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Detection of *Francisella tularensis* within Infected Mouse Tissues by Using a Hand-Held PCR Thermocycler

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The diagnosis of human cases of tularemia often relies upon the demonstration of an antibody response to *Francisella tularensis* or the direct culturing of the bacteria from the patient. Antibody response is not detectable until 2 weeks or more after infection, and culturing requires special media and suspicion of tularemia. In addition, handling live *Francisella* poses a risk to laboratory personnel due to the highly infectious nature of this pathogen. In an effort to develop a rapid diagnostic assay for tularemia, we investigated the use of TaqMan 5′ hydrolysis fluorogenic PCR to detect the organism in tissues of infected mice. Mice were infected to produce respiratory tularemia. The *fopA* and *tul4* genes of *F. tularensis* were amplified from infected spleen, lung, liver, and kidney tissues sampled over a 5-day period. The samples were analyzed using the laboratory-based Applied Biosystems International 7900 and the Smiths Detection-Edgewood BioSeeq, a hand-held portable fluorescence thermocycler designed for use in the field. A comparison of culturing and PCR for detection of bacteria in infected tissues shows that culturing was more sensitive than PCR. However, the results for culture take 72 h, whereas PCR results were available within 4 h. PCR was able to detect infection in all the tissues tested. Lung tissue showed the earliest response at 2 days when tested with the ABI 7900 and in 3 days when tested with the BioSeeq. The results were in agreement between the ABI 7900 and the BioSeeq when presented with the same sample. Template preparation may account for the loss of sensitivity compared to culturing techniques. The hand-held BioSeeq thermocycler shows promise as an expedient means of forward diagnosis of infection in the field.

The potential for use of a biological agent as a weapon has never been greater than it is today. Yet, in many cases, our ability to rapidly diagnose biological warfare agents still relies on classic microbiological and serological methods. *Francisella tularensis* is a putative biological warfare agent and is extremely infectious, with as few as 25 organisms capable of causing disease (1, 2, 13, 19). Tularemia is endemic to many parts of the world, including North America, with documented cases linked to a variety of sources such as ticks, muskrats, water, laboratory handling, and aerosolization during lawn mower use (4, 12, 14, 20, 21). Because *F. tularensis* is fastidious and grows slowly, it can require days to culture. Serology is the most common method used to diagnose tularemia, but a specific antibody response in patient serum is not detectable until 2 weeks or more after infection (1, 13).

The virulence of many of the biological warfare agents generally results in symptomatic disease within hours to a few days, and many of the agents can kill the host shortly after symptoms appear. Development of PCR technology to diagnose these agents from patient samples offers an opportunity to establish a presumptive diagnosis before symptoms are evident or shortly after symptoms appear. Early and rapid diagnosis will allow initiation of agent-specific therapy, offering the best opportunity for recovery of the host (2).

This study evaluated the efficacy of PCR in identifying *F. tularensis* from the liver, lungs, spleen, and kidneys of infected mice. A number of studies identified gene targets within *F. tularensis* affording suitable PCR targets for identification (3, 5, 11, 16, 18). While some studies targeted 16S rRNA or repetitive sequences, others targeted the *tul4* and *fopA* genes to produce gel-based assays for identification of *F. tularensis* from blood, wound scabs, environmental specimens, and tissues of infected animals (3, 7, 8, 10, 11, 17). We developed a TaqMan assay for use on fluorescence-based thermocyclers being used within the biological detection community.

Sensitivity and specificity were determined from these results and compared to those of traditional culturing techniques. Animal challenge groups were dosed with 30-μl volumes of a 1.04×10^4 CFU/ml suspension of *Francisella tularensis* SCHU 4 (total number of organisms was 312 CFU) to simulate an aerosol challenge. Challenge and control groups were euthanatized at 1, 24, 48, 72, and 96 h postchallenge, and the tissues were collected to determine the extent of infection. Each sample was split, with a portion being used for culture, and the remaining tissue was tested by PCR.

The study had two goals. The first was to test two new TaqMan PCR assays directed against different gene targets while determining the bacterial load of *F. tularensis* required in each organ to yield a positive result. The second goal was to compare the BioSeeq, the first of a new generation of portable fluorescence-based PCR thermocyclers entering the commer-

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cial market, with a standard laboratory-based device. The battery-operated BioSeeq was compared to the Applied Biosystems model 7900, a well-established laboratory instrument used in hospitals throughout the world. The efficacy, clinical sensitivity, and specificity of PCR on each of the thermocycler platforms tested were determined by comparison with microbiological culture.

The BioSeeq is a hand-held battery-operated real-time PCR instrument, approximately 12 by 8 by 2 in. in size and weighing approximately 6.5 pounds including batteries. It can be operated using 110 voltage or eight alkaline C batteries that will operate the instrument for approximately 1 h. It contains six independently programmable thermocycler optics modules. The instrument can be operated either in the first responder mode, which allows one to perform assays in the field using preset protocols, or the laboratory mode, which allows one to customize assays and record data using a laptop computer connected to the BioSeeq.

The introduction of hand-held units creates the opportunity to explore the possibility of placing PCR technology into the hands of properly trained and certified first responders for the purpose of presumptive testing. This study is the first step in demonstrating the utility of this unit and determining what the performance of this small field unit may be.

MATERIALS AND METHODS

Infection of mice. Fifty female BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.), ages 6 to 7 weeks and weighing 25 g, were used in this study. The 50 mice were divided equally into a challenge group and a control group that was not exposed to *F. tularensis*. The control and challenge groups both consisted of 25 mice, providing 5 control mice and 5 experimental mice at each of the five time points (1, 24, 48, 72, and 96 h after challenge). Mice were anesthetized, and 30 μl of culture suspension was dripped into the nares. Challenge animals were inoculated with 312 CFU, resulting in respiratory tularemia. The animals were returned to their cages and allowed to recover from anesthesia. To collect samples, animals were euthanized using carbon dioxide vapor. After expiration, the animals were soaked in ethanol and pinned to a dissecting board. The skin was reflected to expose the chest, and blood was collected by cardiac puncture. The abdomen and thoracic cavity were aseptically opened, and the lungs, liver, spleen, and kidneys were removed and placed in sterile 15-ml conical tubes with 1.0 ml of 0.9% saline. The organs were homogenized individually for quantitative culture. The tissue homogenate was diluted 10^{0} through 10^{-3} in sterile saline and cultured on cysteine heart agar (Remel, Lenexa, Kan.). One hundred microliters of each suspension was plated to determine the number of CFU/milliliter of tissue suspension. The plates were incubated at 35°C, non-CO_2, for 72 h before colonies were counted.

DNA preparation. PCR required extraction of DNA from the bacterial cells. The DNA extraction procedure was carried out on the automated Roche MagNA Pure LC instrument (Roche Molecular Biochemicals, Indianapolis, Ind.). Two hundred microliters of homogenized tissue sample was aliquoted into one well of a 32-well cartridge (32 samples per run). After the wells were filled, the cartridge was placed into the MagNA Pure LC. The instrument was run using DNA isolation kit I reagents, and the instrument was set to the DNA I high-performance protocol. DNA was eluted into 100 μl of elution buffer. The eluted DNA was frozen at −20°C until needed for PCR analysis.

Assay design and testing. The TaqMan probe and primer sequences were designed by using Primer Express software (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. The sequence of the probe was selected based on the following criteria: predicted cross-reactivity with currently available GenBank sequences, lack of predicted dimer formation with primers, self-annealing of the oligonucleotide, a 10°C higher melting temperature of the probe than the primers, no stretches of identical nucleotides longer than four, and no guanine at the 5′ end of the probe. *tul4* primers (forward, ATTACAATGGCAGGCTCCAGA; reverse, GCCCAAGTTTTATCGTTCTTCTCA; TaqMan probe, FAM-TTCTAAGTGCCATGATACAAGCTTCCCAATTACTAAGTA-TAMRA) specifically amplified an 89-bp fragment of the *tul4* gene (GenBank accession no. M32059) encoding a 17-kDa lipoprotein (18). *fopA* primers (forward, AACAATGGCACCTAGTAATATTTCTGG; reverse, CCACCAAAGAACCATGTTAAACC; TaqMan probe, FAM-TGGCAGAGCGGGTACTAACATGATTGGT-TAMRA) amplified an 86 bp fragment of the *fopA* gene (GenBank accession no. AF097542) which encodes a 43-kDa outer membrane protein (15). The fluorescent reporter dye at the 5′ end of the probe was 6-carboxy-fluorescein (FAM), and the quencher at the 3′ end was 6-carboxy-tetramethyl-rhodamine (TAMRA). Primer and probe concentrations were determined as a result of optimization experiments (data not shown). In separate experiments, various amounts of primer and probe were tested in a matrix format. Concentrations were determined to be optimal based on which conditions provided the earliest cycle threshold (C_T) and the greatest end point fluorescence. The optimized *fopA* and *tul4* assays were tested against a cross-reactivity panel in order to demonstrate specificity of detection. All DNAs were tested in duplicate in a reaction volume of 25 μl. Cross-reactivity panel DNAs were used at a concentration of 1 ng per well, which is in great excess of the usual 10 pg or less needed to observe a robust fluorescent TaqMan PCR response.

PCR. Amplification, data acquisition, and data analysis were carried out on an Applied Biosystems model 7900 sequence detection system (Applied Biosystems, Foster City, Calif.). PCRs were performed in 50-μl volumes. Each reaction was set up using AmpliTaq Gold Master Mix, which contained AmpliTaq Gold DNA polymerase, AmpErase uracil-*N*-glycosylase (UNG), deoxynucleoside triphosphates with dUTP, ROX passive reference, optimized buffer components (Applied Biosystems, Foster City, Calif.), 400 nM forward and reverse primer, 200 to 250 nM fluorogenic probe, 1.25 U of AmpliTaq Gold DNA polymerase per reaction, and 2 μl of the appropriate mouse DNA per reaction. Reaction plates were incubated at 50°C for 2 min so that UNG could degrade any uracil-containing templates from possible contaminating templates followed by 10 min at 95°C to activate the AmpliTaq Gold polymerase. The reactions were amplified by two-step thermocycling for 45 cycles of 95°C for 15 s each cycle and 60°C for 1 min each cycle. Amplification and data acquisition were carried out in the BioSeeq (Smiths Detection, Edgewood, Md.) in a 25-μl reaction volume. Each sample contained 1.5 U of Platinum *Taq* polymerase (Life Technologies, Gaithersburg, Md.), deoxynucleoside triphosphates, and optimized buffer components, 400 nM forward and reverse primer, 200 to 250 nM fluorogenic probe, and 2 μl of the appropriate mouse organ DNA. Reaction tubes were incubated at 96°C for 90 s to activate the Platinum *Taq* and then amplified for 45 cycles of 96°C and 60°C, each lasting for 10 s per cycle, followed by a final cool-down at 40°C for 30 s.

RESULTS

Validation of the assay's specificity was accomplished by testing against a panel of closely related organisms. Table 1 shows positive detection for *F. tularensis* strains Chataneux, grouse, LVS, SCHU 4 isolates, and *Francisella novicida. F. novicida* is recognized as a human pathogen that has been reclassified as a biogroup of *F. tularensis*. It was expected to produce a positive response with these PCR assays (9). The *fopA* and *tul4* PCR assays specifically recognize all three *Francisella* biogroups: type A tularensis, type B holartica, and Novicida.

Once the *tul4* and *fopA* assays were shown to be specific for *F. tularensis*, they were optimized for performance on the ABI 7900 and the BioSeeq thermocycler platforms. Optimization involved varying the primer ratios, *Taq* enzyme, annealing temperatures, and probe levels in order to get the best performance of the assay on each PCR device.

The limit of detection (LOD) for each of the assays was determined by performing PCR on serial dilutions of target DNA. In order to determine the LOD, a sample was required to rise above threshold and give a positive response for 29 out of 30 samples. The LOD for both the *tul4* assay and the *fopA* assay on the ABI 7900 was 50 fg, which is approximately 25 genome equivalents of *F. tularensis*.

The LOD using purified genomic DNA was slightly higher for each assay when tested on the BioSeeq platform. The *tul4* assay had an LOD of 200 fg on the BioSeeq platform, and the

TABLE 1. Cross-reactivity panel of *fopA* and *tul4* TaqMan PCR assays[a]

Strain or water	Result	
	fopA	*tul4*
Francisella tularensis LVS, biotype B	+	+
Francisella tularensis Chataneux, biotype B	+	+
Francisella novicida	+	+
Francisella tularensis grouse, biotype B	+	+
Francisella tularensis SCHU4, biotype A	+	+
Francisella philomiragia	−	−
Bacillus anthracis vollum	−	−
Brucella suis Thompson bv2	−	−
Brucella melitensis 16M	−	−
Brucella abortus 16C	−	−
Burkholderia pseudomallei	−	−
Burkholderia mallei	−	−
Clostridium botulinum	−	−
Cardiobacterium hominis	−	−
Dichelobacter nodosus	−	−
Enterobacter aerogenes ATCC 13048	−	−
Shigella dysenteriae	−	−
Streptococcus pneumoniae	−	−
Staphylococcus aureus SEA	−	−
Neisseria gonorrhoea ATCC 19424	−	−
Pantoea agglomerans	−	−
Neisseria meningitidis ATCC 13077	−	−
Salmonella enterica serovar Typhi	−	−
Vibrio cholerae EL TOR	−	−
Xanthomonas oryzae ATCC 35933	−	−
Xanthomonas phaseoli ATCC 49119	−	−
Yersinia pestis D27	−	−
Water	−	−

[a] Each assay was tested on the ABI 7900 platform against 1 ng of the purified genomic DNAs listed. Samples were performed in duplicate in a blinded panel. The plus symbol indicates a positive response. Such responses were early and robust, with C_T values ranging from 23.61 to 26.74. A negative response, yielding a C_T value of 45, is indicated by the minus symbol.

TABLE 2. Culturing of tissue homogenates[a]

Tissue	Amt of bacteria (CFU/ml) on day:				
	1	2	3	4	5
Lung	1,500	12,600	819,000	25,700,000	17,000,000
Liver	0	0	2,300	300,000	9,680,000
Spleen	0	0	0	367,000	29,900,000
Kidney	0	0	0	600	4,010,000

[a] Tissues were removed and homogenized in buffer, and a portion was plated on cysteine agar with the remainder being used for DNA extraction. The number of CFU of *F. tularensis* per milliliter of fluid in each of the four target tissues during the time course of the experiment is shown.

fopA assay had an LOD of 300 fg (data not shown). A beta test period which preceded the commercial launch of the product allowed several modifications of the BioSeeq algorithms used to call positive samples. With the new modifications, a positive result is determined when three data points are averaged and three out of four or four out of five consecutive averaged data readings are at least 1.5% greater than the previous signal. In addition, a sample is called positive if, between cycle 1 and cycle 45, there is a 20% increase in fluorescent signal. When comparing the BioSeeq production model with the laboratory based ABI 7900 instrument, the BioSeeq's LODs were approximately four- to six-fold less sensitive than the ABI 7900 using the same assays.

Having demonstrated that the *tul4* and *fopA* assays were both sensitive and specific, we sought to determine their usefulness as diagnostic reagents. To demonstrate this challenge, mice were infected with *F. tularensis* SCHU 4 as described in Materials and Methods in order to induce respiratory tularemia. When the total number of CFU/milliliters was calculated for each of the target tissues, it was not surprising that the bacteria were found within the lungs almost immediately, since the mice were exposed to produce respiratory tularemia through the introduction of bacteria into the nares, where the bacteria rapidly propagated (Table 2). By day 3, the infection within the lungs was significant. *F. tularensis* did not appear in the liver until the third day. The kidney and spleen were the last organs showing infection as detected by culturing. None of the mice, within either the challenge or the control group, died as a result of the infection during the course of the 5-day experiment.

The culture data served as a baseline to facilitate tracking the infection as it intensified during the 5-day period of the study and were used for comparison against subsequent PCR results. The extracted tissues were used as templates for TaqMan PCR using both the *fopA* and the *tul4* assays, and each was run on the ABI 7900 and the Smiths Detection-Edgewood BioSeeq.

The data were scored to indicate samples at day 1 through day 5 for each of the four tissues tested. PCR responses were scored as negative (−) when there was no response after the 44th cycle. If a sample produced a positive response in the run and crossed the established threshold between cycles 24 and 35, it was scored as a three-plus sample (+++). When the threshold was crossed between cycles 35 and 40, the sample was scored as two plus (++), and a C_T between 40 and 44 was scored as a single plus (+), corresponding to a weak positive response. Earlier thresholds for the same PCR assay are indicative of higher starting copy numbers for the target. It was assumed that earlier thresholds were the result of more bacteria colonizing that organ, and the culture data supports that assessment.

Table 3 shows that lung tissue gave the most robust signals throughout the study for both PCR thermocyclers, which was expected since the infection was initiated in the lung. The ABI 7900 was able to detect signal in the lungs on day 2 of the infection using both PCR assays, whereas the BioSeeq gave a response on day 3 for the *fopA* assay and day 4 for the *tul4* assay. A response was seen in the liver and the spleen on day 4, and the kidney tissue DNA demonstrated a response on day 5. There was good agreement between the PCR platforms when the same PCR assay was used, but the assays showed slightly different responses when compared with one another. Assay-to-assay variation is expected since amplification efficiencies and characteristics vary for each primer set. The weak positive responses on the thermocycling units (+) were not always in agreement with the culturing data, as can be seen on day 1 for the ABI response to the liver. Further refinement of the testing procedures in which only thresholds earlier than cycle 40 are used to call a positive would allow greater confidence in the data scoring when comparing PCR and culture data.

TABLE 3. ABI 7900 laboratory-based PCR platform versus BioSeeq portable PCR platform[a]

Tissue and time point	Assay	ABI 7900 result	BioSeeq result
Lung			
Day 1	*fopA*	−	−
Day 2	*fopA*	+	−
Day 3	*fopA*	++	++
Day 4	*fopA*	+++	+++
Day 5	*fopA*	+++	+++
Day 1	*tul4*	−	+
Day 2	*tul4*	+	−
Day 3	*tul4*	++	−
Day 4	*tul4*	+++	+
Day 5	*tul4*	+++	+++
Liver			
Day 1	*fopA*	+	−
Day 2	*fopA*	−	−
Day 3	*fopA*	−	−
Day 4	*fopA*	++	+
Day 5	*fopA*	+++	++
Day 1	*tul4*	+	−
Day 2	*tul4*	−	−
Day 3	*tul4*	−	−
Day 4	*tul4*	+	−
Day 5	*tul4*	++	++
Kidney			
Day 1	*fopA*	−	−
Day 2	*fopA*	+	−
Day 3	*fopA*	−	−
Day 4	*fopA*	−	−
Day 5	*fopA*	++	++
Day 1	*tul4*	−	−
Day 2	*tul4*	−	−
Day 3	*tul4*	−	−
Day 4	*tul4*	−	−
Day 5	*tul4*	++	+
Spleen			
Day 1	*fopA*	−	−
Day 2	*fopA*	−	−
Day 3	*fopA*	−	−
Day 4	*fopA*	+	++
Day 5	*fopA*	+++	+++
Day 1	*tul4*	−	−
Day 2	*tul4*	−	−
Day 3	*tul4*	−	−
Day 4	*tul4*	++	−
Day 5	*tul4*	+++	+++

[a] The C_T values were averaged from the results of five mice for each day. Five negative control mice were run for each time point and tissue, and no false positives were recorded (data not shown). The average point at which the cycle threshold (C_T) was crossed was graded according to the following criteria: A sample with a C_T between cycle 24 and cycle 35 was scored +++, a sample with a C_T between cycle 35 and cycle 40 was scored ++, a sample with a C_T between cycle 40 and cycle 44 was scored +, and a sample with a C_T after cycle 44 was given a score of −. The assay column indicates whether the *fopA* or the *tul4* assay was employed for that sample, and the platform columns show the result for the ABI 7900 and the ETG-Smiths BioSeeq PCR thermocycler.

DISCUSSION

The desire to bring PCR environmental surveillance and detection out of the laboratory has prompted the development of battery-operated hand-held thermocycling platforms compatible with real-time PCR chemistries such as TaqMan. This study represents an effort to compare the newly introduced Smiths Detection-Edgewood BioSeeq with an established laboratory-based instrument and to explore environmental surveillance by targeting pathogen virulence genes.

Traditional agarose-based PCR of *F. tularensis* can be used for determining the ulceroglandular form of tularemia by swabbing the wound of infected patients and extracting the samples using a variety of methods. A 1997 Swedish study compared extraction methods and demonstrated good agreement when PCR was compared with serological confirmation of the disease (17). In that study, the extraction of the pathogen DNA used chaotropic disruption, as did a study by Fulop et al. in 1996 and Junhui et al. in 1996 (6, 11). The use of Whatman FTA paper for spleen and blood samples, carried out in a study by Higgins et al., is a method worth further investigation since the recovery is good and the samples can be preserved for significant periods of time (8).

We presented a study that isolated DNA from infected tissues using a robotic system employing chaotropic disruption and magnetic bead isolation. The data directly compare TaqMan PCR results from two thermal cycling systems with quantitative cultures. Although culturing took 72 h to yield results and PCR could produce results within the same day, PCR was not as sensitive as culturing in detection of the pathogen. A comparison of the data reveals that cultures indicated low levels of bacteria in the liver and spleen a day before the pathogen was detected by PCR. This may be explained in part by the differences in the volume being tested. In the quantitative culture, 100 μl of suspension was plated. In the DNA extraction sample, 200 μl of extracted DNA was eluted into a 100-μl volume and 2 μl was used for PCR. Increasing the volume of the DNA sample and decreasing the water volume may have improved the sensitivity of the PCR.

The LOD data on the *fopA* and *tul4* assays demonstrates that the assays are capable of detecting 25 genome equivalents of purified DNA and should have easily detected the bacterial levels in the liver on day 3 and in the spleen on day 4. Competition of mouse DNA for sites on the magnetic beads appears to have reduced the gene copies of bacterial DNA trapped by the beads. This was an unforeseen problem which may be overcome by adding larger volumes of the DNA preparation to the PCRs.

DNA extraction kits have not addressed the use of mixed DNA sources and selective trapping of one DNA over another, and it may not be possible in a DNA extraction process. The host cell DNA is present in great excess compared to the *Francisella* DNA in the preparations. The PCRs detected *Francisella* DNA in the presence of a 10^6 excess of host DNA (data not shown). Consequently, it is most likely that a collection problem associated with the robotic system is the cause for reduced amounts of bacterial DNA. Since these studies were done, a new extraction kit was released (DNAIII) that appears to be more efficient at lysing and trapping bacterial DNA. In the laboratory, PCR and culturing have comparable sensitivity.

However, in order for PCR to perform as an environmental surveillance tool in the field, more work must be done in the area of DNA sample cleanup and processing. Lessons learned from this study are being funneled into our laboratories' current efforts using high-throughput robotic extraction of environmental samples. However, further investment is warranted if diagnosis is to occur in field medical settings. These field sample preparation units must minimize the logistics associated with sample cleanup and be versatile enough to handle environmental samples for direct introduction into PCR detectors.

The comparison of the ABI 7900 HT and the Smiths Detection-Edgewood BioSeeq provided our first look at the next generation of portable thermocyclers. This study was the result of an 8-month beta test. During this time, software and firmware algorithms were modified, increasing the accuracy of positive and negative responses called on the BioSeeq. As a result, the final software package accompanying the unit, when it is introduced for commercial sale, will be better geared for first responders and technicians with limited experience in performing PCR. Although the software makes interpretation in the field easier for users, mechanisms need to be put in place for calibration and thermocycling upgrades when the units return to the lab after use in the field. Furthermore, the need to analyze empirical data from actual use and the introduction of new assays will require regular upgrades and modifications and will necessitate that firms seeking to market these systems provide a means to download system upgrades without shipping the PCR thermocyclers back to the factory.

The potential use of portable systems with biological organisms requires a plan for system decontamination to minimize personnel exposure after use. First responder assets considering purchasing portable PCR technologies must understand that consumables and PCR assays targeting specific threat agents must be procured. Extensive training on proper handling techniques, storage, and standard operating procedures for PCR must occur simultaneously with the integration of this new detection technology into the operation's doctrine of the unit. In addition, users should be enrolled in a certified training program with annual refresher training and be involved in an active proficiency testing program to ensure that they can document correct use of the instrument.

The BioSeeq is the latest iteration of a trend representing a shift of technology as it moves from the laboratory into the field. It provides another complementary tool in a layered approach for detecting or diagnosing the presence of biological agents. The introduction of these systems could occur rapidly over the next 2 years. The increase in the availability of PCR assays to support their use should be accompanied by a reduction in the price of thermocyclers as the commercial market develops.

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