

In conclusion, rapid advances in diagnostic technology have facilitated real-time multiplex detection of a wide range of human pathogens. A range of platforms are now available in clinical and public health laboratories with the most advanced chip-based detection systems largely confined to academia. Finally, as miniaturization of technology is a rising trend, it is likely that many of the diagnostic platforms will also emerge in deployment of handheld point-of-care devices suitable for rapid detection of agents of bioterrorism or naturally occurring epidemic diseases.

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