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Real-Time PCR in Clinical Microbiology: Applications for Routine Laboratory Testing

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INTRODUCTION

Real-time PCR has revolutionized the way clinical microbiology laboratories diagnose human pathogens (25, 71, 73, 294, 456). This testing method combines PCR chemistry with fluorescent probe detection of amplified product in the same reaction vessel. In general, both PCR and amplified product detection are completed in an hour or less, which is considerably faster than conventional PCR and detection methods. Hence, for some time this technology was referred to as rapid-cycle real-time PCR. Other descriptions of real-time PCR in the early literature included homogeneous PCR and kinetic PCR.

Real-time PCR testing platforms provide equivalent sensitivity and specificity as conventional PCR combined with Southern blot analysis. Since the nucleic acid amplification and detection steps are performed in the same closed vessel, the risk for release of amplified nucleic acids into the environment, and contamination of subsequent analyses, is negligent compared with conventional PCR methods. Real-time PCR instrumentation requires considerably less hands-on time and testing is much simpler to perform than conventional PCR methods. Additionally, accelerated PCR thermocycling and detection of amplified product permits the provision of a test result much sooner for real-time PCR than for conventional PCR. The combination of excellent sensitivity and specificity, low contamination risk, ease of performance and speed, has made real-time PCR technology an appealing alternative to conventional culture-based or immunoassay-based testing methods used in the clinical microbiology for diagnosing many infectious diseases. This review focuses on the application of real-time PCR in the clinical microbiology laboratory.

REAL-TIME PCR INSTRUMENTS

Specifications for commercially available real-time PCR instruments, including the nucleic acid probe formats supported, excitation and detection wavelengths, maximum number of samples per run, reaction volumes, and relative thermocycling times are presented in Table 1. The large capacity (≥96-microwell format) instruments, which include the ABI Prism se-

TABLE 1. Instruments available

Manufacturer	Instrument	Probe chemistries supported	Excitation (nm)	Detection (nm)	No. of samples	Reaction volume (μl)	Rapid thermocycling	Comments
Roche Applied Science (http://www.roche-applied-science.com/lightcycler-online/)	LightCycler 1.0	Hybridization probes, molecular beacons, TaqMan	LED470	530, 640, 710	32	10–20	Yes	Special sample containers.
	LightCycler 2.0	Hybridization probes, molecular beacons, TaqMan	LED470	530, 555, 610, 640, 670, 710	32	10–100	Yes	Special sample containers.
Cepheid (http://www.cepheid.com/pages/home.html)	SmartCycler II	Molecular beacons, TaqMan	LED450–495, LED500–550, LED565–590, LED630–650	510–527, 565–590, 606–650, 670–750	16	25–100	Yes	Special sample containers. Independent modules.
Corbett Research (http://www.corbettresearch.com/home.htm)	Rotor-Gene 3000	Molecular beacons, TaqMan	LED470, LED530, LED585, LED635	510, 550, 580, 610, 660	72	10–150 5–50	Midrange	Standard plastic tubes are used.
ABI (http://www.appliedbiosystems.com/index.cfm)	Prism 7000	TaqMan, molecular beacons	Tungsten/halogen	4-color multiplex	96		No	
	Prism 7300	TaqMan, molecular beacons	Tungsten/halogen	3-color multiplex	96		No	
	Prism 7500	TaqMan, molecular beacons	Tungsten/halogen	4-color multiplex	96		No	
	Prism 7900ht	TaqMan, molecular beacons	Laser 488	500–660	384		No	
BioRad (http://www.bio-rad.com/)	MyiQ	TaqMan, molecular beacons	Tungsten/halogen	Single color	96	15–100	No	
	iCycler iQ	TaqMan, molecular beacons, hybridization probes	Tungsten/halogen	4-color multiplex	96	50	No	
Stratagene (http://www.stratagene.com/homepage/)	Mx4000	TaqMan, molecular beacons	Tungsten/halogen	4-color multiplex	96	10–50	No	
	Mx3000p	TaqMan, molecular beacons	Tungsten/halogen	4-color multiplex	96	25	No	
MJ Research (http://www.mjr.com/)	Chromo 4	TaqMan, molecular beacons	450–490, 500–535, 555–585, 620–730	515–530, 560–580, 610–650, 675–730	96	10–100	No	
	Opticon	TaqMan, molecular beacons	450–495	515–545	96	10–100	No	
	Opticon 2	TaqMan, molecular beacons	470–505	525–543, 540–700	96	10–100	No	
Genetic Discovery Technology (http://www.biogene.com/index.cfm)	SynChron	TaqMan, molecular beacons, hybridization probes	473 laser	520–720	6	10–50	Yes	Electrically conducting polymer technology.

ries (7000, 7300, and 7500), the MyiQ and iCycler, Mx4000, MX3000p, Chromo4, Opticon and Opticon 2, and SynChron, may be particularly useful in laboratories with large numbers of specimens. However, thermocycling on these instruments is slower than on other lower capacity instruments, including the LightCycler 1.0, LightCycler 2.0, and SmartCycler II. This is due to the use of a solid-phase material for heat conductance (heating block principle). The large-capacity instruments support high-volume testing while the rapid, lower capacity instruments permit the work flow flexibility that may be especially useful for laboratories that test fewer samples. In summary, work load and work flow issues may dictate which system is best for different-sized laboratories and test volumes.

The Rotor-Gene instrument uses inexpensive standard plastic tubes for the PCR vessel and air for heat transfer with 72 reactions per run. This instrument is intermediate in speed because time is needed for heat conductance to the center of the tubes. The SmartCycler and LightCycler use specialized vessels for rapid heat transfer and can complete a PCR in 30 to 40 min. An additional few minutes are required for the melting curve analysis on the LightCycler.

All the instruments support all or some of the dyes used for TaqMan probes and molecular beacons. Currently, only the LightCycler supports fluorescence resonance energy transfer (FRET) hybridization probe detection with melting curve analysis. Quantitation of target nucleic acid is possible with any of the instruments and supported detection formats.

Recently, analyte-specific reagents (ASRs) and Food and Drug Administration (FDA)-approved kits have become available in the United States for testing on several real-time PCR instruments. The commercial availability of these reagents now make it considerably easier for many clinical microbiology laboratories to adapt real-time PCR testing platforms into their work flow. Because laboratory-developed (also referred to as in-house developed or home brew) real-time PCR tests required considerable expertise to develop and validate, they are generally limited to larger laboratories, especially referral laboratories. The availability of ASRs and kits will also facilitate the development of common testing protocols and standards so that proper comparative clinical studies can be performed and ultimately reliable test results can be ensured for the patient.

In addition to the usual considerations for new instrument purchase (physical space requirement, cost of instrument, disposables, and reagents, instrument maintenance and service, reliability, upgrades, etc.), selection of a real-time PCR instrument and real-time detection format requires consideration of test volume, probe detection requirements, turnaround time for results, personnel requirements, and software. Also, sample preparation requirements must be considered as this can add to the hands-on time per sample, turnaround time, and expense. Several manufacturers have developed semiautomated nucleic acid extraction instruments for use in tandem with real-time PCR instruments. These include the MagNA Pure LC and MagNA Pure Compact for use with the LightCycler instrument, the GeneExpert for use with the SmartCycler II, and the ABI Prism 6700 for use with the ABI Prism series instruments.

REAL-TIME PCR PROBE TECHNOLOGIES

One detection method for nucleic acid detection with real-time PCR uses SYBR Green to detect the accumulation of any double-stranded DNA product. SYBR Green provides sensitive detection but is not specific. The use of SYBR Green with instruments that can perform a melting curve analysis to determine the melting temperature, T_m, permits detection of different amplification products based upon the %G+C content and length of the amplification product. This is similar but not equivalent to agarose gel electrophoresis where the separation is based primarily on length. Because SYBR Green assays are not specific, they are often used for screening assays where further analysis of specimens is performed to confirm the results.

Sensitive and specific detection is possible with real-time PCR by using novel fluorescent probe technology probes. Three types of nucleic acid detection methods have been used most frequently with real-time PCR testing platforms in clinical microbiology: 5′ nuclease (TaqMan probes), molecular beacons, and FRET hybridization probes (Fig. 1). These detection methods all rely on the transfer of light energy between two adjacent dye molecules, a process referred to as fluorescence resonance energy transfer (500). Collectively, these three types of probes are frequently referred to as FRET probes and this general term has been used in some sections of this review. However, when specifically referring to each of these three types of probes, only FRET appears in the name of one, i.e., FRET hybridization probes. Because FRET hybridization probes consist of two separate probes, the term dual FRET hybridization probes has also been used to describe this specific type of nucleic acid detection method.

For all types of FRET probes, as the distance between adjacent dye molecules increases, FRET decreases. For TaqMan probes or molecular beacons, the two dye molecules are attached to a single probe. In contrast, for FRET hybridization probes, dyes are attached separately to two probes that align in a head–to-tail configuration on target nucleic acid DNA. (For purposes of discussion in this review, the nucleic acid which is targeted for an assay is henceforth referred to as target nucleic acid or simply the target.) The first dye is a fluorescent dye, and the second can be either a quencher dye or another fluorescent dye which can absorb fluorescent light transferred from the first dye and reemit light at a different wavelength. The proximity of the two dyes in the probe(s) is determined by the nucleic acid architecture of the probe(s). However, the mechanisms to achieve a fluorescent signal with the TaqMan, molecular beacon, or FRET hybridization probe format are different.

5′ Nuclease (TaqMan) Probes

The first real-time fluorescent probes developed were 5′ nuclease probes, which are commonly referred to by their proprietary name, TaqMan probes (Fig. 1A). A TaqMan probe is a short oligonucleotide (DNA) that contains a 5′ fluorescent dye and 3′ quenching dye. To generate a light signal (i.e., remove the effects of the quenching dye on the fluorescent dye), two events must occur. First, the probe must bind to a complementary strand of DNA at 60°C. Second, at this tem-

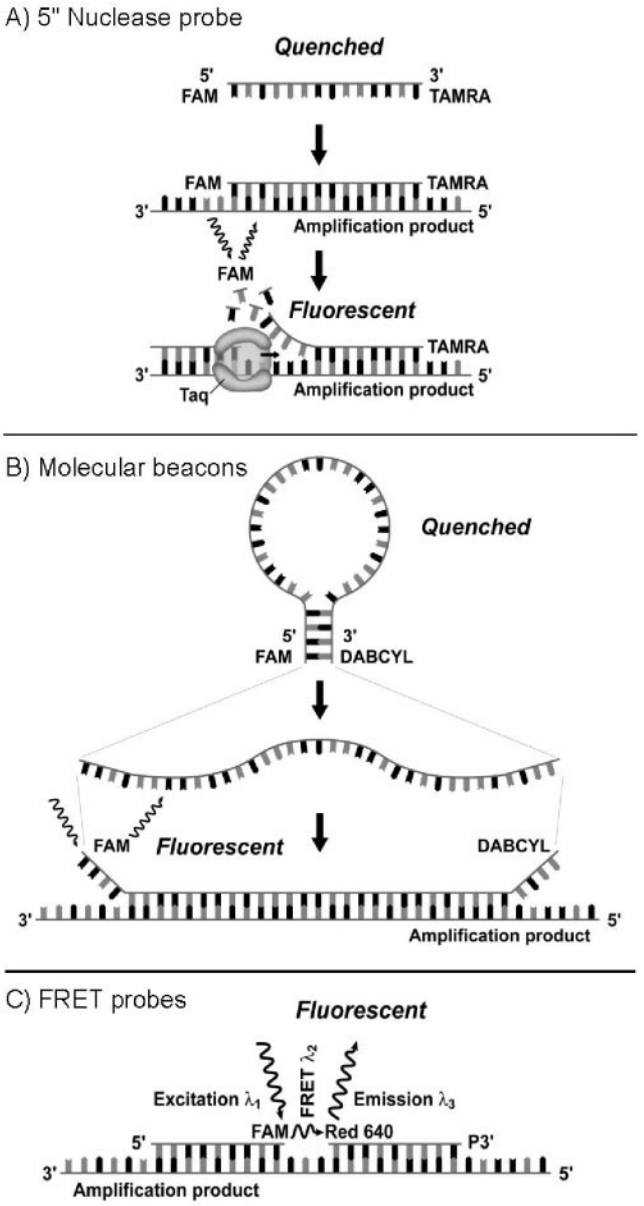

FIG. 1. Real-time probe technologies. (A) 5′ nuclease (TaqMan) probe. (B) Molecular beacon. (C) FRET hybridization probes. (Reprinted from reference 73 with kind permission of Springer Science and Business Media.)

perature, *Taq* polymerase, the same enzyme used for the PCR, must cleave the 5′ end of the TaqMan probe (5′ nuclease activity), separating the fluorescent dye from the quenching dye.

A single TaqMan probe can be used for detection of amplified target DNA. If the intent of the assay is to differentiate a single nucleotide polymorphism from a wild type sequence in the target DNA, then a second probe with the complementary nucleotide(s) to the polymorphism and a fluorescent dye with a different emission spectrum is utilized. Thus, TaqMan probes can be used to detect a specific, predefined polymorphism under the probe in the PCR amplification product. For this application, two reaction vessels are required, one with a complementary probe to detect wild-type target DNA and another for detection of a specific nucleic acid sequence of a mutant strain. Because TaqMan probes require 60°C for efficient 5′ nuclease activity, the PCR is usually cycled between 95 and 60°C for amplification. In addition, the cleaved (free) fluorescent dye accumulates after each PCR temperature cycle, and therefore can be measured at any time during the PCR cycling, including the hybridization step. This is in contrast to molecular beacons and FRET hybridization probes, for which fluorescence can only be measured during the hybridization step.

Molecular Beacons

Molecular beacons are similar to TaqMan probes but are not designed to be cleaved by the 5′ nuclease activity of *Taq* polymerase (Fig. 1B). These probes have a fluorescent dye on the 5′ end and a quencher dye on the 3′ end of the oligonucleotide probe. A region at each end of the molecular beacon probe is designed to be complementary to itself, so at low temperatures, the ends anneal, creating a hairpin structure. This integral annealing property positions the two dyes in close proximity, quenching the fluorescence from the reporter dye. The central region of the probe is designed to be complementary to a region of the PCR amplification product. At high temperatures, both the PCR amplification product and probe are single stranded. As the temperature of the PCR is lowered, the central region of the molecular beacon probe binds to the PCR product and forces the separation of the fluorescent reporter dye from the quenching dye. The effects of the quencher dye are obviated and a light signal from the reporter dye can be detected. If no PCR amplification product is available for binding, the probe reanneals to itself, forcing the reporter dye and quencher dye together, preventing fluorescent signal.

Typically, a single molecular beacon is used for detection of a PCR amplification product and multiple beacon probes with different reporter dyes are used for single nucleotide polymorphism detection. By selection of appropriate PCR temperatures and/or extension of the probe length, molecular beacons will bind to the target PCR product when an unknown nucleotide polymorphism is present but at a slight cost of reduced specificity. There is not a specific temperature thermocycling requirement for molecular beacons, so temperature optimization of the PCR is simplified.

FRET Hybridization Probes

FRET hybridization probes, also referred to as LightCycler probes, represent a third type of probe detection format commonly used with real-time PCR testing platforms (Fig. 1C). FRET hybridization probes are two DNA probes designed to anneal next to each other in a head-to-tail configuration on the PCR product. The upstream probe has a fluorescent dye on the 3′ end and the downstream probe has an acceptor dye on the 5′ end. If both probes anneal to the target PCR product, fluorescence from the 3′ dye is absorbed by the adjacent acceptor dye on the 5′ end of the second probe. The second dye is excited and emits light at a third wavelength and this third wavelength is detected. If the two dyes do not align together because there is no specific DNA for them to bind, then FRET does not occur between the two dyes because the distances between the dyes are too great. A design detail of FRET

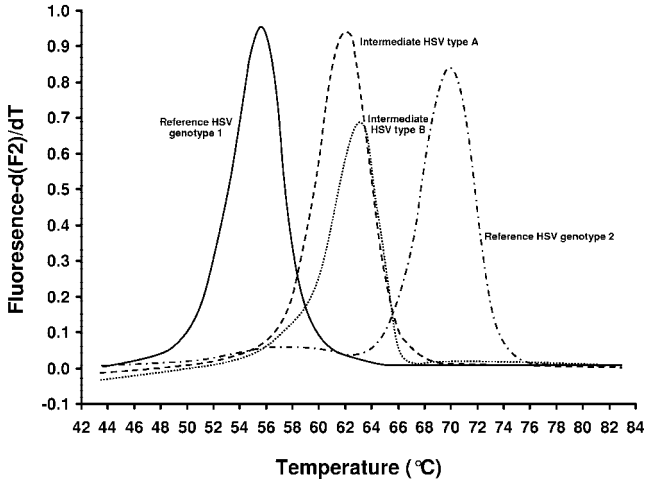

FIG. 2. Melting curves obtained after PCR amplification of HSV DNA.

hybridization probes is the 3′ end of the second (downstream) probe is phosphorylated to prevent it from being used as a primer by *Taq* during PCR amplification. The two probes encompass a region of 40 to 50 DNA base pairs, providing exquisite specificity.

FRET hybridization probe technology permits melting curve analysis of the amplification product. If the temperature is slowly raised, eventually the probes will no longer be able to anneal to the target PCR product and the FRET signal will be lost. The temperature at which half the FRET signal is lost is referred to as the melting temperature of the probe system. The T_m depends on the guanine plus cytosine content and oligonucleotide length. In contrast to TaqMan probes, a single nucleotide polymorphism in the target DNA under a hybridization FRET probe will still generate a signal, but the melting curve will display a lower T_m. The lowered T_m can be characteristic for a specific polymorphism underneath the probes; however, a lowered T_m can also be the result of any sequence difference under the probes. The target PCR product is detected and the altered T_m informs the user there is a difference in the sequence being detected. Generally, more than three base pair differences under a FRET hybridization probe prevent hybridization at typical annealing temperatures and are not detected.

This trait of FRET hybridization probes is advantageous in cases where the genome of the organism is known to mutate at a high frequency, such as with viruses. When a single or limited number (<3) of known polymorphisms occur between two similar targets, FRET hybridization probes can also be used for discriminating strains of organisms. An example of this application is the identification of herpes simplex virus type 1 (HSV-1) and HSV-2 (Fig. 2). Like molecular beacons, there is not a specific thermocycling temperature requirement for FRET hybridization probes. Molecular beacons and FRET hybridization probes, unlike TaqMan probes, are both recycled (conserved) in each round of PCR temperature cycle. Also, for Molecular beacons and FRET hybridization probes, unlike TaqMan probes, fluorescent signal does not accumulate as PCR product accumulates after each PCR cycle.

NUCLEIC ACID EXTRACTION

A critical preanalytical step for real-time PCR assays, as well as any assay in which nucleic acid is analyzed, is nucleic acid extraction. Extraction methods that work for one pathogen in a particular specimen type may not work for another pathogen in another specimen type. For example, herpes simplex virus DNA can be extracted relatively easily from genital swabs (115, 118), whereas extraction of DNA from vancomycin-resistant enterococci in stool samples may be considerably more challenging (451).

A few general comments about extraction of nucleic acid from microorganisms can be made. The thick cell wall of gram-positive bacteria is more difficult to disrupt than the relatively thinner cell wall of gram-negative bacteria. Substances that may inhibit amplification such as heme in blood or bile in stool must be removed. The released nucleic acids should be maintained in an aqueous solution to protect them from degradation. Nucleic acids should be eluted into a small volume in order to maximize detection.

Extraction of clinical specimens can be accomplished either by manual or automated methods. A survey of the literature demonstrates the ability of various commercially available methods to successfully extract a wide variety of specimens for bacterial, viral, and fungal targets (Tables 2 and 3).

Manual Extraction

Phenol-chloroform has been used successfully for the extraction of nucleic acids (290, 396). However, phenol is a caustic and corrosive agent, and its use should be considered a safety hazard by clinical microbiology laboratory. A number of commercial manufacturers have developed manual extraction kits for use by clinical laboratories. Some of the most frequently used manual kits as reported in peer reviewed publications are presented in Table 2. These kits vary as to the method, cost, and time required for extraction (Table 2). This variability permits the flexibility in choosing the kit that best suits the needs of a specific laboratory. Manual extraction kits typically use noncorrosive agents making them safe to use by laboratory personnel. While these kits are generally inexpensive and easy to use, they have several drawbacks.

Processing of samples by manual methods requires multiple manipulations. As the number of samples to be extracted increases, so does the potential for contamination due to increased manipulation. In the United States, Clinical Laboratory Improvement Amendments of 1988 (CLIA) regulations (http://www.cms.hhs.gov/clia/) consider manual extraction high-complexity testing, and therefore this type of testing can only be performed by laboratory personnel with appropriate academic credentials. In order to ensure reproducible results, extensive training is necessary to achieve consistency among laboratory personnel performing manual extraction. Some manual kits use ethanol to precipitate the nucleic acids. If not properly removed, excess ethanol residues can inhibit the PCR (502). Finally, manual extraction is a laborious, time-consuming process which requires the undivided attention of the technologist performing this technique in order to ensure optimal results.

TABLE 2. Manual methods of nucleic acid extraction and purification for rapid real-time PCR assays

Kit (manufacturer)	Technologic principle[a]	Specimen throughput	Specimen type	References[b]
High Pure (Roche Applied Science; www.roche-applied-science.com)	NA capture by glass fiber fleece immobilized in a special plastic filter tube and subjected to centrifugation	24 samples in 1 h	Serum, whole blood, plasma, urine, stool, sterile body fluids, respiratory tract specimens, swabs (genital, dermal)	14, 18, 100, 338, 401, 403
QIAamp (Qiagen; www.qiagen.com)	NA capture by silica gel membrane placed in tube column and subjected to centrifugation or vacuum conditions	24 samples in 1 h for DNA; 24 samples in 1.5 h for RNA	Respiratory tract specimens, plasma, stool, serum, whole blood, urine, sterile body fluids, swabs (nasal, fecal)	18, 19, 36, 120, 147, 148, 184, 193, 198, 210, 212, 214, 225, 226, 234, 235, 238–242, 244, 246, 247, 250, 254, 262, 264, 266, 269, 273, 277, 279, 282, 291, 294, 304, 323, 362, 401, 402, 484, 486, 494, 515, 530, 531
IsoQuick (Orca Research; www.bioexpress.com)	NA is partitioned into an aqueous phase and then precipitated with ethanol and resuspended in water or buffer	24 samples in 1 h for DNA; 24 samples in 2 h for RNA	Plasma, whole blood, stool, respiratory tract specimens, sterile body fluids, swabs (dermal, fecal, genital)	115–118, 120, 184, 213, 256, 259–261, 264–266, 299, 399, 450, 451
IsoCode Stix (Schleichar & Schuell; www.whatman.com)	DNA bound to Matrix and released by simple water and heat elution	Processed individually	Whole blood	561, A, B

[a] NA, nucleic acid.
[b] A, A. Muyombwe, I. Lundgren, L. M. Sloan, J. E. Rosenblatt, P. G. Kremsner, S. Borrmann, and S. Issifou, Program Abstr. 52nd Am. Soc. Trop. Med. Hyg., abstr. 744, 2003; B, J. E. Rosenblatt, A. Muyombwe, L. M. Sloan, P. Petmitr, and S. Looareesuman, Program Abstr. 11th Int. Cong. Infect. Dis., abstr. 14.006, 2004.

Automated Extraction

Automated extraction instruments are manufactured by a number of different companies, and like manual methods vary in method, cost, and time requirements for extraction. Additionally, these instruments vary as to specimen capacity per run and size (footprint) (Table 3). While these systems have not been as widely used as manual methods, a number of studies have reported their utility for extraction of a variety of specimen types (Table 3). Studies which compared manual and automated extraction methods have reported automated extraction to be equivalent and in some instances superior to manual methods (116, 139, 143, 226, 446).

Automated extraction systems have certain inherent advantages over manual methods. Recovery of nucleic acids from automated instruments is consistent and reproducible. Automated extraction systems keep sample manipulation to a minimum, reducing the risk for cross contamination of samples. Many of the instruments are closed systems, further reducing the risk for contamination. Automated systems are typically walk-away systems, and do not require constant attention, which permits personnel to perform other duties. The procedures associated with these instruments could potentially be classified as moderate complexity based on the the Clinical Laboratory Improvement Amendments of 1988 (13). Therefore, laboratory assistants may be able to perform sample extraction with these instruments. Finally once these systems have been validated and proper maintenance procedures are in place, quality control monitoring is less intensive than that required for manual extraction (137).

While the benefits of automated extraction are considerable, there are potential drawbacks. It is most economical when instruments are fully loaded; therefore, a significant number of samples (50 to 100/day) should be processed in order to justify the capital investment that is required for these instruments. The footprints of automated extraction instrumentation may require space that is not currently available in the laboratory. In addition to the cost for equipment, costs for disposables also need to be considered. Some vendors are now manufacturing smaller versions of earlier models of their instruments (Table 3). While these instruments extract significantly fewer samples at a time, they are less expensive and have a smaller footprint than the parent instrument. These smaller versions may be viable options for smaller laboratories which process lower numbers of specimens.

Auxiliary Procedures To Enhance Extraction

Recently, new products have been developed to facilitate the extraction of nucleic acid from clinical samples. Stool transport and recovery buffer (S.T.A.R.; Roche Diagnostics Corporation, Indianapolis, IN) has been used successfully for the extraction of historically challenging specimens such as stool

TABLE 3. Automated systems of nucleic acid extraction and purification for rapid real-time PCR assays

Manufacturer	Technologic principle[a]	Automated platforms	Specimen throughput	Wt (kg) and dimensions (W × D × H, cm)	Specimen type	Literature reference(s)
Roche Applied Science (www.Roche-Applied-Science.com; www.magnapure.com)	NA capture by magnetic silica particles	MagNA Pure LC MagNA Pure Compact	32 samples in 90 min 8 samples in 30 min	151; 90 × 60 × 65 60; 54 × 61 × 57	Serum, plasma, whole blood, stool, respiratory tract specimesns, swabs (dermal, genital)	114, 116, 139, 185, 208, 210, 211, 213, 215, 223, 226, 245, 253, 255, 270, 287, 310, 506
Qiagen (www.qiagen.com)	NA capture by magnetic silica particles	BioRobot EZ1 BioRobot M48 BioRobot M96 BioRobot 9604 BioRobot MDx	6 samples in 20 min 48 samples in 159 min 96 samples in 221 min 96 samples in 2 h 96 samples in 2.5 h	26; 28.2 × 45.5 × 43 130; 107 × 60 × 87.5 130; 107 × 65 × 77.9 95; 120 × 71 × 60 140; 145 × 82 × 81	Swabs (dermal, genital, nasal)	116, 226
Applied Biosystems (www.appliedbiosystems.com)	NA capture by silica fiber membrane under vacuum conditions	ABI Prism 6100 ABI Prism 6700	96 samples in 30 min 96 samples in 75 min; 4 microtiter plates in 3.5 h	20; 53 × 48 × 27 227; 142 × 84 × 99		208, 446
bioMérieux (www.biomerieux-usa.com)	NA capture by silica bound to filter under vacuum conditions	NucliSens Extractor	10 samples in 35–45 min	90; Cylindrical (66 diam. × 79 ht.)	Plasma	143

[a] NA, nucleic acid.

FIG. 3. S.E.T.S. The inner tube will hold standard-sized swabs (approximately 5 to 8 mm in diameter, 10 of 25 mm in length) during spinning procedures. The collection tube is used for collection and storage of liquid from the swab. The screw cap is for tightly closing the collection tube.

(451). S.T.A.R. buffer has three important properties: infectious organisms are inactivated, degradation of nucleic acids is minimized, and the binding of the nucleic acids to magnetic beads, as is used in the extraction process of MagNA Pure (Roche Diagnostics Corporation), is enhanced.

The swab extraction tube system (S.E.T.S.; Roche Diagnostics Corporation) kit, shown in Fig. 3, is a simple method for rapidly and efficiently recovering specimen attached to and absorbed into the fibers of a collection swab. For some organisms, studies have demonstrated that specimen which is retrieved in a microcentrifuge tube by the S.E.T.S. method, can be directly, or after a quick lysis step (boiling), analyzed by a LightCycler real-time PCR instrument (195, 499). Alternatively the centrifuged material can be extracted by the MagNA Pure instrument to obtain a cleaner preparation of nucleic acids.

IsoCode Stix (Schleicher and Schuell, Keene, NH), a method for stabilizing blood samples to be transported long distances, can be used to preserve samples for later testing by real-time PCR. This specimen transport device has been coupled with real-time PCR assays for the detection of bloodborne parasites such as malaria (561). This method is not recommended for use with RNA assays.

REAL-TIME PCR ASSAY DEVELOPMENT

Target Nucleic Acid Selection

The target primer sequences must be unique in order to identify a specific organism or an organism group, (e.g., group

A streptococcus or *Mycobacterium* genus screen), quantitate a microbe (e.g., cytomegalovirus), or identify unique virulence genes (e.g., verotoxin genes) or genes or mutations associated with antimicrobial resistance (e.g., *mecA* gene or mutations in *rpoB* gene associated with rifampin resistance) which can occur across strains or species. Moreover, the PCR primer must be able to identify with high efficiency and specificity the target primer sequences in the specimen of interest (e.g., stool or perianal swab specimens for vancomycin-resistant enterococci). A search for the intended primer sequence in a DNA database such as the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/) may reveal cross-reactivity. However, since the databases currently available represent only a small portion of the nucleic acid sequences for microbes in complex specimen matrices such as stool, specimens and related organisms must also be tested to confirm the lack of cross-reactivity. The target nucleic acid sequence should also be conserved in the organism to be identified or quantitated. If sequence data of the intended target area shows a significant frequency of polymorphisms a more conserved site should be chosen.

PCR Primer and Probe Design

PCR primers provide the first level of specificity for the PCR assay, and primers that only amplify one product will provide the best assay sensitivity. Since real-time PCR also incorporates highly specific homogeneous probe detection, the annealing temperature for probes can be several degrees below the melting temperature of the primers. PCR primers should have a low potential to form secondary structures, including self and crosshybridization with other oligonucleotides in the PCR. This becomes increasingly more difficult as more oligonucleotides are added to the reaction. Details for design of primers and probes are beyond the scope of this review and have been described extensively in two recent publications (191, 500).

Assay Optimization

Optimization of assay conditions can be more challenging for conventional PCR. Due to the numerous manual steps and time requirements for conventional PCR, the assessment of different testing parameters is a painstaking process. For example, several days were frequently required to evaluate the effects of changing a single parameter (e.g., optimal magnesium concentration). Because real-time PCR is more automated and has a shorter test turnaround time, optimization experiments can be performed within hours instead of days.

For real-time PCR a few key components should be optimized in order to achieve maximum results (17, 35, 228, 483). These factors include magnesium concentration, which allows the polymerase enzyme to function at an optimal level; primer and probe concentrations, which affect the sensitivity and specificity of the assay respectively; and the use of additives such as dimethyl sulfoxide, which can aid in the denaturation of nucleic acids with high G+C. The type of polymerase enzyme utilized can also play a significant role, polymerases which permit hot-start PCR are preferrable. These enzymes do not function until a critical maximum temperature is reached, which reduces the generation of nonspecific sequence fragments.

BIOSAFETY CONSIDERATIONS

Clinical microbiology laboratories receive and process a wide variety of specimens, including urine, stool, whole blood, plasma, sputum, and swab materials. These specimens may contain a number of transmissible infectious agents including hepatitis viruses and human immunodeficiency virus (HIV). As molecular testing becomes more commonplace, the question of at what point during the extraction process are specimens rendered noninfectious arises. Many extraction kits contain guanidinium salts as one of their compoenents. Studies have shown that guanidinium salts will disrupt cellular integrity and neutralize inhibitory substances (66). However, there are no published studies that demonstrate treatment with guanidinium will ensure that specimens are not infectious.

The MagNA Pure mixes a guanidinium isothiocyanate-containing lysis solution with the sample and incubates it at room temperature for 2 min. We have found (unpublished observations) that this treatment renders 10^8 *Staphylococcus aureus*/ml nonviable. However, further studies are required to determine if guanidinium has the same effect on other infectious agents. Until these studies are completed individuals using real-time PCR in clinical laboratories should practice universal precautions, i.e., treating all specimens as if they were infectious (10).

In the past, infectious agents, such as anthrax, have been weaponized for use in biological warfare. The intentional release of anthrax spores in the U.S. mail system in the fall of 2001 emphasized the urgent need for rapid and safe laboratory techniques for detecting *Bacillus anthracis* in suspicious powders as well as human specimens (179, 289, 350). The Centers for Disease Control and Prevention (CDC) has issued guidelines for the processing and testing of specimens obtained from a suspected outbreak of bioterrorism, in order to protect first-line workers (direct healthcare providers and laboratory workers) (10). In the case of a smallpox outbreak, rapid and accurate laboratory detection is critical in order to quickly contain the infection, however, this may be difficult as smallpox is a level 4 organism, and as such, must be tested at institutions with specialized biosafety level 4 containment facilities (i.e., CDC or United States Army Medical Research Institute of Infectious Diseases).

Autoclaving has been shown to be an effective way to inactivate potential agents of bioterrorism, while permitting the nucleic acid to remain intact for analysis by PCR assays (119, 125, 179, 289, 350). The authors of these publications demonstrated that autoclaving anthrax spores and vaccinia virus, a close relative of smallpox virus, destroyed their ability to be infectious, while not affecting the integrity of their nucleic acid so it could be detected by PCR techniques.

As indicated in the preceding discussion, S.T.A.R. buffer (Roche Diagnostics Corporation) not only stabilizes nucleic acid during transport at room temperatures, but can inactivate pathogens. We have observed that S.T.A.R. buffer can inactivate many bacteria, including such pathogens as *Mycobacterium tuberculosis* and *Escherichia coli* OH157:H7 isolated from culture, or present in complex matrices such as respiratory and stool specimens without damaging the integrity of the DNA (unpublished data). The pathogen-inactivating properties of S.T.A.R. buffer provides laboratories an added level of safety

TABLE 4. Verification guidelines

Verification	FDA cleared	Modified–FDA, ASR, laboratory developed	Verification requirements[a]
Accuracy	20 positive samples, 50 negative samples	50 positive samples, 100 negative samples	90% agreement with reference method
Precision, qualitative	1 control/day for 20 days or duplicate controls for 10 days	Same as FDA-approved assay	Mean, SD, and C.V.
Precision, quantitative	20 data points at 2 to 3 concentrations. Within run, within day, day-to-day	Same as FDA-approved assay	Mean, SD, and C.V. for each concentration
Analytical sensitivity		Analyze 15–20 low or no concentration specimens	Determine mean and SD; SD × 2 = detection limit
Analytical specificity		Evaluate all interfering compounds (i.e., same chemical or genetic structure, same source)	
Reportable range, quantitative	3–5 concentrations measured in triplicate	3–5 concentrations measured in triplicate	Determine upper and lower limits of linearity
Normal values	Minimum of 20 per category, 100 recommended	Minimum of 20 per category, 100 recommended	

[a] C.V., coefficient of variation.

for processing and transporting pathogens for nucleic acid analysis.

QUALITY CONTROL AND QUALITY ASSURANCE

Verification and Validation

Clinical relevance, cost, instrumentation, and ease of performance should be considered when evaluating a new test procedure (109), but of primary importance is the verification and validation of test performance. The ability of a laboratory test to consistently produce accurate and precise results is not only essential, it is the core of quality assurance programs for clinical laboratories (302). A detailed protocol for the verification of new test methods should be established by the laboratory prior to the verification procedure. Table 4 provides guidelines for verification of new test methods (333). Additionally, documentation of validation is necessary to demonstrate that a verified test continues to perform according to the laboratory's requirements. These procedures help ensure the consistency of the results and that laboratory personnel remain competent to perform tests and report results.

Guidelines developed by regulatory agencies are not current for real-time PCR applications in clinical microbiology. The Clinical Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) published a set of guidelines for molecular diagnostic methods in infectious disease testing in 1995; however, these guidelines were provided before the introduction of real-time PCR technology (332). These are considered guidelines, not standards, for infectious disease testing, and currently are undergoing revision.

The most recent document addressing quality control standards for molecular test systems is the revised CLIA 1988 document published in the *Federal Register*, 24 January 2003 (4). This document addresses requirements for certain quality control provisions and personnel qualifications. It combines and reorganizes requirements for test management, quality control and quality assurance, and also changes the requisite consensus for grading proficiency testing challenges. The CLIA 88 document stipulates that prior to test implementation, clinical laboratories verify the manufacturer's performance specifications and confirm they can be replicated by laboratory personnel when following the procedure. For laboratory developed tests or modification of test systems, laboratories are required to establish their own performance specifications prior to implementation of the new or modified test.

Because nucleic acid test methods are changing and evolving so rapidly, existing guidelines have been difficult to apply. The challenges to clinical laboratories include determining the type of verification experiments required for a real-time PCR assay and an acceptable number and type of specimens to evaluate. Providing a single set of guidelines for real-time PCR which envelops all the necessary verification and validation by all accreditation agencies would be of great benefit to laboratories acquiring this new technology. Along with the need for a well defined quality control program for real-time PCR qualitative assays, there is need for guidelines for quantitative real-time PCR assays. To date such guidelines only exist for a select number of blood-borne viruses (341).

Quality control allows the laboratory to minimize the reporting of inaccurate results, to report results with a high degree of confidence and to decrease costs by detecting errors prior to reporting results (137). One goal of the laboratory quality control program is to reduce the number of controls needed for reporting acceptable results. The following information relates to specific controls used during testing as well as the quality control of reagents used for testing. This discussion is not intended to be all-inclusive nor definitive, and is based to some extent on experience at Mayo Clinic with real-time PCR and our interpretation of published guidelines for generic molecular testing.

Positive and Negative Controls

Ideally, patient specimens containing the target nucleic acid are used as the positive control, but this is often not practical or feasible. An acceptable positive control is pooled negative

TABLE 5. Positive and negative control recommendations for molecular testing

Assay	No. of controls			
	2003 Revision of CLIA 88		NCCLS MM3-A[a]	
	Positive	Negative	Positive	Negative
Qualitative	1	1	1	1
Quantitative	2	1	2	1
Nucleic acid extraction	1	1	NR	NR

[a] NR, no recommendation.

specimens spiked with whole organisms or if that is not available, a representative sample of the nucleic acid to be detected. The positive control should be at a concentration near the lower limit of detection of the assay to challenge the detection system yet at a high enough level to provide consistent positive results.

A blank control such as water or buffer is often used as a negative control. However, an optimal negative control is a sample containing nontarget nucleic acid to demonstrate that nonspecific PCR amplification and detection of amplified product is not occurring. In addition, the negative control is used to demonstrate that the reagents are not contaminated with target nucleic acid and can be used to compensate for background signal generated by the reagents. The recommendation for a negative control every fifth tube to monitor PCR contamination (332) may be excessive with real-time PCR assays. The closed system for amplification and detection used with real-time PCR virtually eliminates the amplicon contamination caused by the opening and closing of reaction vessels which is problematic with conventional PCR and detection methods. Even with the closed system of real-time PCR, the laboratory may still choose to add uracil-*N*-glycosylase to the PCR mix for another level of amplicon contamination control.

The 2003 revisions to CLIA 88 rule does not specifically address real-time PCR assays but recommendations from the molecular testing sections can be applied to real-time PCR assays. Table 5 summarizes the 2003 revised CLIA 88 document (4) and CLSI (332) quality control recommendations. In both of these documents it is recommended that each molecular amplification run of samples contain positive and negative controls. Additionally, in the CLIA document it is indicated that a test system which includes nucleic extraction also include a positive and negative control, with the positive control capable of detecting errors in the nucleic acid extraction procedure. For a quantitative assay, two positive controls representing two different concentrations of target nucleic acid are recommended in both the CLIA 88 and CLSI documents. Laboratories should establish the number and frequency of controls based on manufacturers criteria and agency recommendations.

Internal and Inhibition Controls

An acceptable specimen should be free of inhibitory substances that could produce a false-negative result. Some clinical samples may contain substances which are not always removed by the extraction process and which may inhibit the PCR amplification. Inhibition of amplification can be detected by the introduction of an internal control, also referred to as a recovery template.

Based on the requirements of regulatory or accrediting agencies, individual laboratories should determine when an internal control is required in an assay. For example, the 2003 revision to CLIA 88 document does not address internal controls nor have a requirement for assessment of inhibition of PCR chemistry. In contrast, the College of American Pathologist (CAP) molecular pathology inspection checklist indicates that the laboratory must determine the likelihood of the generated result being a false-negative result due to inhibition when there is no amplification of product (76). If the test is performed according to manufacturer instructions, published data containing the inhibition rate may be used for documentation. Internal controls for laboratory developed assays or modified FDA assays should be determined on a case-by-case basis taking into account the probability that the specimen source contains inhibitory substances. Specimen types such as stool or sputum are generally more inhibitory to PCR chemistry than serum or plasma specimens. Also, the assay performance characteristics (sensitivity, specificity, accuracy, etc.), the implications of a false-negative result and the degree to which a clinical diagnosis depends on laboratory results, require consideration. Internal controls may be naturally present in the original specimen, added to the specimen prior to extraction, or added to the PCR reagent mix before amplification. The simplest way to establish inhibition is to add target nucleic acid to a portion of the sample and perform the test to show that if target nucleic acid were present, the PCR would have been positive. Unfortunately, this approach increases the cost of the assay.

Examples of the different types of internal controls that have been used for real-time PCR assays are shown in Table 6. Homologous and heterologous internal controls are those which do not naturally occur within the specimen source. These have also been referred to as exogenous controls as they must be added to the specimen. Homologous controls are

TABLE 6. Examples of internal controls used in real-time PCR assays

Control	Gene targets[a]	References
Housekeeping genes	Albumin, β-globin, 18S and 28S rRNA, β- and λ-actins, cyclophilins, GAPDH, α- and β-tubulins, hypoxanthine phosphoribosyltransferase or L32	257, 326, 489
Exogenous		
Heterologous control	Neomycin phosphotransferase gene, phocine herpesvirus, bacteriophage λ DNA	432, 466, 468, 485, 516
Homologous control	Control contains the same flanking nucleic acid sequence at the target nucleic acid to which the PCR primers anneal, but a different internal sequence to which a probe anneals	108, 257, 432, 501

[a] GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

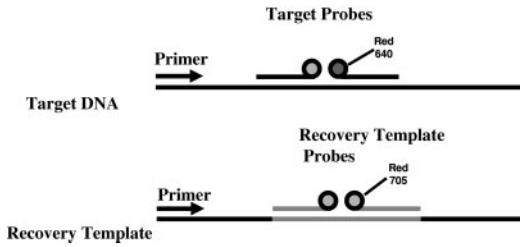

FIG. 4. Recovery template. The recovery template (internal control) has the same sequence as the PCR product except the probe region has been replaced with a sequence complementary to recovery template probes. FRET detection of the target DNA is with a probe labeled with the Red 640 dye in channel 2 of the LightCycler while the recovery template is detected with a probe labeled with the Red 705 dye in channel 3. A small amount of recovery template is added to the PCR and is amplified along with the target DNA by the same primers. Thus, the two reactions compete for the primers. Normally, the recovery template is amplified in all samples, including the negative control. If neither the recovery template nor target DNA is amplified, then it is assumed that inhibition of the PCR has occurred and the test for that sample is not valid. However, if target DNA is amplified but the recovery DNA template is not amplified, then it is assumed that the target DNA is present in a proportionally greater amount. In this situation, partial inhibition of the PCR may be present but the target DNA is successfully amplified or the recovery template may not be able to compete for primers and the recovery template signal may be weak or not present. When this occurs, the positive result is valid because the recovery template amplification result is unnecessary.

coamplified with target DNA using the same PCR primers. However, the internal sequence of the homologous control DNA internal to the PCR primer sites is genetically engineered to be different from the target DNA such that a different product signal occurs with FRET detection.

An example of a homologous internal control is shown in Fig. 4. Heterologous controls consist of separate amplifiable targets. Since these do not contain the target sequence, a separate set of PCR primers and probes are required for amplification and detection respectively. Housekeeping genes occur naturally within the specimen being tested and therefore are referred to as endogenous controls (341). The housekeeping genes occur in all human nucleated cell types and therefore these types of controls are commonly used in human genetic studies. There is no single housekeeping gene that is suitable for all experimental conditions and articles have been published on the variability of certain housekeeping genes in different systems (326, 498).

Real-time PCR assays used in microbiology require optimal sensitivity and the use of internal controls should not decrease the sensitivity of the assay. Performing competitive assays by amplifying serial dilutions of the target DNA with and without the internal control should reveal if the sensitivity of the assay is affected (501). Generally, procedures for synthesis of homolgous internal controls are too complex for the clinical microbiology laboratory (55). A number of manufacturers of real-time PCR ASRs and kits are including homologous internal controls for their assays, which obviates this cumbersome task for the novice user of real-time PCR.

Reagents

The quality control of reagents is extremely important to ensure the success of real-time assays (56). Frequently, commercially available master mix components that contain standardized concentrations of reagents are available, but these do not always include PCR primers and FRET probes.

Whether PCR primers are purchased from vendors or laboratory developed, some method of chromatographic purification should be applied. Purification recovers oligonucleotides of the correct length. Truncated oligonucleotides can affect a PCR by consuming reaction reagents and forming nonspecific amplification products (159). The presence of these irregular oligonucleotides can also falsely elevate the final concentration of the working primers thus affecting the performance of the assay. The CLSI MM3 guideline recommends that laboratories obtain a certificate of analysis from PCR primer vendors. These certificates may contain sequence data, base composition, molecular weight of the sequence, concentration and method of purification (332). Vendors may provide PCR primer concentration, but these concentrations should be verified in the laboratory. Laboratories synthesizing their own oligonucleotide PCR primers should perform chromatographic purification and determine also PCR primer concentration. In compliance with the CAP Molecular Pathology inspection checklist, each new lot of reagent should be tested in parallel with the old reagent lot using both positive and negative patient samples ensuring the same results are obtained with both reagent lots (76).

Purification of FRET probes is especially important to separate both dye and oligonucleotides that have not coupled to form the FRET probes and to remove oligonucleotides with an incorrect length (554). While the quality of probe synthesis has improved greatly over the past few years, quality control is required to avoid probe lots with reduced performance. The CLSI MM3 guideline for PCR primers discussed above should also apply to FRET probes. Some vendors provide a tracing (chromatogram, polyacrylamide gel electrophoresis analysis, etc.) of the purified FRET probe as part of their quality control documentation which may augment quality control. Another quality control service provided at a nominal charge by some vendors is to determine if a particular FRET hybridization probe set is capable of producing FRET. The company will design and synthesize an oligonucleotide complementary to both probes and a melting curve analysis is performed. The production of a melting peak at the predicted T_m will confirm that the FRET hybridization probe set is capable of producing FRET and is acceptable to use. This probe validation process can also be completed in the laboratory by following the method provided on the Idaho Technology website (http://www.idahotech.com) under probe classroom.

Proper storage of reagents can result in an increase in shelf life. FRET probes may arrive in a lyophilized form and the recommendation is to store them at room temperature until resuspended. Some manufacturers state that the hydrated probes should be stored at 4°C for daily use or aliquoted into smaller volumes and stored at −20°C. Numerous freeze-thaw cycles can be detrimental to the FRET probes. The PCR primers can be stored in a similar manner as the probes. The completed master mix (containing primers and probes) can be stored at −20°C for extended periods of time, without degradation of the mix (452). We have also found this to be true with most of our real-time PCR assay master mix reagents used at the Mayo Clinic. The complete mix is stored at either 4°C or

−20°C (assay dependent) for 1 to 6 months without loss of activity. However, we have observed that the length and temperature of storage are assay dependent and conditions of storage require validation for each assay. The advantage of freezing the master mix is assay reproducibility, time savings in setting up assays, and reduced reagent contamination (452).

Quality Assurance

After implementation of the real-time PCR test it is necessary to continue to monitor performance of the assay, equipment, reagents, and personnel. For example, technologists monitor patient specimen positivity rates for all real-time PCR assays used in our institution on a weekly or monthly basis. If a sudden increase in positivity rate occurs, this could reflect seasonal variances of disease frequency (e.g., influenza virus or group A streptococcus), disease outbreak, or specimen-to-specimen or amplification product contamination. Daily quality control of reagents including positive and negative controls and/or extraction controls should be performed. In compliance with accrediting and regulatory agencies, comparable performance of new reagent lots compared with old reagent lots should be verified. Instrument performance should be assessed biannually when multiple instruments are used interchangeably, also as required by accrediting and regulatory agencies. Competency of personnel performing tests must also be evaluated. Examples of competency assessment are included under the Personnel Requirements section below.

Contamination

The risk of contamination is considerably less with real-time PCR compared to conventional PCR, but still can occur (341). Since real-time PCR amplification is performed in a closed system, there is no need for individual air-controlled rooms as is recommended for conventional PCR. In our experience with real-time PCR, specimen to specimen contamination has become a greater challenge than amplified product contamination. The most obvious situation where specimen-to-specimen contamination can occur is with the transfer of specimen to the PCR vessel or to the DNA extraction tube. Care must be taken to avoid contamination of the pipette device with specimen and to avoid the creation of an aerosol by blowing out the specimen from the tip.

Certain types of sample sources are known to contain a high concentration of organisms that may lead to specimen-to-specimen contamination, namely, viral agents. The inclusion of negative controls and continual trend analysis of the assay are used to recognize a contamination event. Additionally, unidirectional work flow should be followed. Separation of procedural steps will require separate work spaces in the laboratory, as detailed below under the ssection on facilities requirements. As with all methods performed in the laboratory, good laboratory practice is critical for accurate results.

IMPLEMENTATION OF REAL-TIME PCR TESTING IN THE CLINICAL MICROBIOLOGY LABORATORY

Implementation of real-time PCR testing platforms in the clinical microbiology laboratory requires careful consideration of facility requirements, personnel requirements, and work flow design. These considerations are similar to those required for implementation of any new type of testing method. A review of these requirements related to our experience at Mayo Clinic with implementing LightCycler technology into the clinical microbiology laboratory is provided in the following discussion. At the Mayo Clinic, some of our real-time PCR assays have been used routinely in the clinical laboratory since early 2000.

Facilities Requirements

As previously mentioned, a physical separation of processes, equipment, and reagents is recommended, to minimize the risk of specimen-to-specimen contamination. Four different work areas are suggested, including a reagent preparation area to prepare PCR master mix, a sample processing area where procedures, including nucleic acid extraction, occurs, a target loading area where the specimen is added to the PCR master mix in the reaction vessel, and an amplification area where thermocycling and probe detection occurs.

The reagent preparation area should be kept free of all patient specimens and DNA extracts. Protocols for the sample preparation area should minimize the number of tubes that are simultaneously open. Each of the work areas should contain dedicated working materials, reagents, and pipetting devices. Reagents should be prepared and aliquoted into single use or small volumes. This ensures ease of use and less chance for contamination.

All working surfaces should be cleaned before and after use, preferably with a reagent that destroys nucleic acid such as a 5% bleach solution. The manufacturer's recommendations should be followed for cleaning of instrument components (e.g., carousels with the LightCycler), processing blocks, and other instrument surfaces and parts.

Gloves should be changed frequently, at least before beginning each of the separate tasks required in a dedicated work area and should always be changed if moving from one work area to another work area. The use of aerosol-resistant pipette tips and pipette tips long enough to prevent specmien contact with the pipetter aids in the prevention of specimen contamination (502). Enzyme contamination control systems such as uracil-*N*-glycosylase can be incorporated into the real-time PCR master mix as an added safeguard to sterilize amplified product that may be carried over to subsequent batches of tests.

Personnel Requirements

Personnel should be trained in both the preanalytical (specimen processing and extraction) and the analytical procedures. Many current laboratory professionals do not have training or experience in molecular methods and also lack theoretical knowledge of molecular microbiology. Based on our experience at the Mayo Clinic, providing a variety of methods for attaining this knowledge is useful. Some vendors are willing to provide overview presentations on molecular biology as well as technical information on their specific testing platform. Appreciation of the fundamentals will help to avoid cookbook

testing and will later allow more careful and focused troubleshooting.

Clinical microbiologists who have not had formal training in molecular microbiology still possess many of the critical skills necessary for success in performing real-time PCR testing. Especially important is meticulous attention to detail, strict adherence to standard operating procedures, and use of aseptic technique.

These skills are easily transferable from culture based conventional microbiologic testing to real-time PCR testing. At the Mayo Clinic we noted that providing training on the basics of accurate pipetting was fundamental, especially for those lacking experience with micropipetting.

Well-written training materials, including training checklists and detailed standard operating procedures for each real-time PCR test, should be available. The training checklist serves to standardize the training of all personnel. At the Mayo Clinic, we believe that identifying a technical expert to provide one-on-one training for real-time PCR is critical. Technologists are required to successfully complete a panel of unknown samples and perform the procedure under direct observation of the technical expert to ensure flawless manipulations throughout the procedure. They also are required to analyze a previous run of samples with a variety of unusual results. This allows them to perfect their skills manipulating the computer software associated with the real-time PCR instrument and ensures consistent analysis and reporting of results. Overall, our technologists have been very enthusiastic about implementation of real-time PCR and excited to learn the new technology.

The availability of resources for troubleshooting is a consideration when selecting a molecular platform for the clinical laboratory. Laboratory-developed tests require that the technical resources to resolve problems related to the assay are available within the laboratory. Use of ASRs and United States Food and Drug Administration-approved tests allow the use of technical support resources available from the manufacturer for troubleshooting problems related to the assay or instrumentation.

Work Flow Design

After selection and successful implementation of a real-time PCR testing platform into the clinical laboratory, efficiencies may be gained by the implementation of additional tests which use the same methodology. Different real-time PCR tests may have subtle variations (e.g., differences in nucleic acid extraction procedures), but overall the methodology is very similar. This attribute reduces the personnel resources required for training and implementation of subsequent tests.

Not unlike other microbiology tests, work flow and testing schedules for real-time PCR tests are determined by the arrival times of specimens into the laboratory, clinical urgency for the results, and laboratory hours of operation. Many of the real-time PCR platforms are most efficiently run in a batch mode. Some vendors provide identical thermocycling protocols for different ASRs for the same instrument. This allows testing for multiple analytes within the same run, which enhances the efficiency of the testing platform.

Example of work flow design: real-time PCR for detection of group A streptococci from throat swabs. At the Mayo Clinic in August 2002 we replaced a conventional testing method (rapid antigen screen with backup culture for rapid antigen negative results) with the LightCycler Strep-A assay (Roche Diagnostics Corporation, Indianapolis, IN) (456, 499). This real-time PCR test is as sensitive as the gold standard method, culture, and provides same-day conclusive results for all patients, whereas the antigen/backup culture method required up to 48 h for a conclusive result for the majority of patients. A simple lysis and extraction method of the swab sample is performed using the S.E.T.S. tube (Roche Diagnostics Corporation) before testing in the LightCycler (Fig. 5).

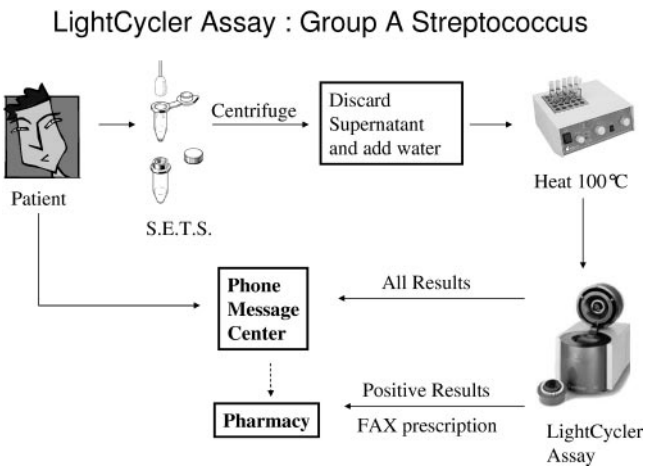

FIG. 5. Work flow algorithm for processing specimens for the laboratory diagnosis of group A streptococcal infections by real-time PCR.

Prior to implementing this real-time PCR assay we worked extensively with our clinical colleagues to review the performance characteristics of this novel testing method, prepare educational materials for patients, determine an appropriate testing schedule, and clarify what health care providers and patients should expect. One misconception of our healthcare providers that we had to clarify was that a majority of patients had conclusive results using the conventional antigen screen. In fact, the sensitivity of the rapid antigen test in our hands was approximately 50% compared to culture. Therefore, during the height of the streptococcal pharyngitis season, when the incidence of true-positive results was ≈30%, ≈85% of patients had to wait up to 48 h for a conclusive result by culture. This was because with ≈30% true-positive cases and ≈70% true-negative cases, half of the true-positive cases, or 15%, were detected by rapid antigen and the other half, or 15% of true-positives, as well as the 70% true-negatives required detection by culture. Cultures are held for 48 h.

In discussions with our health care providers, it became clear that there were other considerations beyond education on test performance. These included, specimen transportation and arrival times, (especially for samples coming from more distant clinics) and issues related to the expeditious provision of antibiotics (i.e., closing times of local pharmacies). Based on these considerations, we implemented testing five times daily (4:00 a.m., 11:30 a.m., 3:00 p.m., 5:30 p.m., and 8:30 p.m.), 7 days/week, with additional batches set up as needed during the peak season.

Additionally, we have eliminated most of the follow-up procedures required by health care providers by using the following innovative processes with our clinical colleagues (456). Patients identify their preferred pharmacy at the time the throat swab is collected. A standardized prescription form (including the antibiotics prescribed and the patient's preferred pharmacy) is completed by the healthcare provider, which then accompanies the specimen to the laboratory. At the conclusion of the test run, results are entered into the laboratory information system and transmitted to the patient's electronic medical record. The results from the electronic medical record are delivered to a computerized message center, allowing patients to obtain their secure results by telephone and pick up the prescription prepared for them at their selected pharmacy. Prescriptions are faxed to numerous local and regional pharmacies by the clinical microbiology laboratory staff (Fig. 5).

In a comparison of the personnel required for performing rapid antigen test and back up culture of antigen negative specimens, we realized a savings of 2.1 full time equivalents. This is based on an annual testing volume of 26,000 tests with the rapid antigen test being performed at four satellite locations in Rochester, Minnesota. Performance of the Roche Strep-A ASR test allowed us to centralize testing in one location. This results in a more efficient process that saved personnel effort.

In summary, the introduction of this rapid real-time PCR assay for detection of streptococcal pharyngitis has streamlined both the testing procedure and resulted in significant personnel savings in the laboratory. Most importantly, we have also implemented new procedures in tandem with this technology that facilitate the expeditious provision of appropriate antimicrobial therapy for our patients.

Example of work flow design: real-time PCR for detection of herpes simplex and varicella-zoster infections. In contrast to PCR testing for group A streptococci, the support of our clinical colleagues implementing this test was less of an issue. This was due in part to the fact that conventional PCR had been used for a number of years at our institution for detection of HSV in spinal fluid as well as other viruses such as hepatis C virus, and human immunodeficiency virus.

We currently use commercially available ASRs for both varicella-zoster virus (Roche Diagnostics Corporation) and HSV (Roche Diagnostics Corporation) testing with the LightCycler instrument. Testing is performed three to six times daily 6 days a week. Nucleic acid is extracted from the specimen using the automated MagNA Pure system (Roche Diagnostics Corporation) by laboratory assistants in our initial processing area. Real-time PCR testing is performed and results entered into the laboratory information system and transmitted to the patient's electronic medical record. In comparison to viral culture, which may take 14 days or longer to complete, the majority of results are available the same day the specimen is received (118). For these real-time PCR virology assays, efficiencies were gained in faster turnaround time and improved sensitivity compared to culture. Downstream, this can lead to a decrease in the number of tests requested on a patient to make a diagnosis, and potentially shorter hospital stays. Additionally the personnel requirements were 2.5 times greater for the standard viral culture method versus the real-time PCR method.

COSTS

Royalties

Developers of laboratory-developed (also called home-brewed or in-house developed) real-time PCR assays for commercial use (i.e., the patient will be charged for the test) should determine whether patents exist for the genetic targets they wish to use for their assays. If such patents exist, licenses and/or royalty fees may have to be executed with the inventors or assignees of the patents. In order to avoid license and royalty fees, some developers may wish to search for alternative nucleic acid targets that are not protected by patent. In situations where alternative targets that are not protected by patent do not exist (e.g., resistance genes associated with antimicrobial resistance), licenses and/or royalties may be too expensive or unattainable (e.g., exclusive license agreements).

Laboratorians should also determine whether a separate royalty is required for performing PCR. Roche Diagnostics Corporation requires that PCR royalties be paid to them if testing is used for commercial (non-research-related) purposes.

Reagents and Instrumentation

The cost for reagents (i.e., polymerase enzymes, PCR primers, fluorescent probes, and internal, positive, and negative controls) may vary according to whether they are laboratory developed, obtained from different wholesale suppliers, or purchased as ASRs or kits from a common manufacturer. Generally, if reagents are purchased as ASRs or kits they are more expensive; however, the quality and performance characteristics of these reagents should be more reliable and consistent because they are produced under Good Manufacturing Practices as mandated by the FDA. Technical support for instrumentation and to some extent for assay reagents should be provided by the manufacturer. In the United States, technical support by manufacturers may be limited for ASRs compared with FDA-approved kits. FDA guuidelines prohibit manufacturers of ASRs to provide standard protocols to U.S. laboratories when ASRs are used for testing. Significant capital outlay for purchase of instrumentation for real-time PCR is also required, though options for creative financing (e.g., reagent rental agreements) may be possible with some vendors.

Frequently, the cost for real-time PCR reagents is significantly more than the cost for culture media used for traditional methods. However, costs for reagents and instrumentation may be obviated by savings in labor requirements in the laboratory and cost savings at the bedside due to higher sensitivity and more rapid turnaround time for results for real-time PCR tests compared with traditional culture-based methods.

Personnel

Often the amount of labor required for performing real-time PCR assays is considerably less than that required for culture-based assays. As previously emphasized, replacement of a rapid antigen/culture method in our laboratory by a real-time PCR assay for the detection of group A streptococcus in throat swabs significantly reduced labor requirements. The antigen-culture method required 4.0 full-time equivalents; in contrast,

TABLE 7. Reimbursement

Technique	Organism	CPT code	Medicare reimbursement (U.S. dollars)	2005 Mayo Clinic list price (U.S. dollars)
Amplified probe	CMV	87496	27–49	228
	EBV	87798	27–49	228
	HSV	87529	27–49	228
	VZV	87798	27–49	228
	Enterovirus	87798	27–49	228
	JC and BK virus	87798	27–49	228
	Group A streptococci	87651	27–49	NA[a]
	Bordetella pertussis/*Bordetella parapertussis*	87801	55–98	271
	Borrelia burgdorferi	87476	27–49	320
	VRE	87798	27–49	271
	Tropheryma whipplei	87798	27–49	321
	Ehrlichia spp.	87798	27–49	222
Quantification	CMV	87497	39–59	307
	EBV	87799	27–59	221

[a] NA, not available through the Mayo Clinic reference laboratory.

a LightCycler real-time PCR assay (LightCycler Strep-A, Roche Diagnostics Corporation) requires 1.9 full-time equivalents. Another example relates to the detection of herpes simplex virus. The personnel time requirement for shell vial culture assay was 2.5 times that required for a real-time PCR assay (LightCycler HSV1/2, Roche Diagnostics Corporation) (456).

Cost Savings at the Bedside

Cost effectiveness studies are required to determine the cost savings at the bedside for real-time PCR compared with conventional testing methods for diagnosis of infectious disease. Intuitively, if a diagnosis can be provided sooner and more reliably (higher sensitivity and specificity), patients who require antimicrobial therapy will receive it sooner. As well, less auxiliary testing should be required (e.g., additional infectious diseases tests such as cultures) and patients should have less morbidity and therefore fewer costs related to supportive therapy (e.g., intensive care related to a delay in diagnosis of sepsis). Providing a negative result sooner can have important implications for the overprescription of antibiotics. For example, if a real-time PCR test result can more quickly rule out the pathogen compared with a culture-based method, then the clinician may be less inclined to use empirical antibiotics or if empirical antibiotics are used the duration of treatment may be shortened. In patients who are suspected of having communicable diseases, such as tuberculosis, expensive infection precaution (isolation) requirements may be discontinued sooner, if the infectious pathogen is ruled out more quickly by real-time PCR than is possible with conventional testing.

Although cost effectiveness studies have not been published for real-time PCR testing, two seminal studies indicate that more rapid provision of microbiology results can result in substantial cost savings. Doern and colleagues showed that same-day versus overnight provision of results for bacterial identification and antimicrobial susceptibility to physicians at their institution resulted in statistically significantly fewer laboratory studies ordered per patient, and a statistically significant savings per patient hospitalization of ~$4,000. This represented an annual cost savings of $2,403,162 for their institution (97). In a similarly designed study, Barenfanger and colleagues demonstrated that provision of more rapid results for bacterial identification and antimicrobial susceptibility decreased length of hospital stay for patients an average of 2.0 days, decreased the mortality rate from 9.6% to 7.9%, and resulted in an annual cost savings of $4,189,500 (26). Studies such as these will be important for verifying whether rapid results generated by real-time PCR testing platforms will have similar financial impacts.

Coding and Reimbursement

Table 7 provides a list of commonly used Current Procedural Terminology codes and respective Medicare reimbursement amounts for real-time PCR tests used for infectious disease diagnosis at the Mayo Clinical Microbiology Laboratory. Although our laboratory has not encountered problems with reimbursement for these tests through our regional Medicare carrier, laboratories are encouraged to check with their local Medicare carriers to determine whether these tests will be reimbursed. In general, Medicare reimbursement is greater for real-time PCR tests than direct antigen immunoassays or culture-based tests.

APPLICATION OF REAL-TIME PCR FOR CLINICAL MICROBIOLOGY TESTING

An increasing volume of published clinical studies demonstrate the utility of real-time PCR for diagnosing microbial pathogens. The high sensitivity (in some instances greatly exceeding sensitivity for conventional testing methods) and high specificity along with a short turnaround time for results and ease of performance, make real-time PCR an attractive replacement method for conventional culture and antigen-based assays.

Table 8 displays a comparison of selected real-time PCR assays developed in our laboratory to gold standard (culture-based) assays for selected pathogens. Shown are the increases in sensitivity and time requirements for performing real-time PCR versus culture-based assays (71). It should be emphasized that specimen preparation (i.e., extraction of nuclic acid) adds

TABLE 8. Comparison of LightCycler assays to culture-based assays for selected pathogens[a]

Organism (reference)	Sensitivity increase for LightCycler (%)	Analytical turnaround time	
		LightCycler[b] (min)	Culture-based assay (days)
Group A streptococci (13)	7	30	1–2
Legionella spp. (5) (BAL[c])	0[d]	45	2–14
Bordetella pertussis (6)	219	50	3–7
Vancomycin-resistant enterococci (fecal surveillance samples) (451)	120[e]	45	2–3
Varicella-zoster virus (skin) (117)	91	45	2–5
Herpes simplex virus (skin, genital) (115)	23	45	1–5
Cytomegalovirus (urine)	88[e]	45	≤1

[a] Adapted and modified from reference 73 with kind permission of Springer Science and Business Media.
[b] The time shown is for the assay only and does not include the time required for extraction of nucleic acid, which may vary from minutes to over 1 hour depending on the specimen type, organism, and whether a manual or automated method is used.
[c] BAL, bronchoalveolar lavage.
[d] Only archived culture-positive samples were available for this comparison.
[e] Based on unpublished clinical studies at the Mayo Clinic, Rochester, Minn.

to the overall time requirements for assays and the time for specimen preparation is not accounted for in Table 8. The time requirements for extraction of nucleic acid can vary from minutes to over an hour and depends on whether a manual or automated method is used, the specimen type, and the organism or organisms targeted.

The following discussion contains a comprehensive summary of peer-reviewed publications and abstracts, which have evaluated real-time PCR assays in the clinical laboratory.

BACTERIA OTHER THAN *MYCOBACTERIA* SPP.

Table 9 displays peer-reviewed publications, which have applied real-time PCR testing platforms for the detection of bacterial pathogens or antibiotic resistance genes. For each publication, the specimen source(s) evaluated is listed. Additionally, specific information on the real-time PCR method is provided including the target nucleic acid sequence, the instrument and detection chemistry employed and whether the assay is available from a commercial manufacturer as an analyte-specific reagent or kit.

General Bacteria

Recent studies have demonstrated the advantages of real-time PCR testing for bacterial agents that traditionally have been identified by direct immunoassay techniques (antigen testing methods: e.g., group A streptococcus from throat swabs, *Clostridium difficile* toxin from feces, or Vero toxin or *Escherichia coli* O157:H7 antigen from feces). Other recent studies have shown the utility of real-time PCR assays for organisms for which the routine culture method is focused on identifying a single pathogen from a specimen (e.g., group A streptococcus from throat swabs, group B streptococcus from vaginal/anal swabs).

Frequently, the sensitivity of these real-time PCR assays equals or exceeds the standard antigen or culture method and the turnaround time for results is much shorter than the culture-based method. One notable example is the time savings, enhanced sensitivity, and reduced personnel requirements previously described in this review for detection of group A streptococcus from throat swabs (499). Recently, commercially produced analyte-specific reagents or kits have become available for real-time PCR for group A streptococcus detection in throat swabs (LightCycler Strep-A assay for use with the LightCycler, Roche Diagnostics Corporation) and group B streptococcus detection in vaginal/anal swabs (IDI-StrepB assay for use with the SmartCycler, Infectio Diagnostics, Inc., Sainte-Foy, Quebec, Canada; LightCycler StrepB *pts*1, Roche Diagnostics Corporation).

Slow-Growing or Poorly Culturable Bacteria

Conventional PCR provided limited applications for bacterial diagnostics due to the technical difficulties required for performing the procedure and the time delay in producing a final result. Moreover, certain specimens, such as sputum and feces, were difficult to test due to intrinsic substances that inhibited PCR chemistry and therefore the sensitivity of the assay was significantly compromised. As a result, conventional PCR testing methods were limited to bacteria that are difficult to culture or grow slowly (e.g., *Anaplasma phagocytophila*, *Bartonella henselae*, *Bordetella pertussis*, *Borrelia burgdorferi* sensu stricto, *Ehrlichia* spp., *Legionella* spp., *Mycoplasma pneumoniae*, or *Chlamydophila pneumoniae*) or for which culture methods did not exist (e.g., *Tropheryma whipplei*). A number of studies have now been conducted which demonstrate the ability of real-time PCR for detecting these fastidious organisms as listed in Table 9.

Agents of Community-Acquired Pneumonia

There has been considerable interest to apply real-time PCR for testing of the bacterial agents of community-acquired pneumonia, especially those agents associated with atypical pneumonias. The main reason for this is that these bacterial pathogens, which include *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella* spp., can be difficult to isolate on culture due to special growth requirements. Additionally, due to the slow growth of these organisms, the time required for a final result may be prolonged. Conventional PCR has been demonstrated to provide excellent sensitivity for detecting these organisms in throat swabs and respiratory secretions (325, 393). Real-time PCR has been shown to be as effective as culture or serologic methods for detecting these pathogens, as witnessed by the relatively large number of published studies presented in Table 9.

Streptococcus pneumoniae, the most common agent associated with typical (lobar) community-acquired pneumonia, is easily detected by PCR in respiratory secretions. A drawback is that *Streptococcus pneumoniae* can colonize the pharynx in the absence of disease, so qualitative detection of this organism by PCR may result in results that are specific for infection, but do not necessarily connote disease (325). As a step towards solving this problem, a recent study, using quantitative real-time

TABLE 9. Testing platforms for bacteria

Category	Organism	Reference(s)	Specimen[a]	Conventional test method[b]	Assay								
					Laboratory developed	ASR	Kit	Target	Instrument	Chemistry	Sensitivity vs. conventional method	Turnaround time vs. conventional method	Comments
General bacteria	Group A streptococcus (*Streptococcus pyogenes*)	499	Throat swabs	Antigen detection/culture		Yes (Roche)[c]		*pts1* gene	LightCycler	Dual FRET hybridization probes	Greater	Faster	Real-time PCR assay required half the personnel
	Group B streptococcus (*Streptococcus agalactiae*)	30, 211, 212	Anal, vaginal or combined anal/vaginal swabs	Culture	Yes			*cfb* gene	LightCycler	Dual FRET hybridization probes	Equal	Faster	Susceptibility testing still required in patients allergic to penicillin
		Abstract[g]	Anal, vaginal or combined anal/vaginal swabs	Culture			Yes (I.D.I.)[d]	*cfb* gene	LightCycler and SmartCycler	Dual FRET hybridization probes (LightCycler), molecular beacons (SmartCycler)	Equal	Faster	Susceptibility testing still required in patients allergic to penicillin
	Shiga (Vero) toxin-producing *Escherichia coli*	387	Feces	EIA, conventional PCR assay	Yes			*stx1*, *stx2*, *stx2e*	LightCycler	Dual FRET hybridization probes	Greater (EIA or conventional PCR)	Faster (conventional PCR)	Differentiation of *stx* genes by melting curve analysis
		28	Feces	Culture	Yes			*stx1*, *stx2*	SmartCycler	Molecular beacons	Greater	Faster	Multiple assay
Slow-growing or poorly culturable bacteria	*Bartonella henselae*	559	Blood (endocarditis)	Culture, conventional PCR assay	Yes			*ribC* gene	LightCycler	SYBR Green I	Greater (culture or conventional PCR)	Faster	Serology may still be used in PCR-negative cases
	Bordetella pertussis	406	Isolates	Culture	Yes			IS*481* (*B. pertussis*)	LightCycler	Dual FRET hybridization probes	Equal	NA[i]	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
		7	Nasopharyngeal swabs	Culture	Yes			IS*481* (*B. pertussis*)	LightCycler	Dual FRET hybridization probes	Greater	Faster	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
		63	Nasopharyngeal swabs	Culture	Yes			IS*481* (*B. pertussis*)	ABI Prism 7700	TaqMan probes	Greater	Faster	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
	Bordetella pertussis and *Bordetella parapertussis*	405	Nasopharyngeal, throat or perinasal swabs, BAL	Culture	Yes			IS*481* (*B. pertussis*), IS*1001* (*B. parapertussis*)	LightCycler	Dual FRET hybridization probes	Greater	Faster	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*

		71	Nasopharyngeal swabs or aspirates, sputa, throat swabs, bronchial washings, pleural fluid, tracheal aspirates	DFA, culture	Yes		IS*481* (*B. pertussis*), IS*1001* (*B. parapertussis*)	LightCycler	Dual FRET hybridization probes	Greater (DFA or culture)	Faster	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
		233	Nasopharyngeal swabs	Culture	Yes		IS*481* (*B. pertussis*), IS*1001* (*B. parapertussis*)	LightCycler	Dual FRET hybridization probes	Greater	Faster	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
		450	Nasopharyngeal swabs	DFA, culture, conventional PCR assay	Yes	Yes[e]	IS*481* (*B. pertussis*), IS*1001* (*B. parapertussis*)	LightCycler	Dual FRET hybridization probes	Greater (DFA, culture, conventional PCR)	Faster (DFA, culture, conventional PCR)	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
		234	Nasal, pharyngeal or nasopharyngeal swabs, nasopharyngeal aspirates	Culture	Yes		IS*481* (*B. pertussis*) IS*1001* (*B. parapertussis*)	ABI Prism 7700	TaqMan Probes	Greater	Faster	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
		233	Nasopharyngeal swabs	Culture	Yes		IS*481* (*B. pertussis*) IS*1001* (*B. parapertussis*)	ABI Prism 7700	TaqMan Probes	Greater	Faster	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
		486	Nasal swabs, sputa and nasopharyngeal aspirates	Culture, conventional PCR assay	Yes		IS*481* (*B. pertussis*), IS*1001* (*B. parapertussis*)	iCycler	Molecular beacons	Greater (culture, conventional PCR)	Faster (culture, conventional PCR)	Assay for IS*481* also detects but cannot differentiate *Bordetella holmesii* from *Bordetella pertussis*
	Tropheryma whipplei	126	Lymph node, duodenal biopsy or cardiac valve	Conventional test method	Yes		16S–23S rRNA gene intergenic spacer region and *rpoB* gene	LightCycler	SYBR Green I	Equal	NA	
Agents of community-aquired pheumonia	*Chlamydophila pneumoniae*	19	Sputa, BAL fluid	Four conventional PCR assays	Yes		*ompA*	ABI Prism 7700	TaqMan probes	See comments	Faster	Real-time PCR assay produced most consistent positive results when replicate testing of samples performed

Continued on following page

TABLE 9—*Continued*

Category	Organism	Reference(s)	Specimen[a]	Conventional test method[b]	Assay								
					Laboratory developed	ASR	Kit	Target	Instrument	Chemistry	Sensitivity vs. conventional method	Turnaround time vs. conventional method	Comments
		494	Peripheral blood mononuclear cells, oropharyngeal swabs	Culture, two nested conventional PCR assays	Yes			*ompA* (two domains, VD2 and VD4)	ABI Prism 7700	TaqMan probes	See comments	Faster	VD4 real-time assay detected most positives
		536	Sputa, nasopharyngeal secretions, throat swabs, bronchial aspirations, BAL fluid, pleural fluid	Conventional PCR assay	Yes			*pst1* genomic fragment	ABI Prism 7700	TaqMan probes	Greater	Faster	
		242	Sputa, nasopharyngeal, throat specimens	Conventional PCR assay	Yes			*ompA* gene	ABI Prism 7700	TaqMan probes	Greater	Faster	
		407	BAL fluid, bronchial secretions, tracheal secretions, gargle water, and throat swabs	Culture, serology	Yes			16S rRNA gene	LightCycler	Dual FRET hybridization probes	Equal	Faster	
		328	Nasopharyngeal aspirates	Conventional PCR assay	Yes			*pmp4* gene	LightCycler	Dual FRET hybridization probes	Equal	Faster	
	Legionella pneumophila	536	Sputa, nasopharyngeal secretions, throat swabs, bronchial aspirations, BAL fluid, pleural fluid	Conventional PCR assay	Yes			16S rRNA gene	ABI Prism 7700	TaqMan probes	Equal	Faster	
		535	BAL fluid, tracheal secretions, bronchial secretions	DFA, culture of urinary antigen	Yes			16S rRNA gene	LightCycler	Dual FRET hybridization probes	Greater	Faster	Results compared to combination of results for DFA, culture, urinary antigen
		547	Sputa, BAL fluid, endotracheal aspirates	DFA, culture	Yes			*mip* gene	LightCycler	Dual FRET hybridization probes	Equal (culture), greater (DFA)	Faster	

Legionella pneumophila and *Legionella* spp.	175	Sputa	DFA, culture of urinary antigen	Yes	23S-5S spacer region	ABI Prism 7700	TaqMan probes	Slightly less sensitive	Faster	Considering only positive result for DFA, culture or urinary antigen real-time PCR was 94% sensitive, equal to culture
	396	Sputa, BAL fluid	Culture, conventional PCR assay	Yes	16S rRNA gene	LightCycler	SYBR Green I	Equal (culture, conventional PCR)	Faster (culture, conventional PCR)	
	391	Induced sputa, BAL fluid	Culture	Yes	16S rRNA gene	LightCycler	Dual FRET hybridization probes	Greater	Faster	
	408	BAL fluid	Culture	Yes	16S rRNA gene	LightCycler	Dual FRET hybridization probes	Equal	Faster	
	167	BAL fluid, open lung biopsy	DFA, culture for BAL, DFA, culture, Warthin starry stain, in situ hybridization	Yes	*mip* gene and 5S gene	LightCycler	Dual FRET hybridization probes	Equal (culture for BAL), greater (DFA for BAL), greater (DFA, Warthin starry stain for open lung biopsy), less (culture and in vitro hybridization for open lung biopsy)	Faster (real-time PCR faster than all methods)	
Mycoplasma pneumoniae	536	Sputa, nasopharyngeal secretions, throat swabs, bronchial aspirations, BAL fluid, pleural fluid	Conventional PCR assay	Yes	16S rRNA gene	ABI Prism 7700	TaqMan probes	Equal	Faster	
	163	Nasopharyngeal secretions, pharyngeal swabs and blood	Serology conventional PCR assay	Yes	16S rRNA gene	ABI Prism 7700	TaqMan probes	Slightly less sensitive (serology, conventional PCR)	Faster (serology, conventional PCR)	95% sensitivity
	501	Sputa, throat washings, throat swabs	Conventional PCR assay	Yes	PI gene	LightCycler	Dual FRET hybridization probes	Equal	Faster	
	484	Throat swabs	Serology, conventional PCR assay	Yes	PhHV sequences	iCycler	Molecular beacons	Greater (serology), equal (conventional PCR)	Faster	
Streptococcus pneumoniae	148	Nasopharyngeal swabs	Culture	Yes	*ply* gene	ABI Prism 7700	TaqMan probes	See comments	Faster	Number of organisms detected by real-time PCR correlated with numbers

Continued on following page

TABLE 9—*Continued*

Category	Organism	Reference(s)	Specimen[a]	Conventional test method[b]	Assay								
					Laboratory developed	ASR	Kit	Target	Instrument	Chemistry	Sensitivity vs. conventional method	Turnaround time vs. conventional method	Comments
													detected by semiqualitative culture. It remains unclear whether these results correlate with pneumococcal lower respiratory tract disease
Agents of meningitis	*Neisseria meningitidis*	77	CSF, plasma, serum, whole blood	Culture	Yes			*ctrA* gene	ABI Prism 7700	TaqMan probes	Less (sensitivity = 80%)	Faster	Additional cases detected in culture-negative samples
		155	CSF, plasma, serum	Culture	Yes			*ctrA* gene, IS*1106*, *siaD* gene	ABI Prism 7700	TaqMan probes	Slightly less (91–93%)	Faster	Additional cases detected in culture-negative samples
		318	CSF	Culture	Yes			16S rRNA gene, *sacC*, *siaD*, *porA* genes	LightCycler	Dual FRET hybridization probes	Equal	Faster	
	Haemophilus influenzae	77	CSF, plasma, serum, whole blood	Culture	Yes			*bexA* gene	ABI Prism 7700	TaqMan probes	Equal	Faster	Additional cases detected in culture-negative cases
	Streptococcus pneumoniae	77	CSF, plasma, serum, whole blood	Culture	Yes			*ply* gene	ABI Prism 7700	TaqMan probes	Slightly less (sensitivity = 92%)	Faster	Additional cases detected in culture-negative cases
Bacteria associated with tick-borne diseases	*Borrelia burgdorferi* sensu stricto	299	Skin biopsies (erythema migrans)	Culture	Yes			*recA* gene	LightCycler	SYBR Green I	Not provided	Faster	Melting curve analysis used to identify different *Borrelia* spp.
	Borrelia garinii and *Borrelia afzelii*	380	Bacterial isolates	Culture	Yes			*recA* gene	LightCycler	SYBR Green I	Equal	Faster	Melting curve analysis used to identify different *Borrelia* spp.
	Ehrlichia chaffeensis	288	Blood, tissue	N.A.	Yes			16S rRNA gene	iCycler	TaqMan probes	See comments	Faster	Previous "positive" samples were all identified by real-time PCR
Potential agents of bioterrorism	*Bacillus anthracis*	29, 119	Bacterial isolates	Culture			Roche[f]	*capB*, *pagA* genes	LightCycler	Dual FRET hybridization probes	Equal	Faster	Autoclaving of bacteria before real-time PCR test did not affect sensitivity of assay

		350	Spiked nasal swab specimens	Culture	Yes	*rpoB*, *lef* genes	LightCycler	Dual FRET hybridization probes	Equal	Faster
		98	Spiked swabs	Culture	Yes	*rpoB* gene	LightCycler	Dual FRET hybridization probes	Equal	Faster
		371	Bacterial isolates	Culture	Yes	*rpoB*, *pagA*, *capC* genes	LightCycler	Dual FRET hybridization probes	Equal	Faster
		389	Bacterial isolates	Culture	Yes	*rpoB* gene	LightCycler	Dual FRET hybridization probes	Equal	Faster
		254	Bacterial isolates			*capC* gene	LightCycler	SYBR Gold	Equal	Faster
		111	Bacterial isolates	Culture	Yes	*rpoB*, *pag*, *capC* genes	ABI Prism 7000, 7700	TaqMan probes	Equal	Faster
		181	Bacterial isolates	Culture	Yes	Undisclosed chromosomal and pXO2, pXO7 targets	ABI Prism 7700, LightCycler, SmartCycler	TaqMan probes	Equal	Faster
	Yersinia pestis	493	Bacterial isolates	Culture		16S rRNA, *pla*, *capI*, *ymt* genes	LightCycler	Dual FRET hybridization probes	Equal	Faster
		306	Bacterial isolates	Culture		Undisclosed target	LightCycler and RAPID[h]	TaqMan probes	Equal	Faster
Bacterial antibiotic resistance genes	MRSA	409	Bacterial isolates	Culture-based susceptibility testing	Yes	*mecA* gene	LightCycler	Dual FRET hybridization probes	Equal	Faster
		448	Blood cultures which showed gram-positive cocci in clusters	Culture-based susceptibility testing	Yes	*mecA* and *sa442* genes	LightCycler	Dual FRET hybridization probes	Equal	Faster
		472	Blood cultures which showed gram-positive cocci in clusters	Culture-based susceptibility testing	Yes	*mecA* and *sa442* genes	LightCycler	SYBR Green I	Equal	Faster
		123	MRSA selective broth (oxacillin) inoculated with specimens from wounds, abscesses, anterior nares, perineum, urine, catheter insertion sites, skin and soft tissues, sputa, trachea	Culture-based susceptibility testing	Yes	*nuc* gene	LightCycler	Dual FRET hybridization probes	Slight decrease (sensitivity = 93.3%)	Faster (selective broth and real-time PCR required ~24 h vs. longer periods for conventional culture)

Continued on following page

TABLE 9—*Continued*

Category	Organism	Reference(s)	Specimen[a]	Conventional test method[b]	Assay								
					Laboratory developed	ASR	Kit	Target	Instrument	Chemistry	Sensitivity vs. conventional method	Turnaround time vs. conventional method	Comments
		150	Bacterial isolates	Culture-based susceptibility testing	Yes			*mecA* gene	LightCycler	Dual FRET hybridization probes	Equal	Faster	
		219	Bacterial isolates	Conventional PCR assay	Yes			*mecA* gene	ABI Prism 7700	TaqMan probes	Equal	Faster	
		130	Nasal or inguinal swabs	Culture-based susceptibility testing	Yes			*mecA* and *femA* genes	ABI Prism 7700	TaqMan probes	Equal	Faster	Prior to real-time PCR, *Staphylococcus aureus* cells are selectively removed from swabs by a unique immunocapture technique
	VRE	361	Bacterial isolates	Conventional PCR assay	Yes			*vanA* and *vanB* genes	LightCycler	Dual FRET hybridization probes	Equal	Faster	
		362	Rectal swabs with and without enrichment broth		Yes			*vanA* and *vanB* genes	LightCycler	Dual FRET hybridization probes	Greater	Faster	
		451		Culture			Yes (Roche)	*vanA* and *vanB* genes	LightCycler	Dual FRET hybridization probes	Greater	Faster	
	Extended spectrum beta-lactamases	394	Bacterial isolates	Culture-based susceptibility testing	Yes			bla_{SHV} genes	LightCycler	Dual FRET hybridization probes	Greater	Faster	
	Penicillin resistance in *Streptococcus pneumoniae*	214	CSF	Culture-based susceptibility testing	Yes			*pbp2b*	LightCycler	SYBR Green I	Greater	Faster	Detection of *php2b* gene associated with susceptibility
	Penicillin resistance in *Neisseria meningitides*	460	Bacterial isolates	DNA (sequencing of *penA* gene)	Yes			*penA* gene codon 566 mutation	LightCycler	Dual FRET hybridization probes	Equal	Faster	*penA* gene codon 566 mutation associated with penicillin resistance detected by melting curve analysis
	Fluoroquinolone resistance in *Staphylococcus aureus*	248	Bacterial isolates	Culture-based susceptibility testing	Yes			*grlA* gene mutations	SmartCycler	Molecular beacons	Equal	Faster	
	Fluoroquinolone resistance in *Yersinia pestis*	275	Bacterial isolates	DNA sequencing of *gyrA* gene	Yes			*gyrA* gene mutations	LightCycler	Dual FRET hybridization probes	Equal	Faster	Mutations detected by melting curve analysis
Obligately anaerobic bacteria	*Clostridium difficile*	27	Feces	Cytotoxicity assay	Yes			*tcdA* and *tcdB* genes	SmartCycler	Molecular beacons	Slightly less (sensitivity = 97%)	Faster	

[a] BAI, bronchoalveolar lavage; CSF, cerebrospinal fluid.
[b] EIA, enzyme immunoassay; DFA, direct fluorescent antibody; N.A., not applicable.
[c] LightCycler Strep-A assay (Roche Diagnostics Corporation, Indianapolis, IN).
[d] ID1-Strep B Assay (Infectio Diagnostics, Inc., Sainte-Foy, Quebec, Canada) for use with SmartCycler (Cepheid, Sunnyvale, CA).
[e] Prototype assay evaluated for this study now available as ASR: LightCycler Bordetella IS481/1001 assay (Roche Diagnostics Corporation).
[f] LightCycler *Bacillus anthracis* detection kit (Roche Applied Science, Indianapolis, IN).
[g] C. Ménard, F. J. Picard, J. Frenett, M. Gagnon, D. B. Ke, M. Ouellette, P. H. Roy, and M. G. Bergeron, Am. Soc. Microbiol. 100th Annu. Meet., abstr. C-193, 2000.
[h] RAPID refers to "ruggedized" advanced pathogen identification device, which is a field-deployable real-time PCR assay platform (Idaho Technology, Salt Lake City, Utah).
[i] NA, not applicable.

PCR, demonstrated that the numbers of *Streptococcus pneumoniae* organisms detected by real-time PCR in nasopharyngeal secretions correlated with the numbers detected by semiquantitative cultures (150). Other prospective clinical studies are required to support these findings. It could be argued that real-time PCR could likewise detect patients colonized but not infected with group A streptococcus. However, a study previously described in this review showed that all patients with group A streptococcus detected by real-time PCR had clinical criteria for streptococcal pharyngitis (499).

Agents of Meningitis

The significant mortality and morbidity associated with bacterial meningitis requires rapid diagnosis. Real-time PCR provides a much more rapid result than culture, which is the gold standard. Additionally, the sensitivities for detecting the major bacterial pathogens associated with meningitis (*Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*) for most studies listed in Table 9 equal culture. Importantly, in cases of meningitis where antibiotics are provided before cultures are obtained, PCR may be particularly advantageous as it can be positive, whereas culture is negative.

Potential Agents of Bioterrorism

The intentional release of anthrax spores in the U.S. mail system in the fall of 2001 catapulted the development of real-time PCR assays for the rapid detection of potential agents of bioterrorism both in human specimens and environmental samples. Scientists at the U.S. Centers for Disease Control as well as scientists in the private sector have developed a number of highly sensitive and specific real-time PCR assays for detection of these agents, including *Bacillus anthracis* and *Yersinia pestis*, as shown in Table 9. Importantly, these assays provide a result much sooner than standard culture methods. Additionally, at least two papers have described the biosafety advantages of using a real-time PCR testing platform versus culture for detection of *B. anthracis*. In these studies the sensitivity of a real-time PCR assay (119) or conventional PCR assay (125) was not affected if samples were autoclaved before testing; the biohazard concern for exposure to *Bacillus anthracis* was obviated because cultures of autoclaved samples were negative. A limitation of real-time PCR studies for agents of bioterrorism has been the lack of testing human specimens. All of the studies listed in Table 9 for *Bacillus anthracis* and *Yersinia pestis* evaluated isolates or spiked human specimens.

Bacterial Antibiotic Resistance Genes

Infections caused by methicillin (oxacillin)-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE) have worse outcomes and higher associated costs than infections caused by methicillin (oxacillin)-susceptible *Staphylococcus aureus* or vancomycin-susceptible *Enterococcus* spp. (74). Unfortunately, the rates of MRSA and VRE continue at an accelerating pace in U.S. hospitals (96). Guidelines published in May 2003 by the Society of Healthcare Epidemiologists of America advise active surveillance programs in health care institutions for detection of MRSA and VRE

TABLE 10. Real-time PCR methods for mycobacteriology[a]

Method or species	Specimen	Technology	Target	Status	Conventional method	Clinical sensitivity vs. conventional method	Turnaround time vs. conventional method	Reference(s)	Comments
Genus screen	Culture	LightCycler FRET HP	16S rRNA	HB	Culture	NA	Faster	243	3 primer/probe sets to detect *Mycobacterium* spp., *Mycobacterium avium*, *Mycobacterium tuberculosis*
Genus screen	Culture	ABI 7700 molecular beacons	16S rRNA	HB	Culture	NA	Faster	449	Also differentiation of *Mycobacterium tuberculosis* and comparison w/ Amplicor PCR
Genus screen	Respiratory and nonrespiratory	ABI 7700 molecular beacons	IS*6110*, *senX3*, *regX3*	HB	Culture	Greater	Faster	50	Genus screen and *Mycobacterium tuberculosis* detection and quantitation
Genus screen	Respiratory	ABI 7700 molecular beacons	16S rRNA	HB	Culture	Lower	Faster	136	Genus screen and *Mycobacterium tuberculosis* detection
Genus screen	Fine-needle aspirates, tissue	iCycler TaqMan	ITS	HB	Culture	Greater	Faster	51	1 primer set/3 probes to detect *Mycobacterium* spp., *Mycobacterium avium*, *Mycobacterium tuberculosis*
Mycobacterium abscessus	Culture	LightCycler FRET HP	*hsp65*, 16S rRNA	HB	Culture, biochemicals or *hsp65* RFLP	NA	Faster	443	Multiplex assay to differentiate *Mycobacterium abscessus* and *Mycobacterium chelonae*
Mycobacterium avium complex	Culture	LightCycler FRET HP	16S rRNA	HB	Culture	NA	Faster	243	3 primer/probe sets to detect *Mycobacterium* spp., *Mycobacterium avium*, *Mycobacterium tuberculosis*
	Fine-needle aspirates, tissue	iCycler molecular beacons	ITS	HB	Culture, nucleic acid probe	Greater	Faster	51	1 primer set/3 probes to detect *Mycobacterium* spp., *Mycobacterium avium*, *Mycobacterium tuberculosis*
	Tissue	ABI 7700 TaqMan	*hsp65*	Expmtl	Culture, nucleic acid probe	Lower	Faster	481	Research in birds
Mycobacterium avium subsp. *paratuberculosis*	Culture	LightCycler SYBR	IS*900*	HB	Culture	NA	Faster	353	
	Fixed tissue	GeneAmp 5700 TaqMan	IS*900*	HB	Culture	NA	Faster	107	Investigation of sarcoid etiology
Mycobacterium bovis	Culture	LightCycler FRET HP	*oxyR*, *narG*, *RD1*	HB	Culture, biochemicals	NA	Faster	462	Differentiation of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG
Mycobacterium bovis	Tissue	LightCycler FRET HP	IS*6110*	Expmtl	Culture, histopathology	Lower	Faster	478	Research in cattle
Mycobacterium bovis BCG	Culture	LightCycler FRET HP	*oxyR*, *narG*, *RD1*	HB	Culture, biochemicals	NA	Faster	462	Differentiation of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG
Mycobacterium chelonae	Culture	LightCycler FRET HP	*hsp65*, 16S rRNA	HB	Culture, biochemicals, or *hsp65* RFLP	NA	Faster	443	Multiplex assay to differentiate *Mycobacterium abscessus* and *Mycobacterium chelonae*
Mycobacterium genavense	Tissue	ABI 7700 TaqMan	*hsp65*	Expmtl	Culture/nucleic acid probe	Lower	Faster	481	Research in birds
Mycobacterium leprae	Tissue	LightCycler FRET HP	*pra*	HB	Microscopy	Lower	Equal	235	
Mycobacterium tuberculosis	Culture	LightCycler FRET HP	*oxyR*, *narG*, *RD1*	HB	Culture, biochemicals	NA	Faster	463	Differentiation of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG

Mycobacterium tuberculosis complex	Culture	LightCycler FRET HP	16S rRNA	HB	Culture	NA	Faster	243	3 primer/probe sets to detect *Mycobacterium* spp., *Mycobacterium avium*, *Mycobacterium tuberculosis*
	Culture	LightCycler FRET HP	ITS	HB	Culture	NA	Faster	236	
	Culture, sputum	LightCycler FRET HP	IS*6110*	HB	Culture	Lower	Faster	169	Idaho Technology LightCycler
	Culture, respiratory	LightCycler FRET HP	ITS	HB	Culture	Lower	Faster	315	Also compared with Amplicor PCR test
	Culture, respiratory and nonrespiratory	ABI 7700 molecular beacons	16S rRNA	HB	Culture	Lower	Faster	449	Differentiation of *Mycobacterium tuberculosis* from NTMs; comparison w/Amplicor PCR
	Respiratory and nonrespiratory	ABI 7700 molecular beacons	IS*6110*, *senX3*, *regX3*	HB	Culture	Greater	Faster	50	Genus screen and *Mycobacterium tuberculosis* detection and quantitation
	Respiratory	SmartCycler TaqMan	IS*6110*	HB	Culture	Equal	Faster	70	Also compared with Amplicor PCR test
	Respiratory and nonrespiratory	ABI 7700 molecular beacons	16S rRNA	HB	Culture	Lower	Faster	136	Genus screen and *Mycobacterium tuberculosis* detection
	Respiratory and nonrespiratory	ABI 7000 TaqMan	IS*6110*	HB	Culture	NR	Faster	260	Comparison with AMTD PCR test
	Sputum	ABI 7700 molecular beacons	IS*6110*	HB	Microscopy and culture	NA	Faster	94	*Mycobacterium tuberculosis* quantitation
	Fine-needle aspirates, tissue	iCycler TaqMan	ITS	HB	Culture	Greater	Faster	51	1 primer set/3 probes to detect *Mycobacterium* spp., *Mycobacterium avium*, *Mycobacterium tuberculosis*
	Fixed tissue	ABI 7700 molecular beacons	GM-CSF, IFN-γ, etc.	Expmtl	Conventional PCR	NA	Faster	562	Gene expression in mouse granulomas
Mycobacterium tuberculosis complex genotyping	Culture	ABI 7700 molecular beacons	*gyrA*, *katG*	LD	IS*6110* RFLP	NA	Faster	412	Genotyping of *Mycobacterium tuberculosis* complex
Mycobacterium tuberculosis complex susceptibility	Culture	LightCycler FRET HP	*katG*, *rpoB*	LD	Broth dilution	NA	Faster	135, 300, 495, 496	INH and RIF resistance
	Culture	LightCycler FRET HP	*rpoB*	LD	Broth dilution	NA	Faster	106	RIF resistance
	Culture	LightCycler SYBR	16S rRNA	LD	Broth dilution	NA	Faster	416	RIF resistance; 2 clinical specimens
	Culture	ABI 7700 molecular beacons	*ahpC*, *oxyR*, *inhA*, *kasA*	LD	Broth dilution	NA	Faster	378	INH and RIF resistance
	Culture, sputum	ABI 7700 molecular beacons	*rpoB*	LD	Broth dilution	Equal	Faster	110, 379	RIF resistance
	Culture, sputum	iCycler TaqMan	*katG*	LD	Broth dilution	Lower	Faster	505	INH resistance
	Culture, smear-positive clinical specimens	iCycler molecular beacons	*katG*, *inhA*, *rpoB*	LD	Broth dilution	Equal	Faster	274	INH and RIF resistance
	Respiratory	ABI 7700 TaqMan	*rpoB*, *katG*, *embB*	LD	Broth dilution	Equal	Faster	523	8 probes to detect INH, RIF, EMB resistance
Mycobacterium ulcerans	Tissue	ABI 7700 TaqMan	IS*2404*	LD	Culture, conventional PCR	Greater	Faster	417	

[a] Abbreviations: EMB, ethambutol; Expmtl, experimental; HP, hybridization probe; INH, isoniazid; LD, laboratory developed; NA, not applicable; NR, not reported; RIF, rifampin; RFLP, restriction fragment length polymorphism; NTM, nontypeable mycobacteria; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; HB, home brew; AMTD, amplified *Mycobacterium tuberculosis* direct test.

carriers (327). Numerous studies have shown that surveillance for and isolation of carriers of MRSA and VRE can significantly reduce the incidence of nosocomial infections by these organisms and be cost-saving (327).

Broad-based surveillance for MRSA or VRE, using culture-based methods, may be especially demanding if not impossible for most clinical microbiology laboratories. Moreover, the time required for a final result may take several days. Real-time PCR testing methods for both MRSA and VRE show great promise for simplifying this process and providing same day results. Notably with VRE, nearly all studies, which use either conventional PCR or real-time PCR, show improved sensitivities for detecting this pathogen from fecal specimens compared with culture. We recently demonstrated a 120% increase in sensitivity using a commercially available ASR for detection of VRE in perianal swabs versus culture (451). Two manufacturers have ASRs or kits available for use for VRE or MRSA testing with real-time PCR testing platforms. Infectio Diagnostic, Inc., has recently received FDA approval for a kit that can directly screen nasal swabs for MRSA using the SmartCycler instrument (IDI-MRSA). Roche Diagnostics Corporation provides separate ASRs for VRE detection (LightCycler *vanA*/*vanB* detection assay) and MRSA (LightCycler *mecA* detection assay), using the LightCycler instrument.

MYCOBACTERIA

The traditional approach for diagnosing mycobacterial infection relies upon the use of stains for detection of acid-fast bacilli and growth in culture on solid and/or liquid media. Mycobacteria isolated from cultures are identified using biochemical analysis, nucleic acid probes, or 16S rRNA gene sequencing. This culture and identification process is time-consuming, labor intensive, and in some cases lacks sensitivity or specificity (236). Real-time PCR has the potential to significantly change the current paradigm for mycobacteria identification by decreasing turnaround time for identification from weeks to hours while maintaining or improving upon diagnostic sensitivity and specificity.

The majority of real-time PCR methods reported to date for mycobacteria focus on detection of the *Mycobacterium tuberculosis* complex and do not differentiate between the species within the complex. Miller et al. developed a real-time PCR assay that rapidly and specifically detected the *Mycobacterium tuberculosis* complex directly from acid-fast smear-positive respiratory specimens and from BacT/ALERT MP culture bottles (315). The same group then demonstrated that a similar *Mycobacterium tuberculosis* complex real-time assay tested on 366 acid-fast smear-positive respiratory specimens had sensitivity and specificity equal to the AMPLICOR PCR assay (Roche Diagnostics Corporation) and required one-half the time (3 h versus 6 h) to complete (69). Stermann et al. successfully designed sets of sequence specific primers and FRET hybridization probes to target polymorphisms within the *narG*, *oxyR*, and *RD1* loci of the *Mycobacterium tuberculosis* complex that allowed differentiation of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium bovis* BCG, respectively (462).

Several publications address the detection of mycobacteria at the genus level (50, 136, 243, 449). In one of the most comprehensive studies, Lachnik et al. designed genus-specific primers to target the 16S rRNA gene and were able to detect 33 species of mycobacteria from culture (243). Two sets of sequence-specific FRET hybridization probes enabled further differentiation of the *Mycobacterium tuberculosis* complex and two members of the *Mycobacterium avium* complex (*Mycobacterium avium* and *Mycobacterium avium* subsp. *paratuberculosis*) from the other 30 species analyzed. *Mycobacterium intracellulare*, another member of the *Mycobacterium avium* complex, was not reliably identified using the assay because it exhibited a melting temperature that was indistinguishable from that of several other species examined.

Detection of antitubercular drug resistance is vital to effective patient management. Real-time PCR offers the potential to detect gene mutations responsible for drug resistance within hours from patient specimens compared with the average of 2 weeks required for traditional susceptibility test methods. The *rpoB* and *katG* genes are the most common *Mycobacterium tuberculosis* targets utilized in real-time PCR methods and well-known mutations in these genes correlate with resistance to rifampin and isoniazid, respectively (106, 110, 135, 353, 379, 495, 496, 505). The significance of other gene targets such as *kasA*, *ahpC-oxyR*, and *inhA* for the prediction of isoniazid resistance is still somewhat controversial (378). Torres et al. used two sets of FRET hybridization probes to detect *rpoB* mutations in 24 rifampin-resistant strains *of Mycobacterium tuberculosis* and another set of FRET hybridization probes to detect *katG* mutations in 15 isoniazid-resistant *Mycobacterium tuberculosis* strains (496). Additionally, Garcia de Viedma et al. used two sets of *rpoB* probes and one set of *katG* probes to detect *rpoB* and *katG* mutations, but in a single tube, for 29 resistant *Mycobacterium tuberculosis* isolates (135). Since not all gene mutations conferring drug resistance are well characterized and are thus not amenable to PCR assay development, traditional culture-based susceptibility testing methods are still required. However, the ability to predict rifampin and isoniazid resistance up to 2 weeks sooner than current methods for some isolates should have significant benefit for patient care.

A number of *Mycobacterium tuberculosis* real-time PCR assays have been performed directly from patient specimens rather than from culture (Table 10). Extraction and amplification of nucleic acids directly from patient specimens can decrease identification turnaround time from weeks to hours. Additional studies focused on extraction optimization from difficult specimen matrices (i.e., sputum, stool) will be required to insure sufficient assay sensitivity when compared with culture. Presently there are approximately 129 currently recognized species and subspecies of *Mycobacteria* (http://www.dsmz.de/species/gn250376.htm), and most have been implicated as human pathogens in the literature. Importantly, in many of the real-time PCR methods published to date, only a fraction of clinically significant mycobacteria species have been tested to determine whether they might be detected or might cross-react when looking for a specific target organism such as *Mycobacterium tuberculosis*.

VIRUSES

The earliest applications of real-time PCR for testing in the clnical microbiology laboratory were reported for the detection

of viruses. This was not unexpected as conventional PCR assays were already recognized as the method of choice for detecting or quantifying some viruses, (e.g., detection of herpes simplex virus in cerebrospinal fluid (CSF) or quantification of cytomegalovirus in blood or plasma). As a result, extensive literature exists describing the application of real-time PCR for detection and quantification of viral pathogens in human specimens. Therefore, this section represents the largest section in this review for any group of pathogens for which real-time PCR has been applied.

Qualitative Viral Assays

Herpes simplex virus. Herpes simplex virus (HSV) produces a wide spectrum of clinical manifestations; including genital, dermal, and central nervous system disease. It is the most common etiologic agent of sporadic focal central nervous system (CNS) disease; the mortality rate in untreated patients is almost 70% but can be reduced to 20% with prompt antiviral therapy with acyclovir (447). Several gene targets have been selected for the detection of HSV DNA by real-time PCR, including genes coding for glycoproteins B, C, D, and G, thymidine kinase, DNA polymerase, and DNA binding protein (317). (Tables 11 and 12).

Herpes simplex virus CNS disease. Several studies published in the early to mid-1990s established PCR as the preferred method for diagnosing CNS disease (244, 384, 420). Collectively, these reports provided the credibility for the routine molecular diagnosis of CNS disease caused by an infectious agent and were the break-through evidence that PCR amplification technology could be applied for the detection of target sequences of other viruses and microbial agents which could not be optimally detected by culture-based methods. Other laboratories, including ours, confirmed these findings in clinical evaluations and recognized that molecular amplification of HSV DNA (replacing brain biopsy inoculation in cell culture) was the new gold standard for the laboratory diagnosis of these infections (23, 90, 386, 431). Indeed, PCR technology, including real-time PCR methods, facilitated our understanding of the clinical spectrum of HSV CNS disease, which can vary from mild meningitis (Mollaret's) to severe necrotizing encephalitis (224, 294, 317, 436, 475, 550).

General experience with these PCR assays indicate that CSF specimens positive for HSV DNA were obtained from neonates to elderly adults, although individuals 30 to 69 years of age infected with this virus predominated (3, 317). The prevalence of HSV-1 versus HSV-2 is likely dependent on the laboratory practice i.e., whether specimens are submitted to a commercial laboratory (likely more severe CNS disease associated with HSV-1) or a local community-based population from which specimens from a wider spectrum of HSV CNS infections are submitted for diagnostic evaluation (3, 317, 374, 477).

The molecular detection by PCR of coinfection due to other herpesviruses and microbial agents in CSF specimens of patients with CNS disease may have important medical implications. Coinfections in the CNS may be associated with more severe disease in patients compared with infection with a single agent. For example, of 30 CSF specimens containing HSV DNA, three samples also had coinfection with human herpesvirus 6 ($n = 2$), and Epstein-Barr virus (EBV) ($n = 1$) DNA. Interestingly, of 22 of these patients with clinically diagnosed encephalitis, two of three patients coinfected with HSV and human herpesvirus 6 died, compared to 1 of 19 (5%) patients infected with only HSV (476).

A recent report indicated the value of using a comprehensive menu of real-time PCR assays (cytomegalovirus [CMV], EBV, HSV-1, HSV-2, and varicella-zoster virus [VZV]) for testing CSF specimens by using a single LightCycler program (466). Compared to conventional PCR, these real-time (LightCycler) assays were rapid, simple, and convenient for testing for herpesvirus DNA in the routine laboratory (467). Because of overlapping clinical symptomatology produced by many of the herpesviruses, in addition to other microbial targets, future testing of CSF samples may incorporate assays for several targets, rather than for a single unique sequence of one organism (3, 224). This would seem both economically and technically feasible since the most labor and time-consuming event is generally the nucleic acid extraction step. Several target assays can be performed after the extraction of a single specimen.

We have used the Roche HSV LightCycler assay (LightCycler herpes simplex virus 1/2 primer/hybridization probes; LightCycler HSV 1/2 Template DNA) since its introduction to the market in early 2003. All CSF specimens are processed and assayed separately from genital and dermal sources to decrease the possibility of specimen to specimen contamination. In addition to primers and probes for PCR detection of HSV target nucleic acid (template DNA), probes specific for an internal control (also called the recovery template) target are also included in the reaction master mix (LightCycler HSV 1/2 recovery template, Roche Diagnostic Corporation). The internal control is amplified by the same PCR primers which are used for HSV target DNA. However, the internal control consists of target nucleic acid which is detected by a second pair of FRET hybridization probes. These probes do not anneal with HSV target DNA.

Theoretically, the internal control added to a sample may be preferentially amplified especially in CSF specimens with low copy levels of HSV DNA. To evaluate this possibility we compared the detection of HSV DNA in CSF in the presence and absence of the internal control reagents. In the presence of an internal control, we found no difference in the detection of dilutions of HSV DNA from clinical specimens. Importantly, both nucleic acid targets were detected with a range of 1 and 2,000 copies of HSV DNA per reaction. At the two higher copy levels excess HSV DNA was preferentially amplified, but not internal control target, by PCR (Table 13).

HSV is detectable in CSF as early as 1 day after onset of clinical signs and symptoms. In most cases, DNA is present for an average of 4 days in CSF specimens of patients with CNS disease; however, HSV DNA may persist for up to 30 days after the onset of CNS disease in some patients who have received antiviral therapy. Persistence of HSV DNA may actually be fortuitous especially in cases that receive empirical treatment before PCR testing is performed (317, 477).

Herpes simplex virus dermal and genital disease. Recognition of conventional PCR as the gold standard for detection of HSV DNA in CSF specimens was readily accepted by microbiologists since the diagnosis of this virus infection was rarely obtained by cell culture techniques with these samples. Con-

TABLE 11. Detection of herpes simplex virus DNA by real-time PCR in cerebrospinal fluid specimens

Reference	No. of specimens	No. positive (%)	Test platform/gene target	Comparison studies	Comments
Ryncarz et al. 1999 (422)	380	42 (11.1)	ABI Prism/glycoprotein B	Of 58 total positive specimens, 13 were positive only by conventional PCR; 3 were positive only by real-time PCR.	Probes specific for HSV-1 or HSV-2 were used to differentiate genotypes in separate reactions.
Kessler et al. 2000 (218)	59	20 (33.9)	LightCycler/DNA polymerase	20 positive samples were detected by both conventional and real-time PCR methods. Four samples yielded discrepant results: two positive by conventional PCR only; two positive by real-time PCR only.	The real-time PCR assay on the LightCycler instrument proved to be very quick and laborsaving (<1 h) compared with conventional PCR (4 h).
Peter et al. 2001 (374)	3,200	62 (1.6)[a]	ABI Prism/DNA polymerase	A preponderance of HSV-2 over HSV-1 infections was obtained in specimens submitted for routine diagnostic testing.	No inhibitors of PCR detected.
Aberle et al. 2002 (3)	576	153 (26.6)	ABI Prism/HSV-1 DNA binding protein; HSV-2 glycoprotein G	Amount of virus varied among the individual diseases, associated with HSV-1, HSV-2, VZV, and CMV. Low levels of EBV and human herpesvirus 6 DNA were detected in CSF specimens.	Overall broad testing for different viruses in CSF clearly leads to a significant increase in the detection rate of viral CNS infections.
Kimura et al. 2002 (220)	28 (from neonates)	20 (71.4)[b]	ABI Prism/DNA polymerase	A real-time PCR assay was applied to quantitate the viral load in the conventional PCR-positive specimens.	Patients with CNS infection had the highest viral loads in CSF. Patients with HSV-2 infection had more CNS involvement and neurological impairment and higher viral load in CSF than did patients with HSV-1.
Stöcher et al. 2003 (467)	30	8 (26.6)	LightCycler/DNA polymerase	Detection limits with herpesvirus type-specific DNA spiked into CSF, serum, or plasma was 250 or 500 DNA copies/ml.	Four LightCycler PCR tests were developed (CMV, EBV, HSV-1 and HSV-2, VZV) with standard test parameters.

[a] Totals, 26 HSV-1 and 36 HSV-2.
[b] Results obtained by conventional PCR: 6 of 7 CSF samples from patients with disseminated HIV disease; 11 of 11 patients with CNS infection; 3 of 10 CSF samples with skin, eye, or mouth infection.

ventional PCR was not adapted for the detection of HSV in dermal or genital sources, because cell culture or direct staining techniques (e.g., fluorescent antibody staining) were relatively more sensitive for detecting HSV in these specimens and conventional PCR would have been too work intense and expensive and require considerable time for a result. In contrast, real-time PCR platforms now make it relatively easy to test dermal and genital specimens for HSV with considerable sensitivity and specificity and results can be available in less than one hour subsequent to nucleic acid extraction.

HSV is likely the most common virus recovered in cell cultures in the diagnostic virology laboratory; this virus accounts for over 70% of the total virus isolates at the Mayo Clinic (454, 456). Combined data obtained for the detection of HSV using

TABLE 12. Comparison of cell culture and real-time PCR for the laboratory detection of herpes simplex virus infections from dermal and genital specimens

Reference	Culture			Real-time PCR			Comments
	No. of specimens	No. positive (%)	Cells	No. of specimens	No. positive (%)	Test platform/gene target	
Ryncarz et al. 1999 (422)	335 (genital tract)	162 (48.4)	Human diploid fibroblast (24-well microtiter plates) rapid culture technique	335	248 (74.0)	ABI Prism/glycoprotein B	First study of laboratory diagnosis of genital tract specimens by real-time PCR
Espy et al. 2000 (118)	200 (genital, 160; dermal, 38; ocular, 2)	69 (34.5)	MRC-5 (shell vials)	200	88 (44)	LightCycler/DNA polymerase, thymidine kinase	Feasibility study performed by developmental technologists
Espy et al. 2000 (115)	500 (genital, 288; dermal, 192; ocular, 20)	158 (31.6)	MRC-5 (shell vials)	500	225 (45)	LightCycler/DNA polymerase, thymidine kinase	Implementation study performed by technologists performing laboratory testing for diagnostic purposes
Koenig et al. 2001 (229)	104 (genital and dermal)	43 (41.3)	Rabbit kidney (tubes)	104	55 (52.9)	LightCycler/glycoprotein D	SYBR Green I dye detection system
Aldea et al. 2002 (4)	118 (genital tract)	28 (23.7)	A-549; MRC-5 (tubes)	118	34 (28.8)	LightCycler/DNA polymerase, thymidine kinase	SYBR Green I dye detection system
Burrows et al. 2002 (57)	262	36 (13.7)		262	75 (28.6%)	LightCycler/DNA polymerase	LightCycler PCR detected and subtyped HSV in 99% (66/67) of HSV-positive specimens, compared to 81% (54/67) by rapid antigen or 57% (36/63) by culture
van Doornum et al. 2003 (506)	668 anogenital (dermal, ocular, oral, bronchoalveolar lavage)	199 (29.8)	Human embryonic lung fibroblasts (24-well microtiter plates), rapid culture technique	688	240 (34.9)	ABI Prism/glycoprotein D, glycoprotein G	17 (culture) and 27 (PCR) specimens were positive for VZV
Wald et al. 2003 (526)	36,471 specimens (mucosal swabs obtained from a variety of anatomic sites) from 296 subjects	1,087 (2.9)	Human diploid fibroblasts (microtiter plates), rapid culture technique	36,461	4,415 (12.1)	ABI Prism/glycoprotein B	Of 4,464 samples positive by more than one test (cell culture and PCR) 3,377 (75.7%) were positive by PCR only; 49 (1.1%) were positive by culture only. This study is the most extensive comparison between virus isolation and detection of a pathogen by PCR. Transport of specimens during summertime likely reduced the detection rate of HSV in cell cultures compared to molecular amplification. This conclusion is consistent with the results of a publication demonstrating the stability of nucleic acids after storage (201)

TABLE 13. Detection of herpes simplex virus DNA by real-time PCR in CSF with and without an internal control

Specimen no.	No. of DNA copies		Internal control result
	With	Without	
1	117,300	108,500	Negative
	15,820	16,500	Negative
	1,959	2,220	Positive
	239	206	Positive
	32	24	Positive
	1	Negative	Positive
2	48	48	Positive
	4	10	Positive
3	1	6	Positive

shell vial cell culture and real-time PCR was highly significant in demonstrating the increased sensitivity and specificity of real-time PCR compared to the cell culture assay ($P \leq 0.0001$) (Table 12). On the basis of these developmental results, the real-time assay replaced the shell vial cell culture assay in May 2000 in our laboratory for the routine detection of HSV infections from these specimens (456). This assay served as a prototype for the ASR assay later commercially developed by Roche Diagnostics Corporation and introduced into our laboratory.

Subsequent trend analysis of the Roche ASR and the shell vial cell culture has demonstrated increased sensitivity (genital specimens, 12%; dermal specimens, 17%) of the Roche LightCycler ASR compared to conventional culture (455, 456). Using the Roche ASR and the melting curve feature of the LightCycler PCR instrument allows differentiation of the two genotypes of HSV. With this HSV assay, about 5% of positive specimens from dermal and genital sources have polymorphisms present so that an intermediate melting curve peak occurs approximately in the middle of the two peaks produced by typical HSV-1 and HSV-2 viruses. We have designated these intermediate strains type A (one polymorphism is present compared with the prototype HSV-1 DNA) and type B (three polymorphisms are present in the probe region compared with HSV-2 DNA) (196). These polymorphisms resulted in an altered FRET probe melting curve, with a peak T_m of 61.8°C for type A and 62.7°C for type B. These fall between the T_m of HSV genotype 1 (55.3°C) and the T_m of genotype 2 (69.7°C) (Fig. 2). These results are consistent with those reported by Anderson and colleagues (8). These intermediate strains obviously represent a unique population of HSV and may have epidemiologic and pathogenic significance compared with wild-type strains of this virus. From a diagnostic standpoint, it is important to recognize that these intermediate strains are identified as HSV but cannot be denoted as either HSV-1 or HSV-2 by this assay without additional testing with intermediate HSV control strains.

Data from at least eight publications, five LightCycler and three ABI and TaqMan, have shown increased detection rates by real-time PCR (range, 20% to 300%) over cell culture methods for diagnosis of HSV infections (Table 12). In our experience, PCR produced a 4.1% increase in the rate of detection of HSV from over 2,500 dermal specimens, representing a 17.2% increase compared with shell vial cell cultures (455).

Varicella-zoster virus dermal disease. Varicella-zoster virus causes both varicella (primary infection, chickenpox) and zoster (reactivated infection, shingles). VZV produces a generalized vesicular rash on the dermis (chickenpox) in unimmunized normal children, usually before 10 years of age. After primary infection with VZV, the virus persists in latent form and may emerge (usually in adults aged 50 years and older) clinically to cause a unilateral vesicular eruption, generally in a dermatomal distribution (shingles). Traditionally, VZV has been detected in the laboratory by the rather slow (2 to 5 days in shell vial cell culture) replication of the virus in cell culture; however, these infections have been more rapidly diagnosed by immunofluorescence and conventional PCR methods (75, 430).

Real-time PCR techniques permit highly sensitive same-day detection of VZV in clinical specimens. At the Mayo Clinic, we compared a LightCycler PCR assay with shell vial cell culture methods for the detection of VZV from dermal specimens in the routine clinical laboratory. This assay served as the prototype for the ASR developed by Roche Diagnostics Corporation (LightCycler VZV ORF29 primer/hybridization probes). VZV DNA was detected in 44 of 253 (17.4%) by real-time PCR, but only 23 isolates of VZV were cultured from these specimens (117) (Table 14). This initial comparison demonstrated a 91% increase in the laboratory diagnosis of VZV infections by real-time PCR compared with cell culture techniques. Subsequent trend analysis of two studies (each spanning a year's period of time), which compared PCR with cell culture confirmed these initial results: 71% increase (455) and 161% increase (456). Two additional studies performed by the ABI TaqMan technology (58.8%) and by LightCycler (240%) methods reported increased detection of VZV DNA compared with cell culture recovery of the virus (437, 506) (Table 14). A real-time PCR assay was developed to differentiate VZV infection due to wild-type virus or vaccine strains of the virus using melting curve analysis (491) (Table 14).

Varicella-zoster virus CNS disease. Neurologic complications after VZV infection, occur most commonly, although not exclusively, in immunocompromised patients, especially those with AIDS and particularly in individuals with a history of or concomitant herpes zoster (92, 224). VZV is a recognized cause of encephalitis, myelitis, radiculitis, and acute meningitis in immunocompromised patients. In a retrospective study, VZV DNA was detected from 5% of CSF specimens (3). Interestingly, real-time LightCycler PCR was positive for VZV DNA (most prevalent herpesvirus detected) in 128 of 1,079 (11.9%) CSF specimens at the Mayo Clinic during a 2-year period (454).

Cytomegalovirus CNS disease. CMV infection can occur in the CNS and clinical presentations are generally in the form of encephalitis, although myelitis has also been described (224). In one study, CMV DNA was detected by conventional PCR in the CSF of HIV patients more frequently than any other herpesvirus (316). CMV DNA is rarely detected in HIV-infected patients without clinical neurological disease. Recently, using a real-time PCR assay, the viral load of herpesviruses, including CMV, was assessed in the CSF (3). Although unclear at this time, assessment of viral load in the CNS may have prognostic

TABLE 14. Detection of varicella-zoster virus DNA in clinical specimens by real-time PCR

Reference	Specimen(s)	Cell culture		Real-time PCR			Comments
		No. of specimens	No. positive (%)	No. of specimens	No. positive (%)	Test platform/ gene target	
Espy et al. 2000 (117)	Dermal	253	23 (9.1)	253	44 (17.4)	LightCycler/gene 28, DNA polymerase; gene 29, DNA binding protein	Real-time PCR was 91% more sensitive than shell vial cell culture assay for detection of VZV DNA.
Furuta et al. 2001 (134)	Saliva (Ramsay Hunt syndrome)			25	13 (52)	ABI Prism/gene 29, DNA binding protein	Analyzed VZV DNA copy number in saliva samples. VZV load in saliva from patients with Ramsay Hunt syndrome peaked near the day of appearance of zoster.
	Saliva (zoster sine herpete)			31	17 (55)		
Aberle et al. 2002 (3)	CSF			576	29 (50)	ABI Prism/gene 31, glycoprotein B	Overall broad testing for different herpesvirus from CSF has led to an increase in the detection rate of those viruses, especially in relation to VZV-associated CNS disease.
Dworkin et al. 2002 (103)	Ocular			10	9 (90)	ABI Prism	Used for detection of VZV DNA from infectious posterior uveitis. PCR target not given.
van Doornum et al. 2003 (506)	Dermal	366	17 (4.6)	366	27 (7.4)	ABI Prism/gene 38	Real-time PCR was 53.8% more sensitive than cell culture (microtiter plates) for detection of VZV infections.
Wiedmann et al. 2003 (530)	CSF, vitreous fluid, dermal, tissue			56 (19 CSF, 6 vitreous, 22 dermal swabs, 9 tissue)	54 (96%)	LightCycler/gene 28, DNA polymerase	Real-time and laboratory-developed nested methods had equal sensitivity for detecting VZV DNA.
Stöcher et al. 2003 (467)	CSF			30	7 (23.3)	LightCycler/gene 28, DNA polymerase	PCR tests were also performed for detection of DNA of CMV, EBV, and HSV-1/2.
O'Neill et al. 2003 (354)	Dermal			68 (some archived specimens used)	29 (42.6)	LightCycler/gene 38	Real-time nested multiplex assay was equal in sensitivity to nested laboratory-developed conventional PCR.
Tipples et al. 2003 (491)	Clinical isolates, Eileen strain, and vaccine strain			18 (14 wild type; 4 Oka vaccine strain)		LightCycler/gene 38	Differentiation of VZV wild-type from vaccine strains was obtained by melting curve analysis of amplified products.
Schmutzhard et al. 2004 (437)	Dermal	110	15 (14)	110	51 (46)	LightCycler/gene 4, transactivator, tegument protein system	Real-time PCR provided 240% increase in detection of VZV infections compared with cell culture.
Campsall et al. 2004 (58)						ABI Prism/open reading frame 62	Assay distinguishes the vaccine strain of VZV (Oka) from wild-type VZV. The assay was 100% concordant with two standard PCR-restriction fragment length polymorphism methods for 136 VZV strains.

implications, may predict distinct CNS manifestations, and may be useful for differentiating between real infection and nonspecific presence of virus in the CSF, especially in severly immunocompromised individuals.

Epstein-Barr virus CNS lymphoproliferative disease. Epstein-Barr virus has been implicated in the development of lymphomas particularly in immunocompromised patients. A review of 26 lymphomas involving the CNS revealed that 9 of 26 (34.6%) occurred in immunocompromised patients after renal transplantation, HIV infection, leukemia, and Wiskott-Aldrich syndrome. EBV sequences were detected in all nine lymphomas, but only 2 of 17 lymphomas occurred in immunocompromised patients (330). In another study, seven of eight patients with posttransplant primary CNS lymphoma had EBV sequences detected by in situ hybridization (377). EBV DNA was detected by conventional PCR in CSF samples from 14 of 49 (27%) of AIDS patients. Eight of the 13 cases had primary CNS lymphoma (49). More recently, real-time PCR assays have been formatted to detect EBV target DNA for the detection of AIDS-related brain lymphoma (3, 39, 488). In one study, of 42 patients, 20 had primary CNS lymphoma and 22 had non-Hodgkin's lymphoma. EBV DNA was detected in the CSF from 16 of 20 (80%) patients with primary CNS lymphoma, 7 of 22 (32%) with systemic non-Hodgkin's lymphoma, and 8 of 12 (67%) with CNS non-Hodgkin's lymphoma (39).

Enterovirus CNS disease. Enteroviruses such as coxsackieviruses A and B, echoviruses, and parechoviruses (previously echoviruses 22 and 23), and poliovirus, are estimated to cause 14% to 21% of all respiratory tract infectious, especially in the summer and autumn months (67). Collectively, these viruses are associated with diverse clinical manifestations ranging from mild febrile illness to CNS (aseptic meningitis, encephalitis), myocarditis, neonatal systemic enteroviral disease, and paralytic poliomyelitis (12, 320). Recovery of enteroviruses in cell cultures is limited by low sensitivity as well as the poor growth characteristics of many serotypes (279). Rotbart described the utility of PCR methods over cell culture methods for rapidly detecting CNS enterovirus infection (419). Several publications have confirmed these results; implementation of this technology in diagnostic virology laboratories has been shown to reduce medical costs incurred by patients by reducing hospitalization and hospital stays and the use of unnecessary antibiotics and antiviral drugs (127, 343, 392, 415, 418).

The recent availability of real-time PCR methods has facilitated the rapid and sensitive detection of enterovirus in the CSF, which is critical for patient care. For example, of 104 CSF specimens, 22 enteroviruses (21.2%) were recovered by cell culture methods, whereas 61 (58.7%) (177% increase) were detected by real-time PCR (319) (Table 15). Real-time PCR assays are directed to amplify conserved target nucleic acid sequences in the 5′-nontranslated region of the virus. However, human parechovirus type 1 (formerly echovirus 22) may not be detected by all PCR assays that use this target (258). Sensitivity for detecting enterovirus cDNA has been shown to be comparable in sensitivity to conventional PCR assays but real-time instruments for PCR were less labor intensive and easier to implement in the clinical laboratory (207, 390) (Table 15).

Polyomaviruses. JC virus (JCV) and BK virus (BKV) were recovered in cell cultures in 1971; BKV was derived from the urine of a renal transplant patient and JCV was from the brain tissue of a patient with Hodgkin's lymphoma complicated by a demyelinating disease, progressive multifocal leukoencephalopathy (298). Present evidence indicates that BKV is strongly associated with nephropathy, especially in kidney transplant patients, as well as patients with hematuria and ureteral stenosis (238, 270). The association of JCV with progressive multifocal leukoencephalopathy in immunocompromised patients, especially those with AIDS, is well documented (102, 272, 278, 521). The clinical significance of these viruses in other diseases such as colorectal cancers and kidney tissues from healthy individuals is controversial (336, 349).

JCV CNS disease. In the early 1990s, conventional PCR of JCV target DNA sequences in CSF specimens replaced histologic examination of brain biopsy tissue for rapid, noninvasive laboratory diagnosis of these infections (479, 480). The simultaneous qualitative, differential detection of JCV or BKV by melting curve analysis of a common target sequence in the VP2 gene was developed with the LightCycler instrument and demonstrated to have performance characteristics comparable to conventional PCR (540). Present evidence indicates that implementation and reporting of qualitative real-time PCR results for JCV in CSF is appropriate even though BKV was found in the CNS of an AIDS patient (43). Nevertheless, additional experience with 400 CSF specimens from immunosuppressed individuals with neurological symptoms has not revealed the presence of BKV DNA by PCR (43).

Simian virus 40 virus, another polyomavirus related to JCV and BKV, has been detected in CNS tissue specimens and may be of more diagnostic significance (perhaps as a coinfection) than BKV in these infections (270, 492).

Parvovirus. B19, previously classified as a parvovirus, is now included in the genus *Erythrovirus* based on preferential replication of this virus in erythroid progenitor cells, as extensively reviewed by Heegaard and Brown (168). Infection with B19 occurs early in life, and the virus is transmitted by respiratory secretions and occasionally by blood products; antibody prevalence ranges from 2% to 15% in early childhood to 85% in elderly adults (87, 168). B19 may result in an asymptomatic infection or produce a wide spectrum of disease ranging from erythema infectiosum (synonyms include slapped cheek syndrome and 5th disease) in children to arthropathy, severe anemia, and systemic manifestations involving the CNS, heart, and liver dependent on the immune competence of the host (54, 410). Infection with B19 in pregnant women may cause hydrops fetalis, congenital anemia, abortion, or stillbirth of the fetus (522).

Most acute infections with B19 are diagnosed in the laboratory by serologically detecting immunoglobulin M (IgM) and IgG class antibodies with enzyme-linked immunosorbent assay (ELISA) testing. PCR detection of target DNA of B19 has had application in the control of transmission of the virus present in blood or blood products such as plasma pools (532). A few real-time PCR assays (ABI TaqMan and LightCycler) have been developed with diagnostic application for detecting B19 DNA in association with infection during pregnancy or assessing the prevalence of the virus nucleic acid in blood products (2, 164, 227, 232, 435, 438).

In one study of 164 nonscreened pools of plasma, 92 (56%) contained B19 DNA as detected by LightCycler DNA; 13 of

TABLE 15. Detection of enterovirus cDNA by real-time PCR

Reference	Culture		Real-time PCR			Comments
	No. of specimens	No. positive (%)	No. of specimens	No. positive (%)	Test platform/ gene target	
Verstrepen et al. 2001 (515)	70	17 (24.3)	70	19 (27.1)	ABI Prism/5′ nontranslated region	Sensitivity of real-time PCR was 100% compared with cell culture.
Read et al. 2001 (403)			50 (originally found positive by conventional PCR)	49 (98)	LightCycler/5′ nontranslated region	Publication described conversion of a conventional multiplex PCR assay that detects HSV-1, HSV-2, VZV, and enterovirus with the LightCycler system.
Corless et al. 2002 (78)	200 (97, CSF; 103, throat swabs) giving previously negative results in cell culture		97 (CSF), 103 (throat swabs)	An additional 33 (15.9) enterovirus and 2 (1) parechoviruses (formerly echoviruses 22 and 23) identified by PCR	ABI Prism/5′ nontranslated region	Real-time PCR was 11.5% more sensitive than cell cultures for the diagnosis of enterovirus infections using CSF specimens. Based on limiting dilutions, the TaqMan enterovirus and parechovirus PCR showed an increase of two orders of magnitude compared to cell culture with a sensitivity of 100% when assessed using enterovirus cell culture-positive samples.
Watkins-Riedel et al. 2002 (528)	60 (stool, 12; CSF, 38; serum, 8; throat swabs, 2)	6/12 feces (50), 2/38 CSF (5.3)	60	6/12 feces (50), 2/38 CSF (5.3)	LightCycler 5′ nontranslated region	Sensitivity of the real-time PCR was 10–100-fold higher compared to AMPLICOR EV test.
Monpoeho et al. 2002 (319)	104 (CSF)	22 (21.2)	104 (CSF)	61 (58.7)	ABI Prism/5′ nontranslated region	Real-time PCR allows a large number of samples to be screened rapidly during an epidemic and its sensitivity, simplicity, and reproducibility make it a highly reliable and suitable tool in the clinical laboratory.
Nijhuis et al. 2002 (344)	41 (feces), 8 (CSF), 43 (bronchoalveolar lavage)	9 (22.0), 3 (37.5), 0	41 (feces), 8 (CSF), 43 (bronchoalveolar lavage)	10 (24.4), 4 (50.0), 2 (4.7)	ABI Prism/5′ nontranslated region	Real-time assay was robust and easily standardized, which make it an excellent alternative for conventional time-consuming viral culture.
Rabenau et al. 2002 (390)			109 (CSF)	23 (21)	LightCycler/5′ nontranslated region	Performance characteristics of real-time PCR were comparable to those using a conventional PCR assay. Compared with the conventional laboratory-developed assay real-time PCR was less labor intensive and easy to use.
Kares et al. 2003 (207)	32	12 (37.5)	55 (CSF, 21; stool, 32; nasopharyngeal aspirate, 3)	32 (58.1)	LightCycler/5′ nontranslated region, SYBR Green I dye detection	Real-time assays were of equal sensitivity to laboratory developed developed real-time PCR test. Fifteen of 20 samples which were negative in cell culture were positive by real-time PCR.
Verboon-Maciolek et al. 2003 (514)	19 (CSF)	4 (21.1)	19 (CSF and serum)	5 CSF (26.3), 9 serum (47.4)	ABI Prism 5′ nontranslated region	Study population was infants ≤60 days old who received a clinical diagnosis of sepsis. Enterovirus infections are an important cause of sepsis in infants admitted to the hospital.

these pools contained more than 10^4 international units (IU)/ml of the *Erythrovirus* genome. Further, of more than 503,000 blood donations, 29 contained more than 5×10^6 IU/ml of B19 DNA (232). Of two real-time PCR kits available commercially (Real Art Parvo B19 LC; Roche Diagnostics) only the Real Art test detected all three genotypes of parvovirus. However, of 140,160 blood units, genotype 1 (detected by both assays), but not genotype 2 or 3, was detected in these plasma specimens (182). Certainly, real-time PCR tests capable of detecting and distinguishing the genotypes of parvovirus B19 will be necessary to determine their clinical imporatance. A recent example is the V9 variant of the virus recovered from skin biopsies from patients with B19-unrelated skin disease (183).

West Nile virus. West Nile virus (WNV), a flavivirus, is transmitted from birds to humans primarily by the *Culex* species of mosquitoes and is responsible for CNS disease, particularly in immunocompromised and elderly patients, with a fatality rate of 7% to 10% (375, 424, 490). Several modes of transmission of WNV have been recognized: blood product transfusion, organ transplantation, and occupational exposure in laboratory workers (4, 5, 424). Serologic detection of IgM (CSF) and IgG (serum) class antibodies to WNV is the standard laboratory procedure for diagnosis infection with this virus, especially in immunologically competent hosts (207, 216). Traditional recovery of the virus in cell cultures for routine laboratory diagnosis is not recommended because of poor sensitivity and safety concerns with the procedures (245, 375).

Detection of target RNA (cDNA) of WNV in CSF or serum specimens can be a valuable adjunctive assay to a serologic diagnosis of infection, especially in patients who do not develop detectable antibodies to the virus (178). In a study of 28 CSF specimens collected during the first 2 weeks of illness from patients with serologically confirmed WNV infections, 16 (57%) were positive by real-time PCR; only 4 of 28 (14%) serum samples from the same patients had detectable WNV RNA (cDNA) (245). In a related study of 10 CSF specimens from confirmed cases of WNV infection, seven were positive by real-time PCR; four of five of these patients died. In this report, no correlation was found between PCR results and either the duration of illness at the time of CSF collection or the presence of IgM class antibody to WNV in that specimen (47).

Even though peak titers of virus in both CSF and serum may be present in the early acute stages of infection and disease, detection of WNV RNA (cDNA) may provide a rapid and early laboratory diagnosis of infection compared with serologic testing (190, 424). Nevertheless, occasionally target nucleic acid of WNV can be detected in blood and CSF specimens several days after the onset of disease symptomatology (245). Further, of 15 blood units tested in a look-back evaluation of blood donors, three were PCR-positive, but all samples were IgM and culture negative for WNV (166).

Detection of WNV target nucleic acid in high volumes of specimens such as insect pools, avian tissues, serum from blood donors, and CSF from patients has been formatted using TaqMan real-time PCR technology (47, 166, 245, 446). At least two commercial sources offer analytic specific reagents or kits for real-time PCR using the LightCycler platform (RealArt WNV reverse transcription-PCR kit, Artus; LightCycler WNV Detection kit, Roche Applied Science) (72).

Respiratory Viruses

Acute respiratory tract infections are a significant cause of morbidity and mortality particularly in the very young and elderly and in immunocompromised patients (33). Predictably, these viruses occur predominantly in the winter and spring seasons of the year (104). In addition to their role in causing common infection of pharynx, eye, and middle ear, these viruses can cause severe systemic complications associated with lower respiratory tract disease, especially in individuals with risk factors such as heart and lung disease and other chronic conditions such as diabetes, kidney disease, asthma, anemia, and other blood disorders.

The classic respiratory viruses have been traditionally identified by inoculation of specimens into a variety of cell cultures. Although early antigenic components of these viruses can be detected as early as 24 h after inoculation of shell vial cultures using monoclonal antibodies, the performance of these tests is dependent on many variables, the most important of which is the lability of these viruses in transit to the laboratory (259, 464). Over a 5-year period at the Mayo Clinic, adenovirus, influenza virus types A and B, and parainfluenza virus represented only 4.2% of the total viruses recovered in this predominantly tertiary-care medical practice. Nevertheless, these viruses required 35% of our total cell culture requirements in the diagnostic virology laboratory (454). Even with the most sensitive and rapid cell culture system, an average of 2 to 3 days were required to detect common viral respiratory infections (101). Because several published comparisons have shown substantial increases in sensitivity of PCR compared with cell culture technology, based on economic factors (expense and labor intensive technology associated with cell culture), and certainly on the performance characteristics of the tests (PCR and culture), laboratories should strongly consider implementation of molecular tests for these respiratory viruses (454, 533).

Influenza viruses. Rapid laboratory diagnosis of influenza is critical for infection control, especially in hospital and nursing home settings. Because of the life-threatening implications of the predicted seasonal occurrence of influenza virus infections, a rapid and accurate identification of both influenza A and B virus genotypes provides the opportunity for intervention with effective antiviral treatment if provided to the patient in the early stages of this viral disease (339). Real-time PCR is considerably more sensitive than cell culture for the detection of influenza virus type A (range, 45.7% to 121% increase) (36, 434).

A recent report from our laboratory indicated that real-time PCR detected 92 of 557 (16.5%) compared to 51 of 557 (9.2%) respiratory specimens inoculated into cell culture. R-mix cell cultures (combined monolayers of human lung carcinoma [A549] and mink lung [Mv1Lu] cells) were stained with monoclonal antibodies between 24 and 48 h postinfection rather than the recommended 24 h. Specimens are batched for several test runs by real-time PCR during the day. The report turnaround time for the real-time PCR method is just a few hours, compared with 24 to 48 h with cell culture technology

(M. J. Espy, S. K. Schneider, P. A. Wright, S. Kidiyala, M. F. Jones, and T. F. Smith. Program Abstr. 20th Annual Clinical Virology Symposium and Annual Meeting of the Pan American Society for Clinical Virology, abstr. M51. 2004).

Rous sarcoma virus. Rous sarcoma virus (RSV) is a major cause of serious lower respiratory tract disease in infants and in adults with underlying cardiopulmonary disease and severely immunocompromised patients especially those individuals with bone marrow transplants and leukemia patients (42, 541). In one study, RSV was the most common virus detected by PCR among children hospitalized for bronchiolitis, pneumonia, or croup (174).

Two major subgroups of RSV are recognized, A and B (538). Similar to PCR for influenza virus, real-time PCR assays for RSV have been shown to be more sensitive compared with direct antigen detection (TestPack) (Table 16). In addition, for three studies, the sensitivity of real-time PCR was 23.6% to 225% greater than that of cell culture systems (122, 154, 508) (Table 16). Immunofluorescence detection of RSV antigen in epithelial cells from the respiratory tract has been an important rapid diagnostic test procedure in the clinical laboratory. For one study evaluating 175 nasopharyngeal specimens, real-time PCR detected 36 (20.6%) RSV-positive samples compared with 32 (18.3%) diagnosed using immunofluorescence (189). In another study, of 75 nasal aspirates from children hospitalized for acute respiratory tract disease, 31 (41.3%) were positive by immunofluorescence and 42 (56%) were positive by real-time PCR (154). These data suggest that PCR could replace cell culture methods and even direct detection of RSV by immunofluorescence for the routine detection of respiratory tract infection caused by RSV (173).

Adenovirus. Limited publications exist which have compared real-time PCR to cell culture for the detection of adenovirus in human specimens (121, 187). Another report indicated the utility for quantifying adenovirus DNA for guiding clinical intervention and assessing response of patients to antiviral therapy (246, 247). Specifically, the real-time PCR assays were designed to detect adenovirus type 4 (subgroup E) in military personnel. The clinical applicability of a real-time PCR assay for adenovirus will require target DNA homologous to the other subgroups of the virus (51 serotypes classified into six groups, A to F) to detect strains associated with respiratory, ocular, and the several other anatomical areas of infection with adenoviruses in immunocompromised patients (230, 414, 513). These retrospective studies demonstrated almost 100% correlation of real-time PCR (ABI and SmartCycler platforms) and cell culture methods with known adenovirus containing specimens (Table 16). Two studies reported equal or greater (35.9%) sensitivity of real-time PCR versus conventional PCR for the detection of adenovirus DNA (151, 170) (Table 16).

Metapneumovirus. In 2001, a new virus from children and adults with acute respiratory tract infections was identified (503, 545). Metapneumovirus is classified among the *Paramyxoviridae*, subfamily *Pneumovirus*, and is closely related to RSV phylogenetically and may have overlapping symptomatology with this virus (63). Reliable detection of this virus may require lengthy incubations times (up to 17 days) after inoculation of cell cultures such as tertiary monkey kidney or LLC-MK2 cells (161). Generally, several types of cells (HEp-2, LLC-MK-2, and MDCK) are required to detect the maximum number of positive specimens containing metapneumovirus. In addition, cytopathic effects are not detected in any of the cells until 10 to 12 days after inoculation. Sometimes subculture (subpassage) is required to confirm cytopathic effects in cell cultures (63).

Molecular detection has indicated that this virus can infect all age groups, producing substantial clinical and economic impact (307). Studies in the Netherlands indicated that by 5 years of age, nearly all individuals have been exposed to metapneumovirus; worldwide, this virus may account for at least 5% to 75% of respiratory tract infections in hospitalized children (203, 504). Real-time PCR assays were found to be as sensitive as conventional PCR for detection of metapneumovirus cDNA (84, 295, 296) (Table 16). Because of the technical advantages of using real-time PCR for the rapid and sensitive detection of metapneumovirus, this method would be preferable to the variable diagnostic results produced by cell culture isolation and identification of this virus.

Parainfluenza virus. Parainfluenza viruses (types 1 to 4) have traditionally been associated with croup, bronchiolitis, and pneumonia in infants and children; however, they also produce significant disease in elderly and immunocompromised patients (20, 33, 160, 483, 537). Ideally, molecular amplification tests for community acquired pneumonia due to the usual viruses (and bacteria) which cause lower respiratory tract involvement with overlapping clinical features, need to be bundled according to clinical practice guidelines using test algorithms developed by clinical and laboratory practice personnel. Recent publication of a rapid and sensitive multiplex real-time PCR assay for the laboratory diagnosis of influenza viruses A and B, RSV, and four serotypes of parainfluenza viruses demonstrated a 30% increase of these respiratory tract viruses compared with cell culture recoveray of those agents (483).

Severe acute respiratory syndrome coronavirus. Severe acute respiratory syndrome coronavirus (SARS-CoV) causes a highly contagious atypical pneumonia which is spread by respiratory secretions and airborne transmission. From November 2002 to July 2003, a total of 8,464 cases were reported, resulting in 799 deaths and a fatality rate of 9.4% (382, 425). Recovery and identification of SARS-CoV in cell cultures is hazardous for routine clinical laboratories because of the risk of laboratory-acquired infections with this virus and biosafety level 3 laboratory facilities are required for cell culture recovery and identification of this virus (548).

Experience from the Chinese University of Hong Kong indicated that the yield of diagnostic virus isolation was much lower than by PCR testing. No specimen was positive by culture but negative by PCR (64). Alternatively, inactivation of the SARS-CoV by autoclaving prior to testing by real-time PCR may provide the potential for the safe processing of the specimen by laboratory personnel (119). Early recognition and containment of a reemergent outbreak of SARS-CoV depends on the vigilance and awareness of physicians and allied health personnel to recognize the clinical, epidemiologic, and laboratory criteria compatible with the published criteria of a case definition of possible infection with this infection (16).

The laboratory can play a critical role to document the etiology of the respiratory tract infection recognizing the overlapping clinical features of SARS-CoV with other viruses such as influenza virus A and B which may be circulating in popu-

TABLE 16. PCR for viruses

Virus	Reference	Culture			Real-time PCR			Comments
		No. of specimens	No. positive (%)	Cells or antigen	No. of specimens	No. positive (%)	Test platform/ gene target	
Influenza virus[a]	Ward et al. 2004 (527)	233 throat swabs	50 (21.5)	MDCK, tertiary cynomolgus	233	140 (60.0)	ABI Prism/M1 matrix gene	Real-time assays were designed for both A and B viruses. Large-scale screening and identification of influenza virus using real-time PCR was carried out as part of the development of zanamivir.
	van Elden et al. 2001 (507)			Tertiary rhesus monkey kidney cells	27 reference strains (A), 9 reference strains (B), and other isolates	A, 36 (37), B, 4 (4)	ABI Prism/matrix protein (A), hemagglutinin gene (B)	Influenza virus could be detected in nasal wash specimens up to 7 days after initial presentation of influenza-like symptoms.
		98 clinical specimens	22 (22.4)	Shell vial	98			
	Spackman et al. 2002 (458)	1,550 cloacal, tracheal, or environmental swabs from various avian species	266 (17.2)	Embryonated eggs	1,550	303 (19.6)	ABI Prism/matrix gene (conserved for all A matrix genes; in addition, primer sets were developed to H5 and H7 strains of avian influenza virus; broad-range detection including both North American and Eurasian lineage avian viruses and isolates of human, equine, and swine origin influenza A virus	Recovery of influenza virus requires 1–2 weeks in embryonated eggs.
	Smith et al. 2003 (453)	58 throat and nasal swabs	35 (60.3) (25 A; 10 B)	Rhesus monkey kidney cells/ tube culture	58	51 (88.0) (41 A; 10 B)	LightCycler/A matrix (300 bp) protein (5′ end of the matrix gene M1), B nucleoprotein (184 bp); LC run parameters were identical for A and B	4 LightCycler (positive only) results sequenced. Analysis of fragment matched the M1 gene of influenza virus A. B strains, frozen supernatants previously known to contain influenza virus B assay tested with H_3N_2 and H_1N_1 strains and two influenza B viruses (Beijing-like).
	Boivin et al. 2003 (37)	Nasal and pharyngeal swabs					LightCycler/hemagglutinin gene	Study assessed kinetics of the influenza virus load in respiratory tract samples of infected individuals receiving early treatment with neuraminadase inhibitors compared to those receiving deferred treatment. The mean pretreatment virus load was significantly lower in 24 patients who initiated treatment within 24 h of the onset of symptoms than it was in 26 patients who initiated treatment between 24 and 48.

	Boivin et al. 2004 (36)	172 (subset of nasopharyngeal aspirates from hospitalized children)	A: 19 (11.0), B: 0	Compared to rapid antigen assays for A and B (Becton Dickinson)	172	42 (24.4)	LightCycler/matrix genes of both A and B	Melting curve feature of LightCycler instrument was used to differentiate influenza strains from RSV. Pediatric study ≤3 yr of age.
	Frisbie et al. 2004 (132)	75 children ≤4 yr; archived nasal aspirates	22 (29.3) archived nasal aspirates	Rhesus monkey kidney cells	75	18 (24)	ABI Prism/A matrix gene; B hemagglutinin gene	Repeat testing by culture of 21 of 22 positive archival specimens revealed only 11 positive results; all 21 were PCR positive by repeat testing.
	Espy et al.[b] 2004	557 respiratory tract specimens (throat swabs, nasal washes, bronchoalveolar lavage, sputum, nasal swabs)	51 (9.2%) R-mix 24 (4.3%) BINAX antigen test	R-mix	557	92 (16.5%)	LightCycler/matrix gene	LightCycler PCR was rapid (3 h extraction and analytic time) and more sensitive than R-mix cell cultures and BINAX for the detection of influenza virus type A (H_3N_2) strains from clinical specimens.
Respiratory syncytial virus	Boivin et al. 2003 (36)	204 nasopharyngeal aspirates from children <3 yr of age	94 (46)	RSV TestPack antigen test	204	104 (51)	ABI Prism/F gene	Authors developed a multiplex test to differentiate RSV and influenza A and B virus amplicons by melting curve analysis.
	Borg et al. 2003 (38)				62 acute respiratory tract infection (children); nasopharyngea1 secretions	29 (46.8)	ABI Prism/F1 gene (subunit of fusion protein)	Real-time (quantitative) assay specific for subgroup A was developed. The median viral load of the specimens from patients with chronic obstructive pulmonary disease was 6.1×10 copies/ml compared with median of 1.2×10^7 copies/ml in children with respiratory tract infection.
					125 adults with chronic obstructive pulmonary disease, nasal lavage fluid and induced sputum	35 (28)		
	Falsey et al. (122)	169, 13 adult volunteers, nasal washes	58 (34)	HEp-2	169	73 (43)	ABI Prism/F gene	Real-time assay detects both RSV A and B subgroups.
	Gueudin et al. 2003 (154)	75 nasal aspirates	34 (45.3)	MRC-5 and A-549	75	42 (56%)	LightCycler/N gene	Direct immunofluorescence was positive in 31/75 (41.3%) of samples.
	Hu et al. 2003 (189)	175 nasopharyngeal aspirates from children	21 (12), immuno-fluorescence		175	36 (20.3) (10 RSV A, 26 RSV B)	ABI Prism/N gene	Assay detects RSV A and B subgroups. Immunofluorescence technique identified 32/75 (42.7%) RSV-positive samples.
	Mentel et al. 2003 (313)				71 71 71	25 (35.2%) (Real-Time) 19 (26.7%) Nested, conventional PCR 10 (14.1) Antigen ELISA (Virion\Serion)	iCycler/F gene	71 consecutive specimens were processed and tested by PCR from hospitalized children with clinical symptoms of acute respiratory distress to obtain a rapid laboratory diagnosis of RSV infection.

Continued on following page

TABLE 16—*Continued*

Virus	Reference	Culture			Real-time PCR			Comments
		No. of specimens	No. positive (%)	Cells or antigen	No. of specimens	No. positive (%)	Test platform/gene target	
	van Elden et al. 2003 (508)	168 (during period of symptoms), combined nose and throat swabs from immuno-compromised adults (autologous or allogenic stem cell transplant patients (n = 73), hematologic malignancies (n = 17)	4 (2.4%)	Shell vial rhesus monkey kidney cells	168	13 (4.9%) (Real-time PCR) 13 (4.9%) Laboratory developed nested PCR	ABI Prism/N gene	Real-time assay detects both RSV A and B subgroups. Detection of RSV cDNA by nested PCR and real-time PCR is equivalent.
Adenovirus	Houng et al. 2002 (187)	96 throat swabs from military personnel with acute respiratory tract disease	72 (previously positive Ad4 strains isolated in cell cultures)	A-549 cell cultures (human lung carcinoma)	96 specimens selected for positive results by previous inoculation in cell cultures from 1953 through 1998 and then tested by PCR in 2001	71	ABI Prism/Ad4 hexon gene	Quantitative real-time PCR assay used to detect Ad4 (subgroup E) DNA in specimens from military recruits during an acute respiratory disease outbreak. The assay did not crossreact with representative members of adenovirus subgroups A, B, C, D, and F.
	Gu et al. 2003 (151)				45	20 (44.4)	ABI Prism/hexon gene	Real-time quantitative PCR was designed to detect adenovirus DNA from all major subgroups of the virus.
					45 (conventional PCR)	20 (44.4)		
	Heim et al. 2003 (170)				234 (real-time PCR)	53 (22.6)	LightCycler/hexon gene	Adenovirus DNA was detected in blood by real-time (quantitative) PCR in 4/27 (14.8%) pediatric and 8/93 (8.6%) of adult stem cell transplant patients but only in 5/306 healthy blood donor controls (1.6%). Detection of high virus loads in blood holds promise for simplified and earlier diagnosis of disseminated adenovirus disease in immunosuppressed patients.
					234 (conventional PCR)	39 (16.6)		
	Lion et al. 2003 (277)				132 (patients)	36 (27.2) (patients); positive results from at least one specimen	ABI Prism/hexon gene	In this series, 8/11 (73%) children with adenovirus DNA in blood specimens, but none of the patients with adenovirus DNA detectable at sites other than blood, developed fatal disseminated adenoviral disease.

	Faix et al. 2004 (121)	140 throat swabs from 86 subjects	99 (70.7)	A-549 cell cultures	140	98 (70%)	SmartCycler/Ad4 hexon gene	Assay developed by Houng et al. (187) was adapted to the SmartCycler instrument for the detection of adenovirus DNA in specimens from military recruits during an acute respiratory disease outbreak. Overall, rapid PCR results had a sensitivity of 100% and a specificity of 100% compared with viral culture.
	Leruez-Ville et al. 2004 (261)				44 plasma	8 (18.1)	ABI Prism/hexon gene	All 8 patients for whom PCR detected adenovirus DNA in blood samples had disseminated adenovirus infection.
	Lankester et al. 2004 (246)						iCycler IQ/hexon gene	Four allogeneic stem cell transplant patients were treated with ribavirin and subsequently with cidofovir. Quantitative real-time PCR determinations indicated that these antiviral drugs did not reduce levels of adenovirus DNA.
Meta-pneumo-virus	Côté et al. 2003 (84)			LLC-MK2 (continuous monkey kidney cell line)	20 specimens selected as probable positive cultures for metapneumovirus 10 nasopharyngeal aspirates	20 (100%) with nucleoprotein, N gene target 10 (100%) with nucleoprotein, N gene target	LightCycler/nucleoprotein, matrix, fusion, phosphoprotein, polymerase gene targets	PCR is method of choice for metapneumovirus laboratory diagnosis because of poor replication of the virus in cell culture.
	Mackay et al., 2003 (295)				62 nasopharyngeal aspirates 6 known PCR-positive and 56 known PCR-negative specimens (conventional methods)	12 (19.4) real-time PCR 6 (9.7) Conventional PCR	LightCycler/nucleoprotein gene target	Real-time PCR was the preferred choice for detecting metapneumovirus and was considerably more reliable than cell culture in the routine clinical laboratory.
	Maertzdorf et al., 2004 (296)				38 clinical samples found positive by conventional PCR 54 clinical samples known as negative by conventional PCR	38 (100) 0	ABI Prism/nucleoprotein gene target	Human metapneumoviruses can be divided into two main genetic lineages (A and B) representing two serotypes and each comprising two sublineages (A1, A2, B1, B2). This assay detects all strains of the virus.
Para-influenza virus	Templeton et al. 2004 (483)	358	11 (3.1%)	HEp-2, HEL, and $LLCMK_2$	358	4 (1.1)	iCycler IQ/accession nos. PI-1-70948, PI-2-AF213352, PI-3-M18760, PI-4-M55976	Rapid real-time multiplex PCR assay was developed for the detection of influenza A and influenza B viruses, RSV, and parainfluenza viruses 1, 2, 3, and 4.

Continued on following page

TABLE 16—*Continued*

Virus	Reference	Culture			Real-time PCR			Comments
		No. of specimens	No. positive (%)	Cells or antigen	No. of specimens	No. positive (%)	Test platform/ gene target	
	Templeton et. al. 2004 (482)	Case report: parainfluenza virus type 3 detected by direct immunofluorescence from nasal wash specimens.					Parainfluenza virus 1 (accession no. 70948); parainfluenza virus 2 (AF213352); parainfluenza virus 3 (M18760); parainfluenza virus 4 (M55976); iCycler IQ (molecular beacon technology)	PCR was found to be far more sensitive than culture or immunofluorescence in an immunocompromised host (post-stem cell transplantation) and results of this assay (real-time PCR) could improve management of patients.
		Parainfluenza virus type 3 detected by culture (child with acute lymphoblastic leukemia who received a hematopoietic stem cell transplant)						
SARS-CoV	Poon et al. 2004 (382)				98 nasopharyngeal aspirates	43 (44)	ABI/TaqMan/ORF16	
					36 stool samples	21 (57)		
	Drosten et al. 2004 (99)				66 samples from 29 confirmed SARS patients; 31 respiratory specimens	47 (70.8)	LightCycler/replicase gene (Real/Art LC kit)	
					35 stool and other specimens	44 (67.1)	LightCycler/nucleocapsid gene	
	Chan et al. 2004 (64)	531 respiratory tract	45 (8.4)	Vero E6	471 respiratory tract	122 (25.9)		No specimen was positive by culture and negative by PCR.
		526 nonrespiratory	4 (0.76)		365 nonrespiratory	83 (22.7)		
	Ng et al. 2003 (337)				36 plasma	15 (41.6)	ABI/TaqMan/ polymerase gene	Samples collected over 2 weeks.
					23 serum	18 (78.2)	ABI/TaqMan/ nucleocapsid gene	Samples collected on day of hospital admission.

[a] In this section, A and B refer to influenza virus types A and B, respectively.
[b] M. J. Espy, S. K. Schneider, P. A. Wright, S. Kidiyala, M. F. Jones, and T. F. Smith, Program Abstr. 20th Clin. Virol. Symp., abstr. M51, 2004.

lations at the same time (16, 68, 72). Currently, real-time PCR reagents are available from at least two commercial manufacturers (Artus: RealArt HPA-Coronavirus RT PCR Kits for LightCycler; ABI Prism 7000, 7700, 7900H; and RotorGene; and LightCycler SARS-CoV, Roche Diagnostics) (72). Early identification and documentation of SARS CoV infection (based on a firm laboratory diagnosis) on a global basis may control the transmission of this highly contagious infection by effective use of isolation and quarantine measures for patients and area contacts (11, 252).

Compared to serology, the use of real-time PCR technology is critical since target nucleic acid of the virus can be detected in specimens from patients in the early stages of infection (Table 16). Poon et al. found that of 50 nasopharyngeal aspirates collected 1 to 3 days after onset of disease, 40 (80%) were positive for SARS-CoV target nucleic acid (383). However, SARS-CoV has been found in sputum, throat swabs, serum, lung, kidney, bone marrow, and feces by real-time PCR targeting sequences in the nucleocapsid and RNA polymerase (*ORF1b*) genes of the virus (46, 240, 252, 297, 337). Specimens (especially feces) obtained about 10 days from symptom onset are associated with the highest yield for all specimen types, which correlates with the timing of peak virus loads (68). Nevertheless, the relative productivity of each specimen type for detection of SARS-CoV needs to be assessed before negative results by real-time PCR assays can be used to rule out the presence of this viral infection (474).

Importantly, the performance of one commercial (RealArt HPA) and six laboratory-developed conventional and real-time (LightCycler) PCR assays were compared for the detection of SARS-CoV in clinical specimens (297). Of 68 clinical specimens (17 respiratory tract specimens, 29 urine samples, and 22 stool or rectal swabs specimen), six of seven assays detected at least 17 of 18 positive results (defined as positive in at least two assays), and two of the assays had a sensitivity of 100%. There was no significant difference in the sensitivity between the assays ($P = 0.5$). In another study, sensitivities of 70.8% (Artus) and 67.1% (Roche) were obtained with 66 specimens from patients with confirmed SARS. The authors emphasized that PCR should not be used to comprehensively rule out SARS (99).

Poxviruses

Variola virus is a large, brick-shaped particle containing DNA and belongs to the *Orthopoxvirus* genus of the family *Poxviridae* (114). Other members of this genus include monkeypox, cowpox, racoonpox, skunkpox, and ectromelia viruses; although very uncommon, recent reports indicate that these infection can occur, especially after human contact with infected animals (95, 131, 267, 322, 404). Almost all dermal lesions due to viruses in routine laboratory practice are caused by HSV and VZV; however, immediate recognition of the clinical features of smallpox and differentiation of variola virus from other virus infections involving the skin is of paramount importance. Most importantly, the finding of a suspected case of smallpox must be considered as an international health emergency and be brought to the attention of national official through local and state health laboratories (171).

The melting curve feature of the LightCycler PCR instrument was particularly adaptable for the differentiation of several members of the orthopoxvirus genes but particularly for the specific identification of variola virus from cowpox and vaccinia virus; this test served as the first real-time assay for the detection of those viruses (114) (Table 17). For this particular assay it is still important to consider clinical and epidemiologic characteristics of the patient to associate vaccinia (previous immunization) or cowpox (animal contact) to the infection since the melting temperature differed for those two viruses by less than 1°C. Subsequently, in a recent study, one LightCycler assay was able to resolve all nonvariola orthopoxviruses by the simultaneous use of four hybridization probe-based real-time PCR assays (345). Separate reaction vessels with specific primers and probes would be required to achieve this level of identification of orthopoxviruses with real-time platforms which do not have melting curve features.

Because of the laboratory safety concerns of infection to individuals processing specimens for the diagnosis of possible variola virus infection from high-risk patients, specific tests to identify this virus should be carried out by trained personnel in a biosafety level 4 facility such as exists at the Centers for Disease Control. However, for clinically evaluated low-risk patients for variola virus infection, specimens could be processed in appropriate facilities (Laboratory Response Network Laboratories) for viruses such as HSV, VZV, enteroviruses, and vaccinia virus. As an additional safeguard for the laboratory, the specimens can be autoclaved, under controlled conditions, before testing the sample for the presence of viral target nucleic acids by real-time PCR (119). Autoclaving had no detrimental affect on the amplification of target DNA from HSV, VZV, and vaccinia virus (119).

QUANTITATIVE VIRAL ASSAYS

Real-time PCR provides a tool in the clinical laboratory for providing quantitative results of viral target nucleic acid present in a clinical sample. The results of quantitation of a viral nucleic acid target determination in a blood specimen, for example, may be applicable for assessing the relationship between the viral load (i.e., copy level) of a viral target and the prediction of the progression of infection to clinical disease. Quantitative test results for nucleic acid targets have become especially relevant with serial specimens from transplant patients to monitor for evolving sypmptomatic infection or for assessing the effectiveness of antiviral therapy.

Technically, quantitative real-time PCR is performed by the addition of standards which have known specified or calibrated levels of target nucleic acid. Three to five dilutions of a standard are included in each test run of each quantitative real-time PCR determination. Using the known copy level of the standard reagent, the software of the instrument generates a standard curve in a plot that relates fluorescence (measure of amplified product) and the cycle number in which the nucleic acid target is detected. Quantitative detection of viral nucleic acid is determined by comparing the cycle number (crossover point or C_p) of the specimen with the standard curve generated with known levels of the target nucleic acid. Quantitative standards (e.g., EBV DNA) from commercial sources are helpful for developing quantitative tests for viral load levels. Alternatively, nucleic acid from viruses cultivated in cell cultures (or

TABLE 17. Detection of othopoxvirus DNA by real-time PCR

Reference	Culture			Real-time PCR			Comments
	No. of specimens	No. positive (%)	Cells	No. of specimens	No. positive (%)	Test platform/gene target	
Espy et al. 2002 (114)				Reference strains. American Type Culture Collection: vaccinia virus (vr-117); cowpox virus (vr-302); monkeypox virus (vr 267). Centers for Disease Control: vaccinia virus; variola virus (smallpox); plasmid insert of a 300-bp segment of the hemagglutinin gene		LightCycler PCR/hemagglutinin gene	Variola virus (T_m, 62.45°C) could be differentiated from vaccinia (T_m, 56.72°C) and monkeypox (T_m, 56.24°C) viruses.
Sofi Ibrahim et al. 2003 (457)				Centers for Disease Control: variola virus, (smallpox): 48 strains of camelpox, cowpox, ectromelia, gerbilpox, monkeypox, rabbitpox, raccoonpox, skunkpox, vaccinia, herpes simplex virus, and varicella-zoster virus		LightCycler, SmartCycler/ hemagglutinin gene	Both PCR platforms were considered acceptable for the detection of variola virus DNA.
				322 coded samples; 48 different stains (above)	SmartCycler: 2 samples with false-positive results among 116 samples not containing variola virus (specificity, 98.3%): LightCycler: 5 samples with false-positive results among 116 samples not containing variola virus (specificity 95.7%)		
Kelly et al. 2004 (217)						ABI Prism, Laboratory Response Network assays for vaccinia virus and for varicella-zoster virus.	Publication reports the detection of vaccinia virus-specific DNA in lesions of patients previously immunized against smallpox.
Kulesh et al. 2004 (241)				16 coded samples: 8 variola virus (smallpox); 5 non-variola virus, orthopox virus isolates; 2 varicella-zoster virus; 1 herpes simplex virus		LightCycler, SmartCycler/ hemagglutinin gene. SmartCycler detected 92.3% of the orthopoxvirus DNAs and between 95% and 93.8% of the variola virus DNAs. LightCycler detected 96.2% of the orthopox DNAs and 93.8% of the variola virus DNAs.	Both LightCycler and SmartCycler had 100% sensitivity with both platforms with samples above the limit of detection (≥12 gene copies).
Nitsche et al. 2004 (345)						LightCycler/genes: *A13L*, *rpo18*, *VETF*	A complete set of four hybridization probe-based real-time PCR assays provide for the specific detection of orthopoxvirus DNA by melting point analysis.
Olson et al. 2004 (352)						LightCycler/fusion protein gene	PCR primers were designed to detected all Eurasian-African species of orthopoxvirus. A single nucleotide mismatch in the target sequence allowed differentiation of variola virus from other orthopoxviruses by melting-curve analysis.

target nucleic inserted into a plasmid) may also be used to generate standard curves for quantitative assays; however, these reagents should ideally be obtained from commercial sources to ensure uniformity of results.

Cytomegalovirus

At the present time, many real-time quantitative tests for CMV DNA have been formatted on either the ABI or the LightCycler instruments. Choice of either instrument depends on work flow issues in the laboratory rather than specific performance characteristics of the platforms. These platforms offer unique performance features of precision and reproducibility of test results; nevertheless, the customized formatting of test procedures using these instruments is highly variable among laboratories (Table 18). For example, of 26 articles in which TaqMan probes were used, there were 10 different gene targets, at least three different units of result reports (CMV DNA/μl, CMV DNA/10^5 peripheral blood leukocytes, CMV DNA/μg of human DNA), and four different specimen compartments of blood (whole blood, plasma, peripheral blood leukocytes, and white blood cell-reduced blood). Nevertheless, compared to conventional PCR, optimization of these important variables can be achieved by real-time instrumentation with common operational profiles, reagents, and standards. The compartment of blood used as the optimal specimen may vary according to the stage of viral replication in an individual patient (400). For example, the presence of CMV DNA in plasma may be associated with active viral replication and disease development relative to other specimens (149).

Several publications using either LightCycler or ABI Prism 7700 (TaqMan) have reported comparisons for the detection of pp65 matrix protein of CMV with real-time PCR (Table 18). Molecular amplification has several important advantages to detection of CMV antigen (the antigenemia test) even though some studies indicated general agreement between the two methods. In general, quantitative real-time PCR has several advantages to the antigenemia test including increased sensitivity for early detection of CMV infection or reactivation, utility for patients with neutropenia, stability of target DNA in blood specimens, wide detection range (7 to 8 $\log_{10}$) of CMV DNA, ability to process large number of specimens, flexibility of time of transport and processing of specimens, and the potential for increased accuracy of results through precision instrumentation (133, 309, 346, 549).

Standardization, implementation, and result interpretation and reporting will ultimately depend on uniform guidelines and availability of commercial products to obtain uniformity among laboratories. Among the important variables are threshold copy levels of CMV DNA significant for low- and high-risk patients to guide antiviral treatment regimens and for different patient populations of organ transplantation patients who have unique demographic and medical management characteristics. Technically, the calculation of the concentration (viral load) is critically dependent on the accuracy of both the copy number of the CMV target in a plasmid standard used to establish a standard curve for quantitation, but also on the calculations of CMV DNA/ml of specimen introduced into the PCR mixture prior to amplification. Interpretation of results need to be guided by the compartment of blood sampled (serum, plasma, leukocytic) which may yield maximal copy numbers of CMV DNA at different stages of this viral infection. Finally, probit regression analysis, (probability of achieving e.g., 95% positive results at a low copy level of target DNA), and an electronic display of trend analysis results for ease of interpretation by attending physicians should be integrated into this laboratory practice (149). It is recognized, however, that graphic trend analysis of sequential quantitative results is an unmet challenge for most laboratory information systems.

Epstein-Barr Virus

Several malignancies have been associated with EBV infections, especially in immunosuppressed patients who lack antibody to the virus. These include posttransplant lymphoproliferative disorders, Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, gastric carcinoma, breast cancer, and hepatocellular carcinoma (158, 239). As previously discussed, some EBV-associated malignancies can occur in the central nervous system especially in HIV patients. A recent report indicates that EBV may contribute to the pathobiology of multiple sclerosis in children (6).

Quantitation of EBV DNA in these patients provides the potential for the designation of viral load (threshold) levels generally associated with healthy or subclinical carriers of EBV (reactivated infection) compared with those levels of virus that produce disease states such as posttransplant lymphproliferative disorder in transplant patients (158). Viral load levels obtained during the posttransplantation course may also provide the clinician with information for initiating and monitoring response to therapy. From a clinical perspective, quantitative viral load information may guide a preemptive strategy to reduce the incidence and level of EBV reactivation in transplant patients by administration of antiviral agents when target EBV DNA or significant levels of EBV DNA are detected. Similar to quantitative CMV assays, the majority of real-time PCR quantitative assays for detection of EBV DNA, have been developed and formatted using the ABI 7700 Prism instrument and TaqMan probes. Dilution of target EBV DNA (mostly DNA polymerase, BALF-5) inserted into plasmids have been prepared and amplified by real-time PCR to produce the standard curves for quantitative assays. The expected linear range of detection by either the ABI or LightCycler real-time PCR platform spans 10^7 to 10^8 $\log_{10}$ copies/ml.

Practical clinical applications of EBV viral load determinations by real-time PCR to reduce the incidence of EBV reactivation and replication and the subsequent development of EBV related lymphomas (posttransplant lymphproliferative disorder) have been demonstrated. Generally, quantitative assays are oftentimes performed three to five times each week enabling the investigators to determine the patterns and trends of EBV replication in solid organ transplant patients (342, 509). For example, several publications from the University Medical Center, Rotterdam, The Netherlands, have shown a correlation between EBV viral DNA loads and the likelihood of development of posttransplant lymphproliferative disorder (341, 342, 509–511). A threshold of 1,000 copies of EBV DNA/ml plasma was chosen to begin each treatment with rituximab, a monoclonal antibody directed against the CD20 receptor binding site for EBV (510). This resulted in a com-

TABLE 18. Quantitative detection of cytomegalovirus DNA by real-time PCR[a]

Platform	Reference	Specimen	Target	Quantitative standard	Reporting units	Comparison to other systems	Comments
ABI Prism	Machida et al. 2000 (293)	16 patients (bone marrow transplant); 136 blood samples from patients: 70 blood specimens from healthy volunteers	US17 gene	Range: 10 to 10^7 CMV DNA copies/well	Copies of CMV DNA/500 mg of DNA (blood), copies CMV DNA/100 μl plasma	Real-time PCR compared with antigenemia assay and by shell vial cell culture.	CMV DNA was not detected in blood specimens of the 55 healthy patients seropositive for CMV. The results of PCR (plasma) did not correlate well with those obtained by the antigenemia test; however, PCR (nucleated cells from blood) had high correlation with the antigenemia test results.
	Nitsche et al. 2000 (347)	Plasma; bone marrow transplant patients	Major immediate-early gene	As a positive control, a plasmid containing the target sequence from the target gene was used with 10^1 to 10^7 plasmids/assay	Copies CMV genome/ml plasma	CMV load was higher in CMV antigen-positive (antigenemia) patients than in antigen-negative patients.	CMV DNA was detected from 5/27 healthy blood donors seronegative for CMV.
	Tanaka et al. 2000 (473)	Peripheral blood leukocytes, plasma	Immediate-early gene	Range from 6 to over 10^6 copies of CMV DNA, Plasmid containing the IE gene used to develop a standard curve for quantitative results.	Copies CMV DNA/10^6 cells	Real-time PCR compared with antigemia assay.	The correlation between the CMV DNA copy number and the pp65-positive cell count (antigenemia test) was statistically significant ($P = 0.01$). CMV DNA copy level/10^6 cells was higher in symptomatic patients (blood, plasma specimens) than in asymptomatic patients.
	Funato et al. 2001 (133)	Whole-blood samples from infants with hepatitis	Major immediate-early gene	A plasmid containing the target gene of CMV was constructed and used as a quantitative standard. Reference value for the CMV DNA copy numbers was determined as 10^9 molecules/3.7 ng/μl based on the molecular weight of the DNA inserted into the plasmid.	Copies CMV DNA/μg DNA	Conventional PCR and real-time PCR were compared for the detection of CMV DNA in this patient population.	No CMV DNA was detected in 97 healthy patients by conventional or by real-time PCR methods. Eighteen samples tested negative by conventional PCR: 14 samples were negative; four samples contained CMV DNA with low copy levels of CMV DNA (10–80).
	Gault et al. 2001 (138)	Blood (peripheral blood leukocytes) (46 samples)	UL83 (pp65 gene)	Plasmid containing one copy of the UL83 target sequence used as a quantitative standard plasmid containing human genomic DNA (albumin gene) coamplified with specimen DNA.	Copies CMV DNA/2 × 10^5 leukocytes	Known pp65 antigenemia-positive (PBL samples) collected from solid organ transplant patients were amplified by real-time PCR.	The results of the real-time PCR assay correlated with those of the antigenemia assay ($P < 0.0001$).
	Griscelli et al. 2001 (149)	Peripheral blood leukocytes of bone marrow transplant patients	UL83, pp65 gene (phosphorylated matrix protein)	A plasmid containing both the CMV and the glyceraldehyde-3-phosphate dehydrogenase genes was used as a standard.	Copies CMV DNA/200,000 leutkocytes	Real-time PCR was compared to the antigenemia test.	Study quantified CMV DNA in the glyceraldehyde-3-phosphate dehydrogenase gene using a plasmid containing both sequences of 16 patients, CMV DNA was detected by PCR in 13/16 (91.3%), a mean of 15 days prior to the appearance of antigenemia.

Guiver et al. 2001 (156)	Blood, 362 samples from 25 patients; 12 single lung, 13 heart transplant	Glycoprotein B gene	Plasmid standard of CMV DNA diluted to contain 10 to 10^6 copies/PCR	Copies CMV DNA/ml (limit of sensitivity was 500 copies/ml blood based on sampling volume of 2 μl of EDTA treated blood following extraction.	Real-time PCR was compared to CMV antigenemia test.	Viral loads of real-time PCR showed a highly significant linear correlation with antigemia levels.
Limaye et al. 2001 (271)	Plasma samples from stem cell transplant patients	Immediate-early gene		Copies CMV DNA/ml		CMV DNA was detected a median of 13 days before onset of CMV disease (range, 0–35 days). Monitoring plasma CMV DNA loads may be useful in the prevention of pre-engraftment CMV disease.
Najioullah et al. 2001 (329)	Blood (serum) (positive for CMV in cell cultures) from transplant patients	HXFL4 gene	Plasmid dilutions from 5×10^2 to 2×10^8 copies of CMV DNA/ml were tested. (four dilutions were used to develop a standard curve in the quantitative test). CMV DNA obtained from Advanced Biotechnologies used as a positive control for real-time PCR.	Copies CMV DNA/μg DNA	Real-time PCR and conventional PCR compared with positive cell culture results obtained with blood specimens.	The qualitative PCR was positive in 48 samples and the quantitative real-time assay was positive in 46 samples from a total of 50 cell culture-positive specimens.
Sanchez et al. 2001 (426)	Blood specimens from lung transplant recipients	DNA polymerase gene, human apoprotein B gene		Copies CMV DNA/μg human DNA		Risk of CMV pneumonitis after lung transplantation is related to the level of CMV DNA in blood.
Satou et al. 2001 (429)	Whole blood	Major immediate-early gene	Target gene inserted into a plasmid.	Copies CMV DNA/μg DNA	Results of real-time PCR correlated with conventional PCR and with serology in several specimens from one liver transplant patient.	Forty specimens from normal subjects were negative for CMV DNA. Real-time PCR was useful for the quantitative detection of CMV DNA from serial samples of blood from a patient post-liver transplantation.
Dworkin et al. 2002 (103)	Aqueous vitreous specimens from patients with posterior uveitis	Not provided	CMV DNA obtained from Advanced Biotechnologies, Inc., Columbia, MO; SYBR Green I dye fluorescein detection system	CMV genome/μl vitreous		Real-time PCR may be applied to infectious agents responsible for posterior uveitis; the technique will likely be useful for the diagnosis of this entity and the linkage of viral pathogens to the disease.
Greenlee et al. 2002 (146)	Blood (RBC, WBC-reduced), 100 blood donors seroprositive for CMV; 93 blood donors seronegative for CMV	Immediate-early gene of CMV	ABI standard at stock concentration of 10^4 copies/ml. The standard was used as a template for the TaqMan assay at 5, 50, 500, and 50,000 copies/50 μl PCR.	Copies CMV DNA/ml		CMV DNA was not detected in any of the RBC samples.

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TABLE 18—*Continued*

Platform	Reference	Specimen	Target	Quantitative standard	Reporting units	Comparison to other systems	Comments
	Mori et al. 2002 (321)	Plasma allogeneic hematopoietic stem cell transplant patients	US17 gene	A standard ranging 10^1 to 10^7 copies/well was used for a standard curve for quantitation of CMV DNA	Copies CMV DNA/100. μl	Qualitative results of the real-time PCR were compared with the determinations obtained with the antigenemia test.	Authors used real-time PCR results to guide preemptive therapy (ganciclovir) for CMV disease. Qualitative results of real-time PCR were detected earlier than antigenemia in the posttransplantation period in 18/30 (60%) patients. Antigenemia was detected before PCR results in only 3/30 (10%) patients.
	Sanchez et al. 2002 (427)	Plasma and leukocyte lysate preparations	DNA polymerase gene. Primers and probes for amplification of the human apoprotein gene were included in the assay.	CMV quantitation was linear over a range of 10^1 to 10^6 copies.	Copies CMV DNA/ml	Authors created a multiplex, quantitative real-time PCR assay that amplifies CMV DNA and human DNA in the same reaction tube.	Applied to whole blood, the assay provides a measurement of CMV DNA in reaction to cellular content without a need for cell-counting procedures.
	Yakushiji et al. 2002 (549)	Plasma from stem cell transplant patients	US17		CMV DNA copies/ml	CMV antigenemia and quantitative real-time PCR were compared for monitoring CMV reactivation after allogenic stem cell transplantation.	The number of CMV antigen-positive cells by the antigenemia assay and the level of CMV DNA by real-time PCR correlated well.
	Hänfler et al. 2003 (162)	Buffy coat leukocytes	pp150 IL-32 gene	Linear range 10^1 to 10^7 copies standard curve data prepared from a plasmid with target insert diluted from 0.5×10^1 to 0.5×10^7 copies/μl	CMV DNA copies/2-μl sample	Authors developed a duplex real-time PCR that was capable of quantifying CMV DNA and β-actin DNA as internal control simultaneously within one reaction.	A high degree of conformity was attained between PCR viral load and antigenemia tests using leukocyte specimens.
	Ikewaki et al. 2003 (193)	Plasma	US17 gene	Plasmid containing the target sequence was diluted (seven 10-fold dilutions) corresponding to 10^1 to 10^7 plasmid copies/reaction. Dynamic range: 5×10^2 to 5×10^8 copies/ml	CMV DNA/ml	Study compared real-time PCR assay for detection of CMV DNA with nested conventional PCR and antigenemia tests.	Real-time PCR was more sensitive than the antigenemia and nested PCR assays. In addition, real-time PCR was able to detect CMV reactivation earlier in the clinical course than the antigenemia and nested PCR. CMV viral loads of 5,000 copies/ml were proposed as the cutoff for initiating therapy in adult T-cell leukemia-lymphoma patients.
	Li et al. 2003 (269)	Whole blood	Glycoprotein B	Plasmid standard concentration calibrated by spectrophotometry at 260 nm.	Copies CMV DNA/ml	A laboratory-developed PCR assay and a semiquantitative pp65 antigenemia assay were compared to a quantitative real-time assay for the detection of CMV.	High correlation between antigenemia value and CMV DNA loads. Antigenemia values of 11 to 100, 101 to 1,000, and over 1,000 positive cells/2×10^5 leukocytes corresponded to median CMV DNA levels of 1,000, 4,000,

							and 10,000 copies/ml and are proposed as cutoff points for initiating antiviral therapy in patient groups with high, intermediate, and low risk of CMV disease, respectively.
	Nitsche et al. 2003 (346)	Blood leukocytes	Major immediate-early gene			Authors compared real-time PCR with antigenemia test using blood specimens from patients who underwent stem cell transplantation.	The PCR and antigenemia tests were positive in 19 of 77 patients. An additional 26% (22 patients) were positive exclusively by PCR. in 5/20 patients. Febrile episodes in two patients with fever of unknown origin may have been caused by a reactivation of CMV. These results imply that CMV infection can be expected not only in transplant patients but also in chemotherapy treated neutropenic patients.
	Persson et al. 2003 (373)	Neutropenic patients following chemotherapy	UL65 (pp67 protein)		CMV DNA genomes/ml		CMV DNA but not HHV-6 (or HHV-7) was detected
	Boeckh et al. 2004 (35)		A double primer assay was designed consisting of two sets of primers and probes to amplify a portion of the gB region (UL55) and UL123-exon 4 region		Copies CMV DNA/ml	The double-primer (UL55/UL123-exon 4) was superior to pp65 antigenemia and viremia by culture with regard to sensitivity, specificity, and predictive values.	
	Nesbitt et al. 2004 (335)	Plasma, 78 EDTA-anticoagulated whole blood samples	Junction between glycoprotein B and UL123		Copies CMV DNA/ml	Tested the effects of storing whole blood for 24 h before separating plasma	Study indicated that valid results can be obtained from EDTA-anticoagulated blood stored at room temperature for at least 24 h before separation of plasma.
	Visconti et al. 2004 (520)	Peripheral blood mononuclear cells experimentally infected with CMV	Immediate early	Linear range over 4 $\log_{10}$	CMV DNA genome copies/ml	Authors experimentally determined the efficiency of removal of CMV using leukocyte depletion filters in a model system of PBMC's infected with the virus.	CMV DNA load reduced but was not eliminated in whole blood and platelets after treatment with leukocyte depletion filters.
LightCycler	Nitsche et al. 1999 (348)		Major immediate-early gene	Linear range: 10^1 to 10^7 CMV DNA genome equivalent/assay. MIE gene inserted into plasmid vector; DNA concentration determined by	CMV DNA genome equivalent/assay		LightCycler and ABI Prism 7700 were compared for the detection of CMV DNA. The ABI Prism 7700 appears to be useful for the processing of

Continued on following page

TABLE 18—*Continued*

Platform	Reference	Specimen	Target	Quantitative standard	Reporting units	Comparison to other systems	Comments
				a spectrophotometer at 260 nm and the corresponding copy number calculated.			large numbers of samples under standard conditions, whereas the LightCycler has its strength in smaller sample numbers and the use of various reaction conditions.
	Schaade et al. 2000 (433)	Plasma, urine	Glycoprotein B gene	Amplicon cloned in plasmid. Concentration of DNA calibrated by spectrometry at 260 nm. Dynamic range, 10^2 to 10^8 plasmid copies of CMV DNA/ml	DNA copies/ml		LightCycler detected higher levels of CMV DNA than the COBAS instrument; however, both tests had comparable sensitivities for detecting CMV DNA in clinical specimens.
	Kearns et al. 2001 (213)		Glycoprotein B gene	Range: 2×10^3 to 5×10^8 CMV DNA copies/ml (copies/reaction converted to copies/ml)		LightCycler assay compared to TaqMan real-time PCR. Of 50 samples, the majority of results (36/50, 72%) were within 0.5–1 $\log_{10}$ and 3 (14%) by $\log_{10}$.	Data confirm and extend those of Schaade (433). The LightCycler assay was shown to be more sensitive than the detection of early antigent fluorescent foci testing for urine and respiratory specimens.
	Kearns et al. 2001 (215)	Blood	Glycoprotein B gene	Range: 10 to $>2 \times 10^5$ CMV DNA copies EcoRI plasmid quantified and linearized and used as quantitative standard. A series of five $\log_{10}$ dilutions corresponding to 2×10^1 to 2×10^5 copies/2 μl were prepared and run as external standards.	DNA copies/μl	LightCycler results comparable to TaqMan (blood). TaqMan could reproducibly detect down to 20 plasmid copies. Detection level of LC, ≤10 copies CMV DNA. Of 51 positive samples (blood) the quantitative results using TaqMan ranged from 10^2 to 3×10^6 copies/ml. LightCycler results ranged from 1.1×10^2 to 1.7×10^6 copies/ml.	PCR product identity confirmed by melting curve analysis. Runs acceptable if the external quantitative standard within 0.5 $\log_{10}$ of the target value and the standard curve gave a mean squared value of $>10^{-2}$.
	Ando et al. 2002 (9)	Aqueous humor; 6 patients with clinically diagnosed CMV retinitis	Glycoprotein B gene	Range: 10^1 to 10^4 copies/μl. Plasmid with target insert used to develop standard for quantitation			SYBR Green I dye CMV DNA was detected at levels up to 1.6×10^4 copies/μl of aqueous humor obtained from patients with retinitis. Study revealed correlation between levels of CMV DNA and the extent of the area affected by CMV retinitis before antiviral treatment and the prolonged retention of CMV genome after antiviral treatment.
	Kearns et al. 2002 (216)	Urine and respiratory samples	Glycoprotein B gene	Range: 2×10^3 to 5×10^8 CMV DNA copies/ml. Note: copies/reaction (215) converted to copies/ml in the publication	DNA copies/ml		LightCycler PCR provided a 3-fold increase in sensitivity compared to detection of CMV early antigen in cell culture (urine samples). Urine

						viral loads were higher in congenitally CMV-infected infants (median, 1.6×10^5 copies/ml) compared with 15 transplant recipients (median, 9×10^3 copies/ml). Urine samples did not require extraction prior to PCR testing.
Stöcher et al. 2002 (465)		US17 gene of CMV	Linear range: 500 to 50,000 CMV DNA copies/ml	CMV DNA copies/capillary		Authors developed a normalized quantitative competitive real-time PCR assay using a PCR competitor as heterologous DNA. The results obtained with conventional real-time quantification on the LightCycler instrument were almost identical to those obtained with the normalized based quantification assay.
Cortez et al. 2003 (79)	Whole blood from 51 stem cell transplant recipients	Plasmid with target insert used to develop standard for quantitation (5000, 500, 50, 5 copies/reaction). Conversion to copies/ml: DNA extracted from 200 μl whole blood and then suspended in 50 μl water; 5 μl was used in each of two reactions. The sum of these two reactions represented 50 μl of whole blood for which the sum was multiplied by 25 to obtain copies/ml.	CMV DNA copies/ml			Real-time PCR results were positive earlier than antigenemia results in 30/39 (77%) episodes of CMV infection detected by antigenemia. Real-time PCR remained positive after treatment was discontinued in 14/39 (36%) episodes and predicted the return of CMV reactivation in 4/13 (31%) episodes.
Mengelle et al. 2003 (309)	14 patients (solid organ transplant), 198 blood specimens (leukocytes)	UL83 gene (codes for lower matrix protein pp65)	Plasmid with CMV target sequence used to develop standard for quantification. Detection limit was 1 $\log_{10}$ genome copy/capillary Range: 1 $\log_{10}$–5 $\log_{10}$ copies/reaction.	CMV DNA copies/ml		CMV DNA was detected before pp65 antigen in three patients, whereas the two tests were positive simultaneously for eight patients. Molecular method of real-time PCR could be useful for monitoring infections and antiviral treatment in recipients of solid organ transplants.
Pang et al. 2003 (365)	404 plasma specimens from 66 solid organ transplant patients	Glycoprotein B gene	Range: 2.5×10^2 to 10^7 DNA copies/ml plasma. Positive control was prepared (10^6 genome copies/tube) from CMV-infected cell cultures.	CMV DNA copies/ml plasma	LightCycler PCR was most sensitive (54% of specimens positive) compared to the COBAS Amplicor	The costs per sample were highest with COBAS ($104.7) compared with LightCycler ($39.8) and antigenemia ($35.8) (Canadian dollars).

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TABLE 18—*Continued*

Platform	Reference	Specimen	Target	Quantitative standard	Reporting units	Comparison to other systems	Comments
						CMV monitor (48.6%) and the pp65 antigenemia (26%) assay.	Because of its sensitivity, specificity, cost effectiveness, and simplicity, the LC-PCR assay would replace the antigenemia and COBAS assay as the preferred technique for the surveillance, diagnosis, and monitoring of response of CMV diseases in high-risk populations.
	Gouarin et al. 2004 (145)	Whole blood from 21 renal transplant patients; 248 specimens	UL83 gene (codes for lower matrix protein pp65)	Plasmid with target insert used to develop standard for quantitation. Range: 5 to 5 $\times$ 10^6 copies/reaction	CMV DNA copies/ml		CMV DNA was detected earlier than antigenemia in the posttransplantation period. Real-time PCR with whole blood can be used to monitor renal transplant patients at risk of developing CMV disease and to assess response to antiviral therapy. Study found that >4 $\log_{10}$ copies/mL of whole blood indicates extensive CMV infection and leads to initiation of antiviral treatment.
	Hong et al. 2004 (186)	147 plasma specimens from bone marrow and stem cell transplant patients	Glycoprotein B or EcoRID region	β-Globulin gene was amplified in parallel to control for the efficiency of nucleic acid extraction and amplification steps. Range: 125 copies/ml to 5 $\times$ 10^9 copies/ml. A CMV gB plasmid containing the whole 2.7-kb gB gene subcloned into a pCR2.1 vector was used as a quantification standard for the assay.	CMV DNA copies/ml plasma	gB target provided a sensitivity of 96% and specificity of 100% compared to the EcoRID target (real-time) and conventional (gel and Southern blot)	The combination of automated DNA preparation and real-time PCR detection allows a sensitive, precise, accurate high-throughput assay of CMV viral load that can be used as a laboratory trigger for preemptive antiviral therapy.
iCycler iQ	Aberle et al. 2002 (3)	CSF	us 17 gene		Copies CMV DNA/ml (corresponding to 2 copies of DNA per TaqMan PCR mixture).		Virus load was 2.0 $\times$ 10^2 to 1.9 $\times$ 10^6 CMV DNA/ml in patients with encephalopathy in immunosuppressed patients. Quantitation of viral loads in CSF may be important regarding prognosis of disease and prediction of distinct CNS manifestation.
RotorGene	Herrmann et al. 2004 (176)	138 plasma specimens from 44 patients with suspected CMV infection	DNA polymerase gene; glycoprotein B gene	Linear range of 10^3 to 10^8 copies/ml. Target DNA used for standard curves was purified genomic DNA from CMV strain AD169.	CMV DNA copies/ml	Of 138 samples, 105 were positive by real-time PCR and 71 were positive by COBAS assay (34 samples exclusively positive by real-time PCR) (PCR was 48% more sensitive than COBAS).	Two quantitative CMV assays with different gene targets were developed. The duplex real-time PCR system had a higher sensitivity than the COBAS Amplicor Monitor test system and the linear measure level was at least 3 orders of magnitude higher.

Kalpoe et al. 2004 (205)	409 specimens from solid organ transplant (SOT) and stem cell transplant (SCT) patients; 295 corresponding whole-blood samples were selected to address the correlation between CMV DNA loads in plasma and whole blood	Immediate-early antigen gene	Clinical samples were spiked with a fixed amount of phocine herpesvirus DNA as an internal control.	CMV DNA copies/ml plasma	Based on a comparison with the pp65 antigen assay, quantification of CMV DNA in plasma appeared to be capable of guiding the clinical management of transplant patients.	CMV DNA load in whole blood tends to be slightly but not significantly higher than that in plasma. A CMV DNA level in plasma of 10,000 copies/ml provided a threshold of 81% sensitivity and 90% specificity for initiating treating in transplant patients (SOT and SCT) at risk for CMV disease.

[a] Abbreviations: IE, immediate-early; RBC, red blood cells; WBC, white blood cells; IL, interleukin; HHV, human herpevirus; MIE, major immediate-early; LC-PCR, Light Cycler PCR.

plete abrogation of posttransplant lymphproliferative disorder mortality after 6 months of therapy (341, 342, 510). Further, EBV DNA was not detected in 14 of 17 (82.4%) of these patients posttreatment. In another report, nine transplant patients (eight bone marrow and one kidney) developed posttransplant lymphproliferative disorder associated with a rapid rise in EBV viral load exceeding 10^5 EBV genomes/μg of peripheral blood mononuclear cell-derived DNA compared with $\leq 10^4$ EBV genomes/peripheral blood mononuclear cell in patients who did not have posttransplant lymphproliferative disorder (355).

Reports of real-time PCR assay for detection of EBV DNA have appeared mainly in the last 6 years; over 80% were published from 2001 to 2004 (Table 19). The focus of these reports has been the development of individual assays to provide quantitative EBV DNA results to support specific medical practices. Consequently, these laboratory developed assays in each institution have been customized and the results evaluated in patient populations (e.g., solid-organ transplant patients) which may be unique regarding demographic characteristics (age and gender), pretransplant diseases, type of transplant (lung, kidney, heart, pancreas), and immunosuppression regimen and other medications. In contrast to assays based totally on biological variables, real-time PCR instrumentations provide the basis to develop and standardize the many technical components of these platforms. For example, sample extraction could be monitored to achieve maximum yields of nucleic acids and provide for effective removal of PCR inhibitions.

For the assay, the idealized PCR target gene could be selected that would allow maximum efficiency of the amplification process. Further, a plasmid insert of this gene with appropriate calculations to determine nucleic seed and target concentration could be used as a quantitative standard and the units of reporting would be the same in all laboratories and obviously dependent on the analysis of a common sample compartment of blood (whole blood, peripheral block, mononuclear cells, plasma, or serum). Real-time PCR assays have the potential for controlling these technical variables in the laboratory. Ultimate utility of these assays for EBV quantitation as well as quantitation for other viruses such as CMV will be the application of accurate, reproducible results in each patient population. While empirically establishing local practice guidelines such as beginning antiviral treatment according to threshold levels of DNAemia are practical and necessary for appropriate medical management of patients, it is also important to acknowledge that each patient may have their own individual set point, that is, the viral load level which leads to symptomatic infection.

BK Virus

As previously mentioned under Qualitative Viral Assays, BK virus can cause tubulointerstitial nephritis and ureteric stenosis in renal transplant recipients and hemorrhagic cystitis in patients who have undergone bone marrow transplantation (359). Renal biopsy specimens may be examined histologically for the presence of BKV inclusion bodies which is a more specific diagnostic criterion compared with detection of the virus in urine specimens. In addition, the presence of characteristic decoy cells in the urine is a morphological marker for

TABLE 19. Quantitative detection of Epstein-Barr virus DNA by real-time PCR[a]

Platform	Author (reference)	Specimen	Target	Quantitative standard	Reporting units	Comparison to other systems	Comments
ABI Prism	Kimura et al. 1999 (221)	Peripheral blood mononuclear cells (PBMC)	*BALF5* gene (DNA polymerase)	Linear range, 2 to 10^7 copies EBV DNA/μg DNA	Copies EBV DNA/μg of PBMC DNA	Real-time PCR detection of EBV DNA was compared with in situ hybridization techniques.	The virus load in peripheral mononuclear cells was $10^{3.7}$ copies/μg of DNA in patients with EBV-related lymphoproliferative disorders, $10^{4.1}$ copies/μg DNA in patients with chronic active EBV infections, and $10^{2.3}$ copies/μg DNA in patients with infectious mononucleosis. The copy numbers of EBV DNA in PBMC from symptomatic EBV infections was correlated with the EBV-positive cell number determined by the in situ hybridization assay ($P < 0.0001$).
	Lo et al. 1999 (281; also 265, 280)	Plasma	BamHI-W; EBNA-1	A calibration curve was run in parallel and in duplicate with each analysis, using DNA extracted from the EBV-positive cell line Namalwa (ATCC) as a standard. A conversion factor of 6.6 pg of DNA/diploid cell was used for copy number calculation.	Copies EBV DNA/ml		Using real-time PCR cell-free EBV DNA was detectable in 55/57 (96%) median concentration, 21,058 copies of nasopharyngeal carcinoma (NPC) patients and 3/43 (7%) controls (median concentration, 0 copies/ml). Results suggest quantitative analysis of plasma EBV DNA may be a useful clinical and research tool in the screening and monitoring of patients with nasopharyngeal carcinoma.
	Niesters et al. 2000 (342)	Plasma	*BNRF1* p143 gene (nonglycosylated membrane protein)	A standard containing 6.68×10^9 EBV particles/ml (Advanced Biotechnologies) was used as a standard. Serial half-log dilutions of this standard ranging from 10^7 to 10 copies/ml were made to characterize linearity, precision, specificity, and sensitivity of the assay.	Copies EBV DNA (genome equivalents)/ml		EBV DNA could be detected in all transplant patients diagnosed with PTLD, with a mean load of 544,570 copies/ml. No EBV DNA could be detected in healthy individuals and in immunosuppressed control groups. A mean of 6,400 copies/ml could be detected in patients with infectious mononucleosis.
	Orii et al. 2000 (356)	22 transplant patients	*BALF5*	A plasmid that contained *BALF5* was constructed and diluted to prepare a standard source curve for quantitation.	Copies EBV/μg DNA	Quantitative real-time PCR was compared to qualitative DNA in plasma by PCR and EBV-encoded mRNA	Real-Time PCR and EBER-1 (in situ hybridization) results exceeded the cut-off level of $10^{2.5}$ copies/μg DNA

					(EBER1) by in situ hybridization for the detection of posttransplant lymphoproliferative disease	
Dehee et al. 2001 (91)	Blood, peripheral blood mononuclear cells	*BNRF1* gene	A plasmid with the target gene was developed to produce a standard curve for quantitative determinations. All virus DNA quantification was carried out simultaneously in order to determine the input cellular DNA for each sample and was used as an endogenous reference to normalize the variations due to differences in the PBMC count or DNA extraction. The Namalwa cell line (ATCC CRL1432) containing two integrated copies of the EBV genome/cell was used as a positive control.	Copies EBV DNA/10^6 PBMC		Significantly higher EBV loads were found in HIV-infected patients compared with EBV-seropositive healthy group ($P < 0.00001$). EBV loads were not correlated with the clinical stages of HIV infection or HIV replication
Jabs et al. 2001 (198)	Peripheral blood mononuclear cells	BamHI-K (BKRF1) encoding EBNA-1	A pCMV EBNA plasmid which carries the complete EBNA nuclear gene (EBNA-1) was used for calibration of the Namalwa DNA standard (CRL-1432, ATCC)	Copies of EBV DNA/μg of PBMC DNA	Aim of study was to develop a rapid and reliable PCR protocol for the quantification of cell-associated EBV DNA associated with PTLD in transplant recipients.	Single-tube coamplification of EBV and genomic coreactive proteins allowed normalization of EBV DNA copy levels that provided a more accurate quantification (than amplification of EBV DNA alone) of cell bound target DNA.
Lo et al. 2001 (282)	Serum	BamHI-W	A calibration curve was run in parallel and in duplicate with each analysis, using DNA extracted from the EBV-positive cell line Namalwa as a standard. A conversion factor of 6.6 pg of DNA/diploid cell was used for copy number calculation (281).	Copies EBV DNA/ml	Patients with gastric carcinoma, gastritis, and healthy controls were evaluated for the presence of EBV DNA in serum by real-time PCR. Resected tumor specimens from patients with gastric carcinoma were tested for EBER (in situ) and by real-time PCR (serum)	Serum EBV DNA reflects tumoral EBER status and presents the possibility that circulating EBV DNA may be used as a tumor marker for the EBER-positive gastric carcinomas.
van Esser et al. 2001 (509)	Plasma (stem cell transplant patients)	*BNRF1* p143 nonglycosylated membrane protein gene	A standard containing 6.68×10^9 EBV particles/ml (Advanced Biotechnologies) was used as a standard. Serial half-log dilutions of this standard, ranging from	Copies EBV DNA (genome equivalents)/ml		Qualitative monitoring of EBV DNA levels from the start of and during therapy for EBV-lymphoproliferative disease rapidly and

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TABLE 19—*Continued*

Platform	Author (reference)	Specimen	Target	Quantitative standard	Reporting units	Comparison to other systems	Comments
				10^7 to 10 copies/ml were made to characterize linearity, precision, specificity, and sensitivity of the assay.			accurately predicts for response to therapy as early as within 72 h.
	Wagner et al. 2001 (525)	Blood, peripheral blood mononuclear cells plasma	BamHI-K region coding for EBNA1 BamHI-W	The EBV-positive Burkett's lymphoma cell line Namalwa was used as a standard for quantification of EBV DNA. Namalwa cells contain two integrated EBV copies/cellular genome. Other cell lines (Daudi and Raji) were also used as positive controls.	EBV genomes/μg PBMC DNA		Patients with PTLD had a median viral load of 19,200 EBV genomes/μg PBMC DNA or 3,225 EBV genomes/100 μl plasma. Although both PBMC and plasma were useful specimens for laboratory diagnosis of PTLD, the specificity was higher if the EBV viral load was determined in plasma.
	Leung et al. 2002 (264)	Whole blood	*BALF5* gene (DNA polymerase)	Raji cells used as a positive control for detection and preparation of a standard curve for EBV DNA (dilutions to 7.5 10^5 genome/reaction; β-globulin used as a housekeeping gene.	Copies EBV DNA/μg amplified DNA (EBV DNA copy number was normalized against β-globulin)	Aim was to develop a rapid and reliable PCR protocol for quantitation of the cell-associated EBV genome.	The mean copy levels of EBV DNA/μg amplified DNA in patient populations were: healthy controls, 0.5; hemodialysis, 27; renal transplant, 40; and infectious mononucleosis, 782. The percentage of individuals whose EBV levels were >35 copies/μg amplified DNA: healthy, 0; hemodialysis, 38; renal transplant, 40%; infectious mononucleosis, 87%.
	Matsukura et al. 2002 (305)	Blood (PBMC)	*BALF5* gene (DNA polymerase)	A standard curve for quantitative detection of EBV DNA was obtained by measuring dilutions of a plasmid containing the target gene.	Copies EBV DNA/μg DNA	Aim of study was to demonstrate the significance of serial monitoring of EBV DNA by real-time PCR after liver transplantation.	In 15 patients, the mean values of the highest EBV DNA levels from patients who had the following clinical features: fever (36,232); upper respiratory syndrome (16,040); diarrhea (15,968); ascites (2,485); lymphadenopathy (336,858); and PTLD (60,486).
	Teramura et al. 2002 (487)	Plasma; serum	*BALF5* gene (DNA polymerase)	A standard curve for quantitative detection of EBV DNA was obtained by measuring dilutions of a plasmid containing the target gene.	Copies EBV DNA/ml	Study compared the detection of EBV DNA in specimens from patients with hemophagolytic lymphohistiocytosis with patients with infectious mononucleosis.	EBV DNA copy levels from serial specimens from 10 patients demonstrated decreasing or undetectable levels of target DNA following appropriate therapy.

van Esser et al. 2002 (510)	Plasma (stem cell transplant patients)	*BNRF1* p143 gene (nonglycosylated membrane protein)	A standard containing 6.68×10^9 EBV particles/ml (Advanced Biotechnologies) was used as a standard. Serial half-log dilutions of this standard, ranging from 10^7 to 10 copies/ml were made to characterize linearity, precision, specificity, and sensitivity of the assay.	Copies EBV DNA (genome equivalents)/ml	Authors studied whether preemptive therapy with rituximab prevents EBV lymphoproliferatifive disease in patients undergoing stem cell transplantation.	Preemptive therapy was given to patients with viral reactivation more than or equal to 1,000 copies of EBV DNA (genome equivalents)/ml; 14/17 (82.4%) patients treated with rituximab had complete clearance of EBV DNA from plasma.accurate quantification (than amplification of EBV DNA alone) of cell bound target DNA.
Orentas et al. 2003 (355)	Blood, peripheral blood mononuclear cells	*EBER1* gene	A plasmid containing the *EBER1* gene was used to prepare a standard curve for the quantitative detection of EBV DNA.	EBV genomes/μg PBMC		In 9 patients (8 bone marrow, 1 kidney transplant), PTLD was associated with a rapid rise in viral load exceeding 10^5 EBV genomes/μg of PBMC-derived DNA. The threshold for normal EBV viral load (compared to the levels in patients with PTLD) based on a combined experience with viral load analysis is defined as 10^4 EBV genomes/μg PMBC.
Pitetti et al. 2003 (381)	Serum, Patient population was children (mean age, 9 yr) with primary EBV infection (n = 28); EBV seronegative patients (n = 25); EBV-seropositive patients (n = 26)	*BALF5* gene (DNA polymerase)	Plasmid with target insert was used to provide quantitation standards.	Copies EBV DNA/ml		None of the purified DNA sample inhibited the amplification of the internal positive control. Twenty-one (75%) of the patients with primary EBV, one (4%) of the seronegatives, and none of the seropositives had detectable EBV DNA loads. Viral loads varied widely in patients with primary EBV infection (10^1 to 10^3). EBV posttransplant lymphoproliferative disease was diagnosed in a seronegative patient with an EBV DNA load of 1,450 copies/ml.
Wadowsky et al. 2003 (524)	Whole blood; plasma from 44 transplant patients	*BALF5* gene (DNA polymerase)	The gene target was inserted into a plasmid and determinations made in 10-fold increments ranging	Plasma: copies EBV DNA/ml	Whole blood: EBV DNA copies/ml, EBV copies/μg DNA based on spectrophotometric	Comparing real-time PCR and conventional PCR, whole-blood PBL loads correlated highly (r^2 =

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TABLE 19—*Continued*

Platform	Author (reference)	Specimen	Target	Quantitative standard	Reporting units	Comparison to other systems	Comments
				from 20 to 2,000,000 copies/reaction		measurement, and EBV copies DNA/10^5 PBLs based on absolute count. EBV DNA load levels were measured in whole blood (n = 60), plasma (n = 59), and samples of peripheral blood lymphocytes (n = 6) by competitive PCR.	0.900), whereas plasma and PBL loads correlated poorly (r^2 = 0.512). Data provide outoff levels of EBV DNA in blood compartments (whole blood, plasma, PBLs) for various risk groups of patients.
	Leung et al. 2004 (266)	Plasma	BamHI-W EBNA-1	A calibration curve was run in parallel and in duplicate with each analysis, using DNA extracted from the EBV-positive cell line Namalwa as a standard. A conversion factor of 6.6 pg of DNA/diploid cell was used for copy number calculation (281).	Copies EBV DNA/ml	The sensitivities and specificities of IgA-VCA and EBV DNA for diagnosis of NPC were determined in 139 new cases of NPC and 178 healthy individuals.	The sensitivities of EBV DNA and IgA-VCA for diagnosis of NPC were 95% and 81%, respectively. The specificities of EBV DNA and IgA-VCA were 98% and 96%, respectively.
	Lin et al. 2004 (273)	Plasma specimens from 99 patients with biopsy-proven nasopharyngeal carcinoma	ABI Prism/ BamHI-W	β-Globulin gene served as a control for activity of *Taq* polymerase. A calibration curve was obtained using DNA extracted from the EBV-positive cell line Namalwa as the standard.	EBV DNA/ml plasma		The median concentrations of plasma EBV DNA were 681 copies/ml among 25 patients with stage III disease, 1,703 copies/ml among 74 patients with stage IV disease, and 291,940 copies/ml among 19 control patients with distant metastasis. Patients with relapse had a significantly higher plasma EBV DNA concentration before treatment than those who did not have a relapse. Quantification of plasma EBV DNA was useful for monitoring patients with nasopharyngeal carcinoma and predicting the outcome of treatment.
	Yu et al. 2004 (555)	Plasma	EBER	All plasma DNA samples were also subjected to real-time PCR analysis for the β-globin gene.	Copies EBV DNA/ml	Tumor tissue samples were tested for the presence of EBV by in situ hybridization and compared to the detection of EBV DNA in plasma by real-time PCR.	Plasma EBV DNA concentrations in patients with EBV-encoded RNA (EBER)-positive tumors nonnasopharyngeal and neck carcinomas (squamous cell carcinoma; lymphoepithelial carcinomas) (median, 3,827 copies) were significantly

							higher than those in the controls (median, 0 copy/ml, $P = 0.0001$). Plasma EBV DNA was detected in all of the patients with EBER-positive tumors.
EBV LightCyder	Aritaki et al. (21)	Peripheral blood cells; 16 patients undergoing stem cell transplantation.	GenBank accession no. V01555	Raji cells that contained 50 copies EBV genome/cell used as standard	Copies/μg of EBV DNA	Other herpesvirus targets (HHV-6 and CMV) were measured in this study.	No EBV-related disease was found in the study patients. Viral loads were low ($<10^3$ copies/μg of EBV DNA). High numbers of CMV genome were detected in 3/13 patients after transplant and reactivation of HHV-6 was frequently seen.
	Brengel-Pesce et al. (45)	Peripheral blood mononuclear cells from 88 patients: 32 healthy EBV-seropositive; 34 EBV-associated disease; 22 HIV infected.	*BXLF-1* gene (thymidine kinase)	*BXLF-1* cloned into plasmid and quantitative standard run from 2 to 2×10^7 copies.	PBMC: copies EBV DNA genome/μg DNA. Serum: copies EBV DNA/ml.	LightCycler results were compared with a routinely used ELISA-PCR of 150 samples and a good correlation was found ($R = 0.956$)	12/32 (37.5%) PBMC from healthy EBV-seropositive individuals were positive at very low copy levels. EBV DNA was detected in 80% of classical infection mononucleosis patients (mean, 288 copies/ml of serum). In 5 cases of EBV-related posttrans plantation lymphoproliferative disease, the viral load was >10,000 copies EBV DNA/μg DNA at the diagnosis of lymphoma.
	Stevens et al. (463)	Whole blood	*EBNA-1* gene	EBNA was inserted into plasmid DNA and quantified in duplicate in each PCR run. For a quantitative standard the plasmid was used in concentrations ranging from 10 to 10^4 copies/reaction.	Copies EBV DNA/ml	Real-time PCR was compared with quantitative competitive PCR for EBV DNA.	Aim of study was to develop a LightCycler-based real-time PCR assay for monitoring EBV load in whole blood. In 253 blood samples from patients with Burkitt's lymphoma, infectious mononucleosis, or human immunodeficiency virus infection, a weak but significant correlation between the two methods was found ($P < 0.001$).
	Patel et al. (370)	Whole blood; plasma	*BZLF-1* gene, which codes for the ZEBRA protein	Namalwa EBV cell line (ATCC 1432) used as a standard, which contains two copies EBV genome/cell. *BZLF-1* gene was incorporated into a plasmid for use as a quantitative control (10^0 to 10^9 copies/reaction).	Copies CMV DNA/ml	Intra- and interassay variability studies using external quantitative standards and DNA extracted from whole blood and plasma samples were shown to be within 0.5 $\log_{10}$.	Quantitative EBV DNA assay was developed to investigate the natural history of EBV infection in immunosuppressed patients to identify those at greatest risk of developing disorders and monitor response to therapy.

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TABLE 19—*Continued*

Platform	Author (reference)	Specimen	Target	Quantitative standard	Reporting units	Comparison to other systems	Comments
	Stöcher et al. (466, 467)	Plasma	GenBank accession numbers 109336–109351 (EBV). Note: numbers for CMV, HSV-1/2 and VZV also included.		Copies EBV DNA/ml	Internal control DNA contained a stretch of the neomycin phosphotransferase gene which was flanked by the four forward and reverse primer binding sites that were specific for each herpesvirus type-specific PCR of the set.	Authors developed a set of automated LightCycler PCR assays for the detection of CMV, EBV, HSV-1/2, and VZV DNA in plasma samples and complementation of the assays with internal amplification controls.
LightCycler	Balandraud et al. (24)	Peripheral blood mononuclear cells	IR-1	Raji cell line, which harbors 50 copies of EBV genome per cell, was used as an external EBV standard. EBV copy number was calibrated by serial dilutions of Raji cell DNA and ranged from 10^5 to 0.064 copies/ml of standard EBV DNA dilution.	Copies EBV/500 ng DNA (1.5×10^5 cells)		In patients with rheumatoid arthritis, the EBV DNA load in PBMCs is increased almost 10-fold compared with that in normal controls.
iCycler iQ	Yuge et al. (556)	Blood, PBMC, and plasma	*BALF5* gene (DNA polymerase)	Linear range, 2 to 10^7 copies EBV DNA/μg DNA.	Copies EBV DNA/μg DNA	Case report of a 3-year-old, previously healthy boy who developed a chronic active EBV infection.	Viral loads of EBV DNA in PBMC and plasma remained high with therapeutic interventions. PCR of a liver tissue sample was positive for EBV DNA $CD56^+$ cells infected with EBV.
	Jeblink et al. (200)	Whole blood	*EBER* gene (glycoprotein B gene for CMV)	Both EBV and CMV assays were able to detect viral DNA over a linear range of 10^1 to 10^7 copies/well, which was equivalent to 10^3 to 10^9 copies/ml	EBV DNA copies/ml	EBV and CMV viral loads in patient samples obtained by gel-based and by real-time PCR were very similar.	The real-time PCR assays showed increases in viral load before clinical measures of viral disease and decreases in viral load during antiviral therapy in two of six pediatric patients.

[a] Abbreviations: ATCC, American Type Culture Collection; PTLD, posttransplant lymphoproliferative disease; HIV, human immunodeficiency virus; PBL, peripheral blood leukocytes; IgA, immunoglobulin A; VCA, viral capsid antigen.

viral replication (359). Although PCR detection of BKV DNA in urine specimens of patients with clinically suspect nephritis is a sensitive test, a positive test does not necessarily reflect the etiology of this condition since asymptomatic reactivated infection may occur in 10% to 45% of kidney transplant patients. Conversely, a negative PCR result can be informative to reduce or eliminate the likelihood of BKV-associated nephritis.

Randhawa et al. developed a real-time PCR LightCycler assay to quantitate BKV DNA in renal transplant patients (395). Viral loads were measured in urine, plasma, and kidney biopsy specimens in three clinical conditions: (i) patients with asymptomatic BKV viruria, (ii) patients with active BKV allograft nephropathy, and (iii) patients with resolved BKV nephropathy. Active BKV nephropathy was associated with quantitative levels of 5×10^3 copies/ml plasma of BKV DNA. All of these active cases had BKV target DNA at levels greater than 10^7 copies/ml of urine. Resolution of nephropathy was correlated with decreased viruria levels, disappearance of viral inclusions, and persistent but low-level target DNA in biopsy specimens. Viral loads in patients with asymptomatic viruria were generally lower but sometimes overlapped with levels typical for patients with BKV nephritis (395).

Establishment of general threshold levels of BKV DNA in urine may be useful. For example, the occurrence of hemorrhagic cystitis in allogeneic bone marrow transplant patients was associated with BKV DNA levels in urine above 10^4 copies/μl; similarly, all four patients with acute BKV-related nephropathy had copy levels of $> 10^5$ copies/μl (263, 512). BKV was also detected in the plasma of three of four (75%) patients (512). The quantitative levels of BKV DNA in urine and blood may not always be directly correlated; this difference may reflect independent reactivation of the virus in different tissues during immunosuppression (263).

Viral Hepatitis Agents

Almost all of the reported rapid real-time PCR assays for hepatitis viruses are quantitative tests that measure viral load in serum or plasma for monitoring therapeutic responses of patients with hepatitis A, B, C, D, or E infection. These assays were laboratory developed and showed various dynamic ranges of results and reproducibility (Table 20). The chemistries used include SYBR Green I, FRET hybridization, TaqMan, and molecular beacon probes used with the LightCycler, ABI PRISM sequence detection system, and Stratagene Mx4000. Assays to quantify hepatitis B and C virus load in liver tissue have also been described (542, 558). Presently, there is only one commercially available quantitative assay, which has a research use-only label for the measurement of hepatitis A viral DNA in serum or plasma. Assays for qualitative detection of hepatitis B and C viruses in serum and plasma have been reported with high analytical sensitivity necessary for screening of blood donors (314) and diagnosis of infection prior to the appearance of serologic markers. However, many of the quantitative assays for hepatitis B and C viruses are as sensitive as these qualitative assays and may be applicable for diagnostic purposes. Some qualitative assays were developed to determine hepatitis B virus polymerase gene mutants at various levels of subpopulation and analytical sensitivity (60, 388, 539, 544, 553, 560).

Human Immunodeficiency Virus

HIV-1 and HIV-2 RNA levels in the plasma of infected individuals can be determined reliably by laboratory-developed quantitative rapid real-time PCR assays (Table 21). These assays differ in the probe chemistry and amplification/detection systems used, and dynamic ranges of results and assay precision are comparable to those of commercially available assays. Quantitative assays for measurement of HIV-1 and HIV-2 proviral DNA have also been developed. Qualitative rapid real-time PCR assays developed for HIV-1 and HIV-2 have been reported for the detection of proviral DNA. They are highly sensitive, and the analytical sensitivities of the HIV-1 proviral DNA assays were comparable to that of the commercially available Amplicor HIV-1 DNA test, v1.0 (Roche Molecular Systems, Inc., Branchburg, NJ).

FUNGI

Aspergillus Species

Of all the fungal genera, *Aspergillus* has been the one most extensively targeted for the development of real-time PCR assays. The rationale behind this effort is that timely detection of *Aspergillus* spp. may decrease the extreme morbidity and mortality associated with invasive aspergillosis. Currently, there are at least 167 recognized species and species variants of *Aspergillus* (http://www.ncbi.nlm.nih.gov/Taxonomy) but most cases of aspergillosis are attributed to *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*. A few other species, including *Aspergillus nidulans*, *Aspergillus terreus*, and *Aspergillus versicolor*, have been reported to cause clinically significant disease (413).

The vast majority of real-time PCR methods reported to date for *Aspergillus* target *Aspergillus fumigatus*. Second in frequency are reports describing real-time PCR methods for *Aspergillus flavus*. The variety of extraction methods, targets, primers and probes, source material, and amplification protocols makes direct comparison of the methods difficult. In addition, the number of specimens examined from patients with proven or probable invasive aspergillosis is low, making the assessment of method sensitivity and specificity difficult. Notably, Rantakokko-Jalava et al. (397) and Pryce et al. (385) compared the results of their real-time PCR assays for *Aspergillus fumigatus* in bronchoalveolar lavage fluid, tissue biopsy specimens, or blood to a clinical diagnosis for invasive aspergillosis that was based on recently published consensus criteria (22).

Rantakokko-Jalava et al. used a LightCycler assay targeting a mitochrondrial gene for *Aspergillus fumigatus* and observed positive PCR results from bronchoalveolar lavage fluid in six of seven, two of four, and four of five patients with proven, probable, and possible invasive pulmonary aspergillosis, respectively. The diagnostic sensitivity of the assay was reported to be 73% with a specificity of 93% and positive and negative predictive values of 73% and 95%, respectively. Use of a crossing point > 35 cycles as a cutofffor a positive result improved the ability to discriminate between colonization and invasion but decreased the sensitivity of the assay to 45%. Importantly, this report established analytical specificity by testing against a

TABLE 20. Comparison of rapid-cycle real-time PCR assays for the detection of hepatitis virus types A, B, C, D, and E in clinical specimens[a]

Hepatitis virus type	Reference	Specimen	Test platform/probe chemistry	Target	Specimen volume input	Range of quantitative results	Comparison to other assays	Comments
HAV	Costa-Mattioli et al. 2002 (83)	Serum	ABI Prism 7700/TaqMan probe	87-bp fragment in 5′-noncoding region	140 μl	360 to 3.6×10^8 copies/ml	100% sensitivity and specificity in 41 patient sera and 200 healthy blood donor sera tested by anti-HAV IgM.	CV varied from 0.98% to 6.46%; HAV RNA remained detectable for >60 days after clinical diagnosis.
	Rezende et al. 2003 (411)	Serum	LightCycler/TaqMan probe	77-bp fragment in 5′ noncoding region	140 μl	100 to 10^8 copies/ml	72% sensitivity in 50 patient sera tested by anti-HAV IgM.	
	Roche Applied Science (http://www.roche-applied-science.com/lightcycler-online/)	Plasma, serum	LightCycler/FRET hybridization probes	5′ noncoding region	300 μl serum	5,000 to 10^8 geq/ml		Commercially available for research use only.
HBV	Abe et al. 1999 (1)	Serum	ABI Prism 7700/TaqMan probe	174- or 241-bp fragment of *S* gene or 331-bp fragment of *X* gene	100 μl	200 to 2×10^9 copies/ml	Correlated with Quantiplex HBV DNA 1.0 Assay (R^2 ranged from 0.93 to 0.97 for the three targets) and quantified eight clinical sera more than Quantiplex among 46 patient sera; 100% specificity in 23 healthy donor sera.	Interassay CV varied from 0.7% to 9.1%.
	Aliyu et al. 2004 (5)	Serum	LightCycler/FRET hybridization probes	259-bp fragment of *S* gene	200 μl	250 to 5×10^8 copies/ml	Detected 26 positive sera more than conventional laboratory-developed PCR assay among 89 patient sera	Intra- and interassay CV varied from 2.6% to 75.3% and from 9.2% to 70.2%, respectively.
	Brechtbuehl et al. 2001 (44)	Plasma, serum	LightCycler/SYBR Green I	100-bp fragment of *S* gene	150 μl	1.3×10^4 to 1.3×10^{10} copies/ml for single-round PCR; 400 to 1.3×10^8 copies/ml for nested PCR	Quantification of HBV DNA levels in 21 (58%) of 36 HBsAg-positive blood donor sera were higher than those obtained by Amplicor HBV Monitor test, with mean titer difference of 0.64 log (range, 0.14 to 0.96 log) among samples of $>10^6$ copies/ml.	Interassay CV ranged from 1.2% to 2.9%.
	Candotti et al. 2004 (59)	Plasma	Stratagene Mx4000/TaqMan probe	81-bp fragment of *S* gene	200 μl	30 (95% detection rate) to 10^5 IU/ml	Correlated with single-virus real-time PCR assay (R^2 = 0.94) among 24 samples	Intra- and interassay CV ranged from 0.8% to

						with HBV DNA titers ranging from 15 to 10^5 IU/ml; 100% specificity among 266 healthy blood donor sera tested by HBsAg EIA.	1% and from 3.4 to 3.6%, respectively; equivalent detection of HBV genotypes A to F; a multiplex assay which also detects HCV RNA and HIV-1 RNA.
Cane et al. 1999 (60)	Serum	LightCycler/FRET hybridization probes	104-bp fragment of polymerase gene	10 μl	10^5 copies/ml minimum	100% agreement with direct DNA sequence analysis and/or cloning assay in 20 sera from 8 patients (HBV genotypes A to H).	Detect polymerase gene mutations encoding resistance to lamivudine in HBV minor species comprising 20% of total population.
Hennig et al. 2002 (172)	Plasma	ABI Prism 7700/TaqMan probe	132-bp fragment of *C* gene	1 or 2 ml	28 IU/ml for 1-ml sample; 14 IU/ml for 2-ml sample	Detected 14 of 16 (88% sensitivity) HBsAg-positive sera and 3 of 189 (98.4% specificity) HBsAg-negative sera among 205 anti-HBc-positive patients	Developed for screening of blood donors.
Ho et al. 2003 (180)	Serum	LightCycler/FRET hybridization probes	130-bp fragment of *C* gene	200 μl	250 to 2.5 × 10^{10} copies/ml	Detected 114 of 120 (95% sensitivity) HBsAg-positive sera and none of 45 (100% specificity) HBsAg-negative sera; correlated with Digene Hybrid Capture II HBV DNA test (R^2 = 0.90) on 67 patient sera.	Intra- and interassay CV, were 6% and 16%, respectively.
Ide et al. 2003 (192)	Serum	ABI Prism 7700/SYBR Green I	52-bp fragment of *C* gene	150 μl	50 to 316 copies/ml		Utilizes amplified products from the Amplicor HBV Monitor test as templates; intra-assay CV was 1.4% at 316 copies/ml.
Jardi et al. 2001 (199)	Serum	LightCycler/FRET hybridization probes	144-bp fragment of *C* gene	200 μl	10^3 to 10^8 copies/ml	Detected 128 of 193 (66%) HBsAg-positive sera vs. 84 detected by Quantiplex HBV DNA 1.0 assay; correlated with Quantiplex assay (R^2 = 0.94) in 30 patient sera.	Intra- and interassay CV, varied from 2% to 4% and from 4% to 6%, respectively.

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TABLE 20—*Continued*

Hepatitis virus type	Reference	Specimen	Test platform/probe chemistry	Target	Specimen volume input	Range of quantitative results	Comparison to other assays	Comments
	Leb et al. 2004 (253)	Plasma	LightCycler/FRET hybridization probes	139-bp fragment of *C* gene	200 μl	200 to 8×10^8 copies/ml	Quantified 107 vs. 100 samples by COBAS Amplicor HBV Monitor test among 123 clinical plasma simples, with good correlation (R^2 = 0.95) between the two assays in 97 samples.	Intra- and interassay CV, were 0.7% and 1.5%, respectively.
	Loeb et al. 2000 (283)	Serum	ABI Prism 7700/TaqMan probe	105-bp fragment of overlapping region between *X* and polymerase genes	200 μl	10 to 10^9 copies/ml	Quantified 119 vs. 55 sera by Quantiplex HBV 1.0 assay among 157 patient sera; 100% specificity in 119 sera negative for all HBV serologic markers.	Detected HBV DNA in 10 (9%) of 109 patients who were positive only for anti-HBc total antibodies in 84 (78%) of 108 HBsAg-positive/HBeAg-negative patients.
	Mercier et al. 1999 (314)	Plasma, serum	ABI Prism 7700/TaqMan probe	113-bp fragment of *C* gene	200 ul	50 copies/ml (analytical sensitivity)	100% sensitivity and specificity in 50 patients and 50 blood donors tested by Quantiplex HBV 1.0 (bDNA) assay; 1.5% false-positive rate in screening 274 healthy blood donors.	A multiplex assay which also detects HCV RNA
	Mukaide et al. 2003 (324)	Serum	ABI Prism 7700/TaqMan probe	120-bp fragment of overlapping region between core and polymerase genes	300 ul	3 to 10^8 IU/ml	Correlated with Amplicor HBV Monitor test at R^2 = 0.94 with a commercially available transcription-mediated amplification-hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan) at R^2 = 0.94 in 156 and 100 patient sera, respectively.	Intra- and interassay CVs varied from 1.1% to 7.8% and from 1.6% to 4.8%, respectively.
	Pang et al. 2004 (364)	Serum	ABI Prism 7700/3′ minor groove binder-conjugated TaqMan probe	108-bp fragment of core promoter gene, or 107-bp fragment of precore gene	?	200 to 10^9 copies/ml	Correlated with COBAS Amplicor HBV Monitor Test in 110 sera containing wild-type/core promotor mutant HBV (R^2 = 0.86) and in 71 sera containing wild-type/precore mutant HBV (R^2 = 0.85).	Capable of quantifying HBV DNA in samples containing from 10% to 90% core promotor mutants or samples with 20% to 90% precore mutants

Paraskevis et al. 2002 (367)	Serum	LightCycler/FRET hybridization probes	156-bp segment of overlapping region of polymerase and *S* genes	200 μl	250 to 10^{11} copies/ml	Quantified 292 vs. 282 sera by Amplicor HBV Monitor test among 302 HbsAg-positive patients ($R^2 = 0.86$); quantified 63 vs. 36 sera by Quantiplex HBV DNA 1.0 assay among 66 HbsAg-positive patients ($R^2 = 0.88$).	Intra- and interassay CVs varied from 4.6% to 30.1% and from 5.1% to 53%, respectively.
Pas et al. 2002 (369)	Serum	ABI Prism 7700/TaqMan probe	90-bp fragment of pre-*S* gene	200 μl	46 to 1.25×10^9 IU/ml		
Punia et al. 2004 (388)	Serum	LightCycler/SYBR-Green I	rt204- and rt180-containing fragments of polymerase gene	100 μl	-	Detected HBV rt204 and rt180 mutants in two more patients than conventional DNA sequencing assay among 10 patients treated with lamivudine.	Detect and quantify HBV rt204 and rt180 mutant populations at 0.01% of 10^5 to 10^9 copies of wild-type HBV DNA using amplification-refractory mutation system PCR.
Schaefer et al. 2003 (434)	Serum	LightCycler/SYBR-Green I	336-bp fragment of *S* gene	200 μl	20 to 2×10^9 IU/ml	Yielded quantification differences of <0.5 log IU/ml in 25 of 30 (83.3%) sera tested by a similar LightCycler assay amplifying a *X* gene target.	Primers amplified *S* gene fragments of all well-characterized HBVs in humans, primates, squirrels, and woodchuck.
Stelzl et al. 2004 (461)	Serum	LightCycler/FRET hybridization probes	120-bp fragment of HBV genome (? gene)	200 μl	2.5×10^2 to 10^9 IU/ml	100% sensitivity and specificity compared to COBAS Amplicor HBV Monitor test among 117 clinical sera; results of 76% (37 of 49) of positive sera were within ±0.5 log IU/ml by both tests and 18%, respectively.	Commercially as RealArt HBV LC PCR. Reagents available from Artus GmbH, Hamburg, Germany; intra- and interassay variation ranged from 9% to 40% and from 16% to 73%, respectively.
Sum et al. 2004 (469)	Serum	ABI Prism 7000/molecular beacon probe	112-bp fragment of *S* gene	200 μl	10^2 to 10^9 copies/ml	Detected 128 (73%) vs. 119 (68%) positive sera by COBAS Amplicor HBV Monitor test among 175 HBsAg-positive patients ($R^2 = 0.90$).	<7% intra-assay and <5% interassay variation in testing HBV *ad* and *ay* subtype DNA standards.

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TABLE 20—*Continued*

Hepatitis virus type	Reference	Specimen	Test platform/probe chemistry	Target	Specimen volume input	Range of quantitative results	Comparison to other assays	Comments
	Takaguchi et al. 2002 (470)	Serum, liver tissue	ABI Prism 7700/TaqMan probe	272-bp fragment of *X*–pre-*C* gene region	100 μl serum, 10 mg tissue	200 copies/ml (analytical sensitivity for serum)	Detected HBV DNA in 12 (33%) of 36 patients with HBsAg- and anti-HCV Ab-negative chronic liver disease; 5 of 6 serum HBV DNA-positive patients also had detectable HBV DNA in liver tissue.	Unknown analytical sensitivity of assay for liver tissue.
	Weinberger et al. 2000 (534)	Serum	ABI Prism 7700/TaqMan probe	81-bp fragment of *S* gene	200 μl	10^2 to 10^9 geq/ml	Good correlation of results with laboratory-developed semiquantitative conventional PCR assay (R^2 = 0.83).	
	Whalley et al. 2001 (539)	Serum	LightCycler/FRET hybridization probes	130-bp fragment of polymerase gene	200 μl	400 copies/ml (analytical sensitivity)	Good agreement with results of direct DNA sequence analysis.	Utilize melting curve analysis to detect and differentiate rtM204 mutants at ≥25% level of minor population.
	Wightman et al. 2004 (544)	Serum	ABI Prism 7700/molecular beacon probe	rtM204-containing fragment in C domain of polymerase gene	200 μl	10^2 copies/ml (analytical sensitivity)	Good agreement with results of direct DNA sequence analysis.	Able to detect 1% minor populations of rtM204V and rtM204I mutants.
	Yeh et al. 2004 (553)	Serum	LightCycler/FRET hybridization probes	340-, 368-, and 416-bp fragments of *S* gene	200 μl	10^2 to 10^{11} copies/ml	Good correlation with quantitative results obtained by Amplicor HBV Monitor test (R^2 = 0.99), NGI Super-Quant assay (R^2 = 0.98), and Quantiplex HBV bDNA assay (R^2 = 0.99); good agreement of genotype results with direct DNA sequence analysis.	Intra- and interassay CVs were 8.9% and 14.3%, respectively, for quantification of HBV DNA; able to differentiate HBV genotypes A to G and detect mixed genotype infections at 10% level of minor population.
	Zanella et al. 2002 (557)	Serum	GeneAmp 5700/TaqMan probe	123-bp fragment of *S* gene	200 μl	10^2 to 10^{10} copies/ml	Detected HBV DNA in 38 (35%) more samples than Digene Hybrid Capture HBV DNA test (R^2 = 0.76)	Intra- and interassay CVs ranged from 8.4% to 35.8% and from

							among 108 HBsAg-positive sera; 100% specificity on sera from 20 healthy seronegative blood donors.	6.2% to 53.1%, respectively.
	Zanella et al. 2002 (558)	Liver tissue	GeneAmp 5700/TaqMan probe	123-bp fragment of *S* gene	200 mg tissue	50 to 10^7 copies/ml	No comparison with other assays.	Higher HBV DNA titers in liver tissues from HBsAg-positive than HBsAg-ngative patients ($P < 0.01$).
	Zhang et al. 2002 (560)	Serum	LightCycler/FRET hybridization probes	217-, 586-, and 650-bp fragments of basal core promoter, pre-*C*, and *S* genes, respectively	200 μl	100 copies/ml (analytical sensitivity)	Good agreement with results of direct DNA sequence analysis.	Utilize melting curve analysis to detect amd differentiate core/precore and HBsAg mutants at 5% level of minor population
HCV	Bullock et al. 2002 (53)	Serum	LightCycler/FRET hybridization probes	324-bp fragment in 5′ noncoding region for initial PCR; 284-bp fragment for second-round PCR	200 μl	2×10^4 copies/ml minimum	Concordance with line probe assay (INNO-LiPA) in 110 (99%) of 111 patient sera.	HCV genotyping assay differentiating among genotypes 1, 1a, 1b, 2, 2a/c, 2b, 3a, and 4.
	Candotti et al. 2004 (59)	Plasma	Stratagene Mx4000/TaqMan probe	68-bp fragment in 5′ noncoding region	200 μl	167 (95% detection rate) to 10^5 IU/ml	Correlated with single virus real-time PCR assay ($R^2 = 0.95$) among 14 samples with HIV-1 RNA titers ranging from 300 to 10^5 IU/ml; 99% specificity among 266 healthy blood donor sera tested by anti-HCV EIA.	Intra- and interassay CVs ranged from 0.8% to 2.4% and from 2.6 to 2.9%, respectively; equivalent detection of HCV genotypes 1 to 6; a multiplex assay which also detects HBV DNA and HIV-1 RNA.
	Enomoto et al. 2001 (112)	Serum	ABI Prism 7700/TaqMan probe	161-bp fragment in 5′ noncoding region	250 μl	10 to 10^8 copies/ml	Correlated with Quantiplex HCV 2.0 ($R^2 = 0.84$), Amplicor HCV Monitor v2.0 ($R^2 = 0.85$), and HCV core	

Continued on following page

TABLE 20—*Continued*

Hepatitis virus type	Reference	Specimen	Test platform/probe chemistry	Target	Specimen volume input	Range of quantitative results	Comparison to other assays	Comments
							Ag EIA (R^2 = 0.55) in 50 patient sera.	
	Kawai et al. 1999 (209)	Serum	ABI Prism 7700/TaqMan probe	199-bp fragment in 5′ noncoding region	50 μl	2×10^3 to 2×10^8 IU/ml	Correlated with Amplicor HCV Monitor v1.0 (R^2 = 0.81) in 138 patient sera; 10- to 100-fold higher titers than those of Amplicor HCV Monitor.	
	Kleiber et al. 2000 (222)	Plasma, serum	ABI Prism 7700/TaqMan probe	250-bp fragment in 5′ noncoding region	200 μl	64 to 10^7 IU/ml	100% specificity in 100 HCV-seronegative blood donor sera tested by Cobas Amplicor HCV test, v2.0.	CVs varied from 21.6% to 30.4%; equivalent detection of HCV genotypes 1 to 5.
	Komurian-Pradel et al. 2001 (231)	Serum	LightCycler/SYBR Green I	220-bp fragment in 5′ noncoding region	200 μl	4 to 4.05×10^6 IU/ml	Correlated with Quantiplex HCV RNA 2.0 assay (R^2 = 0.79) and quantified 2 more clinical serum specimens than Quantiplex among 33 patient sera.	CVs varied from 0.7% to 3.7%.
	Martell et al. 1999 (304)	Serum	ABI Prism 7700/TaqMan probe	194-bp fragment in 5′ noncoding region	140 μl	330 to 10^7 copies/ml	Correlated with Quantiplex HCV RNA 2.0 assay (R^2 of 0.71 to 0.88) and National Genetics Institute Superquant assay (R^2 of 0.74 to 0.99) in 79 patient sera.	CVs varied from 1% to 6.2%.
	Mercier et al. 1999 (314)	Plasma, serum	ABI Prism 7700/TaqMan probe	168-bp fragment in 5′ noncoding region	200 μl	50 copies/ml (analytical sensivity)	100% sensitivity and specificity in 50 patients and 50 blood donors tested by Quantiplex HBV 1.0 assay; 0.7% false-positive rate in screening 274 healthy blood donors.	CVs varied from 8.7% to 74.7%; a multiplex assay which also detects HBV DNA.
	Ratge et al. 2002 (398)	Serum	LightCycler/FRET hybridization probes	235-bp fragment in 5′ noncoding region	200 μl	109 to 2,500 IU/ml	100% agreement in results with a qualitative conventional assay for 156 patient sera.	CVs varied from 11.5% to 12.9%; two rounds of rapid-cycle real-time PCR in this assay.
	Schroter et al. 2001 (440)	Serum	LightCycler/SYBR Green I	124-bp fragment in 5′ noncoding region	200 μl	900 to 7×10^6 copies/ml	Correlated with Amplicor HCV Monitor v1.0 and in-house conventional PCR assays in 81 patient sera and 30 healthy blood donor sera.	

	Schroter et al. 2002 (439)	Serum	LightCycler/FRET hybridization probes	143-bp fragment in 5′ noncoding region	200 μl	10^3 IU/ml (analytical sensitivity)	100% concordance with conventional HCV NS5B gene sequencing assay in 190 patient sera.	HCV genotyping assay differentiating among genotypes 1 to 4 (not subtypes).
	Takeuchi et al. 1999 (471)	Serum	ABI Prism 7700/TaqMan probe	161-bp vs. 256-bp fragment in 5′ noncoding region	250 μl	10 to 10^8 copies/ml	256-bp target amplification correlated with Quantiplex HCV 1.0 assay ($R^2 = 0.84$) and Amplicor HCV Monitor v1.0 ($R^2 = 0.61$) in 15 patient sera; 10- to 100-fold more sensitive than and Amplicor HCV Monitor v1.0, and 100% specificity in 50 patient sera.	CVs varied from 0.4% to 4.7%; 161-bp target amplification showed higher sensitivity (e.g., higher RNA titers) than 256-bp target amplification.
	White et al. 2002 (542)	Serum, liver biopsy tissue	LightCycler/SYBR Green I	244-bp fragment in 5′ noncoding region	200 μl serum; 0.8 to 8.1 mg liver tissue	10^2 to 10^6 copies/ml serum or copies/ug of total RNA in tissue	Correlated with Amplicor HCV Monitor v2.0 ($R^2 = 0.86$) in 16 patient sera.	Assay conisted of an initial 15-cycle real-time PCR followed by 40-cycle real-time PCR.
	Yang et al. 2002 (552)	Plasma, serum	ABI Prism 7700/molecular beacon	182-bp fragment in 5′ noncoding region	100 μl	10^4 (60% detection rate) to 10^9 copies/ml	100% sensitivity and specificity in 17 patients and 14 healthy blood donors tested by anti-HCV EIA.	Equivalent detection of HCV genotypes 1 to 6.
HDV	Yamashiro et al. 2004 (551)	Serum	LightCycler/SBYR Green I	134-bp fragment; 98% homologous for genotypes I and IIa, 100% homologous for genotype Iib	150 μl	100 to 10^6 copies/ml	Good correlation with HBV DNA levels in 48 patients.	
HEV	Orru et al. 2004 (357)	Feces	LightCycler/SYBR Green I	77-bp fragment in ORF2 region	300 μl of 10% stool suspension in PBS	10 to 10^6 copies/PCR	Correlated with anti-HEV antibody by EIA and conventional real-time PCR in one clinical sample.	

[a] HAV, HBV, HCV, HDV, and HEV, hepatis virus type A, B, C, D, and E, respectively; CV, coefficient of variation; geq, genome equivalent; EIA, enzyme immunoassay; Ab, antibody; PBS, phosphate-buffered saline.

TABLE 21. Comparison of rapid-cycle real-time PCR assays for the detection of HIV-1 and HIV-2 in clinical specimens[a]

HIV type	Reference	Specimen type(s)	Test platform/probe chemistry	Target	Specimen volume input	Range of quantitative results	Comparison to other assays	Comments
HIV-1	Brussel et al. 2003 (52)	PBMC in whole blood	LightCycler/FRET hybridization probes	Overlapping region between LTR and chromosomal *Alu* element in initial PCR; LTR gene in second round of PCR	10^6 PBMC	6 to 10^5 copies per 5×10^4 PBMC	No comparison with other assays.	A nested PCR assay designed to quantify HIV-1 proviral DNA for in vitro infectivity experiments.
	Candotti et al. 2004 (59)	Plasma	Stratagene Mx4000/TaqMan probe	79-bp fragment in LTR region	200 μl	680 (95% detection rate) to 2×10^5 IU/ml	Correlated with single virus real-time PCR assay (R^2 = 0.87) among 39 samples with HIV-1 RNA titers ranging from 200 to 2×10^5 IU/ml; 100% specificity among 266 healthy blood donor sera tested by anti-HIV-1/2 EIA.	Intra- and interassay CV ranged from 1.6% to 3.4% and from 3.4 to 7%, respectively; equivalent detection of HIV-1 group M subtypes A to G; a multiplex assay which also detect HBV DNA and HCV RNA.
	Desire et al. 2001 (93)	PBMC in whole blood	ABI Prism 7700/TaqMan probe	199-bp fragment of *pol* gene	5×10^6 PBMC	5 to 10^5 copies per 10^6 PBMC	No correlation with HIV-1 RNA levels in 21 clinical plasma samples tested by Amplicor HIV-1 Monitor v1.5 or Quantiplex HIV RNA 2.0 assay.	Quantify HIV-1 proviral DNA; intra- and interassay CVs were 13% and 27%, respectively.
	Eriksson et al. 2003 (113)	PBMC in whole blood	ABI Prism 7700/TaqMan probe	89-bp fragment of *pol* gene	5×10^6 $CD4^+$ cells	2 to 10^5 copies per 10^4 $CD4^+$ cells	No comparison with other assays.	Quantify HIV-1 proviral DNA; intra-assay %CV ranged from 30% to 34%
	Ghosh et al. 2003 (140)	Breast milk	ABI Prism 7700/TaqMan probe	Fragment of LTR gene	1 ml	10^2 to 10^7 copies/ml	No comparison with other assays.	Quantify HIV-1 proviral DNA in breast milk.
	Gibellini et al. 2004 (141)	Plasma	LightCycler/SYBR Green	142-bp fragment of *gag* gene	1 ml	50 to 5×10^5 copies/ml	Detected 4 (7%) more positive samples than Quantiplex HIV-1 RNA 3.0 bDNA assay (R^2 = 0.91)	Both intra- and interassay CVs were <4%.

						among 56 plasma specimens from HIV-infected patients; 100% specificity on 25 samples from seronegative blood donors.	
Gibellini et al. 2004 (142)	PBMC in whole blood	LightCycler/SYBR Green	266-bp fragment of *pol* gene	5×10^6 PBMC	5 to 10^4 copies per 10^6 PBMC	No correlation with HIV-1 RNA levels in 50 clinical plasma samples tested by Quantiplex HIV RNA 3.0 assay; 100% sensitivity and specificity on 50 HIV-1-seropositive and 20 seronegative clinical sera tested by conventional PCR assay.	Quantify HIV-1 subtype B proviral DNA; intra- and interassay CVs were <5%.
Gueudin et al. 2003 (153)	Plasma	LightCycler/TaqMan probe	111-bp fragment of LTR gene	200 μl	1.5×10^3 to 5×10^5 copies/ml	No comparison with other assays.	Specifically quantify HIV-1 group O RNA; both intra- and interassay CVs were <20%.
Ito et al. 2003 (197)	Lymphoid tissue (tonsillar tissue)	ABI Prism 7700/SYBR Green	Fragments of various bp sizes in V3–V5 region of gp120 gene	5 mg	10 to 10^5 copies/reaction	Good correlation with levels of HIV-1 p24 Ag in lymphoid tissue ($R^2 = 0.99$).	Quantify HIV-1 RNA in lymphoid tissue; both intra- and interassay CVs were <10%.
Palmer et al. 2003 (363)	Plasma	ABI Prism 7700/TaqMan probe	Fragment of *gag* gene	1 to 7 ml	10^2 to 10^8 copies/ml	Correlated with results of VERSANT HIV RNA 3.0 bDNA assay ($R^2 = 0.89$) on 22 clinical specimens, but detected titers in 15 additional samples that were negative by bDNA test; 100% specificity on samples from seronegative blood donors.	Intra-assay variability ranged from 13% to 37%.

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TABLE 21—*Continued*

HIV type	Reference	Specimen type(s)	Test platform/probe chemistry	Target	Specimen volume input	Range of quantitative results	Comparison to other assays	Comments
	Saha et al. 2001 (423)	Plasma	LightCycler/scorpion probe	104-bp fragment of *pol* gene	?	1 to 10^8 copies/reaction	Good correlation with results of rapid PCR assay using molecular beacon probe ($R^2 = 0.99$).	Intra- and interassay variability ranged from 1% to 46% and from 5% to 20%, respectively.
	Vet et al. 1999 (518)	Plasma	ABI Prism 7700/molecular beacon probe	Fragment of *gag* gene	?	10 to 10^6 copies/reaction	Correlated with results of Amplicor HIV-1 Monitor test v1.0.	A multiplex assay which also quantifies HIV-1, HTLV-I, and HTLV-II RNA levels.
	Victoria et al. 2003 (519)	PBMC and lymphoid cell lines	Smart Cycler/SYBR Green	Fragment of LTR gene	5×10^6 cells	10 to 5×10^4 copies per 10^6 cells	No comparison with other assays.	Quanitfy HIV-1 RNA for in vitro infectivity experiments.
	Weber et al. 2003 (529)	Cell culture supernatant	Smart Cycler/SYBR Green	199-bp fragment of *pol* gene	200 μl	10 to 10^7 copies/reaction	No comparison with other assays.	Quantify HIV-1 proviral DNA for in vitro infectivity experiments.
HIV-2	Damond et al. 2001 (88)	Whole blood	LightCycler/TaqMan probe	160-bp fragment of *gag* gene	1 ml	5 (100% detection rate) to 500,000 copies/10^5 PBMC	HIV-2 proviral DNA levels correlated with positive cell cultures for HIV-2 virus ($U = 26$; $P < 0.02$) but not with rate of plasma HIV-2 RNA detection ($U = 61$; $P < 0.07$) among 29 HIV-2 antibody-positive patients.	Requires nested RT-PCR and quantifies HIV-2 proviral DNA, with intra- and inter assay CVs ranging from 11.8% to 41% and 30% to 40%, respectively; equivalent detection of HIV-2 subtypes A and B.
	Damond et al. 2002 (89)	Plasma	LightCycler/TaqMan probe	89-bp fragment of *gag* gene	1 ml	250 to 5×10^5 copies/ml	HIV-2 RNA detected at >250 copies/ml in 21 (54%) of 39 patients with detectable HIV-2 proviral DNA, with 100% specificity among 25 HIV-negative blood donors and 25 HIV-1 antibody-positive patients.	Intra- and interassay CVs varied from 1% to 4.4%; equivalent detection of HIV-2 subtypes A and B.

Ruelle et al. 2004 (421)	Plasma	LightCycler/SYBR Green I	173-bp fragment in LTR region	1 ml	250 to 2.5 × 10^7 copies/ml	HIV-2 RNA detected at >250 copies/ml in 12 of 13 untreated HIV-2 antibody-positive patients but none in two HIV-1 antibody-positive and 1 HIV-negative sera.	Intra- and interassay CVs varied from 0.2% to 5.6% and from 0.7% to 3.1%, respectively; equivalent detection of HIV-2 subtypes A and B.
Schutten et al. 2000 (442)	Plasma	ABI Prism 7700/ TaqMan probe	61-bp fragment of region between LTR and *gag/pol* ORF, or 67-bp fragment of *gag* gene	400 μl	500 (100% detection rate) to 5 × 10^6 copies/ml	HIV-2 RNA detected in 13 (81%) of 16 HIV-2 antibody-positive (by Western blot) patients.	Interassay CVs varied from 2% to 7.5%; differential quantification of HIV-2 subtype A and B RNAs.
Vet et al. 1999 (518)	Plasma	ABI Prism 7700/ molecular beacon probe	Fragment of *env* gene	?	10 to 10^6 copies/reaction	Correlated with qualitative results of anti-HIV-2 antibody assay.	A multiplex assay which also quantifies HIV-1, HTLV-I, and HTLV-II RNA levels.

[a] PBMC, peripheral blood mononuclear cells; LTR, long terminal repeat; EIA, enzyme immunoassay; CV, coefficient of variation; Ag, antigen; HTLV, human T-cell lymphotropic virus; RT, reverse transcription.

broad-range panel of potentially cross-reacting organisms, all of which showed negative results.

The assay developed by Pryce et al. (385), targeted the 18S rRNA gene of *Aspergillus fumigatus* isolated from whole blood samples and compared the results to the clinical features of eight patients at high-risk of invasive aspergillosis. Their assay was positive for 1 patient with clinically proven invasive pulmonary aspergillosis and 1 patient with probable invasive pulmonary aspergillosis. The PCR assay was also positive in 2 patients with no clinical evidence of fungal infection and therefore it was not possible to distinguish between a false-positive PCR result and subclinical fungemia in these patients. The PCR results were negative for 1 patient with proven disseminated invasive *Aspergillus terreus* infection, highlighting the limitations of assays targeting only *Aspergillus fumigatus*.

A number of studies have compared the sensitivity of real-time PCR methods to the galactomannan ELISA test for *Aspergillus* antigen recently approved by the FDA. Kami et al. (206) compared a real-time PCR assay targeting the 18S rRNA gene to the galactomannan test (Platelia Aspergillus, Pasteur Diagnostic) and a test for (1→3)-β-D-glucan (Fungi-Tec, Seikagaku Corporation), which serves as a marker of fungal infection. They examined 323 blood samples from 122 patients with hematologic malignancies, including 33 patients with invasive pulmonary aspergillosis and 89 control patients. The reported sensitivity for the PCR, galactomannan, and (1→3)-β-D-glucan assays for the diagnosis of invasive pulmonary aspergillosis were 79%, 58%, and 67% respectively; specificities were 92%, 97%, and 84%. The positive PCR findings preceded those of galactomannan and (1→3)-β-D-glucan measurements by 2.8 ± 4.1 and 6.5 ± 4.9 days, respectively. Other studies comparing the galactomannan ELISA to laboratory developed real-time PCR assays suggest that a combination of the two methods may provide improved diagnosis of invasive aspergillosis (61, 80, 428)

The molecular mechanisms of drug resistance in fungi have traditionally been difficult to elucidate due to the cumbersome nature of susceptibility testing for these organisms. Nascimento et al. (331) developed a real-time PCR method to detect *Aspergillus fumigatus* mutations that confer high-level resistance to itraconazole. Their results demonstrated that overexpression of two genes, *AfuMDR3* and *AfuMDR4*, which encode drug efflux pumps, and the selection of drug target site mutations can be linked to high-level itraconazole resistance.

Candida Species

The majority of real-time PCR assays developed to date for *Candida* species have focused on the identification of the six or seven most common species isolated from clinical specimens and most assays analyzed isolates growing in pure culture (157, 188, 268). If separation of the various species was attempted, it required multiplexed sets of primers and probes or multiple sets of species-specific probes.

Candida species are the fourth leading cause of nosocomial bloodstream infections and are associated with a mortality rate of 40 to 50% so rapid and reliable detection of candidemia has attracted significant interest (105, 152, 366). Selvarangan et al. (444) reported the identification of six *Candida* spp. directly from growing blood cultures using the internal transcribed

TABLE 22. Real-time PCR methods for fungi[a]

Genus	Specimen	Technology	Target	Status	Conventional method	Clinical sensitivity vs. conventional method	Turnaround time vs. conventional method	Reference(s)	Comments
Aspergillus	BAL fluid	iCycler TaqMan	18S rRNA	HB	Culture, histopathology	Greater	Faster	428	Compared with antigen detection
	BAL fluid, blood	LightCycler FRET HP	18S rRNA	HB	NA	NA	Faster	287	MagNA Pure vs. manual extraction
	BAL fluid, lung tissue	LightCycler FRET HP	ITS	Expmt1	Culture, histopathology	Greater	Faster	358	Research in rabbits
	BAL fluid, tissue	LightCycler FRET HP	mito. tRNA	HB	Culture, histopathology	Greater	Faster	397	
	BAL fluid, blood	LightCycler FRET HP	cyto. *b*	HB	Culture, histopathology	NR	Faster	459	Quantitative; nested PCR was more sensitive
	Blood, serum	ABI 7700 TaqMan	5.8S rRNA	HB	Culture, histopathology	Lower	Faster	376	Quantitative
	Blood, plasma	ABI 7700 TaqMan	18S rRNA	HB	Culture, histopathology	Greater	Faster	206, 210	Compared with antigen detection
	Blood, serum	ABI 7700 TaqMan	28S rRNA	HB	Culture, histopathology	Greater	Faster	61	Compared with antigen detection
	Serum, plasma, cell pellet	ABI 7700 TaqMan	*fks*	HB	Culture, histopathology	NR	Faster	81	Evaluation of serum, white cell pellet, plasma
	Blood	LightCycler FRET HP	18S rRNA	HB	Culture, histopathology	NR	Faster	285, 385	Quantitative
	Blood, tissue	LightCycler FRET HP	18S rRNA	Expmt1	Culture, histopathology	Greater	Faster	286	Research in mice and rabbits
	Serum	LightCycler FRET HP	mito. rRNA	HB	Culture	Greater	Faster	80	Comparison with antigen detection
	Culture	ABI 7700 TaqMan	*atr*	HB	Conventional PCR	NA	Faster	445	Monitoring of efflux pump transcript levels
	Culture	ABI 7700 Molecular Beacons	*mdr*	HB	Conventional PCR	NA	Faster	331	Monitoring of expression levels
	Tissue	ABI 7700 TaqMan	18S rRNA	Expmt1	CFU quantitation	NA	Faster	41	Caspofungin efficacy monitoring in mice
	Tissue	LightCycler FRET HP	18S rRNA	HB	Culture, histopathology	Equal	Faster	194	3 clinical specimens
Candida	Blood	ABI 5700 TaqMan	ITS2	HB	Culture	Greater	Faster	290–292	Quantitative
	Blood	LightCycler FRET HP	18S rRNA	HB	Culture	NR	Faster	285, 385	Quantitative
	Blood	LightCycler FRET HP	18S rRNA	HB	Culture	Greater	Faster	543	Detects seven species with one probe set
	Blood	LightCycler FRET HP	ITS	HB	Culture	Equal	Faster	444	Detects/differentiates six species with four probe sets
	Culture	ABI 7700 TaqMan	ITS2	HB	Culture	NA	Faster	157	Detects/differentiates six species with three probe sets
	Culture	ABI 7700 TaqMan	*erg11*	HB	Broth dilution	NA	Faster	65, 144	Fluconazole resistance genotyping
	Culture	ABI 7700 molecular beacons	ITS2	HB	Culture, phenotypic assays	NA	Faster	368	*C. dubliniensis* identification
	Culture	LightCycler FRET HP	*erg11*	HB	Broth dilution	NA	Faster	284	Fluconazole resistance genotyping
	Culture	LightCycler FRET HP	*erg11, cdr, mdr*	HB	Broth dilution	NA	Faster	129, 256	Fluconazole resistance genotyping
	Culture	LightCycler FRET HP	*act*	Expmt1	Culture	NA	Faster	351	Cutaneous *C. albicans* viability model
	Culture	LightCycler SYBR	5.8S rRNA	HB	Culture, biochemicals	NA	Faster	188	Detects/differentiates six species with six primer sets

	Oral rinse	LightCycler SYBR	18S rRNA	HB	Culture	Lower	Faster	543	
	Tissue	LightCycler FRET HP	18S rRNA	HB	Culture	Greater	Faster	194	1 clinical specimen
Coccidioides	Culture	LightCycler FRET HP	Ag2/PRA	HB	Culture, nucleic acid probe	NA	Faster	31	Extraction procedure safety evaluated
Conidiobolus	Tissue	LightCycler FRET HP	18S rRNA	HB	Culture, microscopy	Equal	Faster	194	1 clinical specimen
Cryptococcus	Culture	LightCycler SYBR	5.8S rRNA	HB	Culture, biochemicals	NA	Faster	188	
	Tissue	LightCycler FRET HP	18S rRNA	Expmt1	Culture	Lower	Faster	32	Research in mice; quantitative
Histoplasma	Culture, BAL fluid, tissue, bone marrow	LightCycler FRET HP	ITS	LD	Culture, nucleic acid probe	Equal except for bone marrow	Faster	303	3 clinical specimens
	Tissue	LightCycler FRET HP	18S rRNA	LD	Culture, nucleic acid probe	Equal	Faster	194	1 clinical specimen
Paracoccidioides	Culture	ABI 7700 TaqMan	20 genes	LD	Conventional PCR	NR	Faster	301	Yeast and hyphal phases differential gene expression
Pneumocystis	Oral swabs, tissue	iCycler SYBR	mt LSU	Expmt1	Stains, microscopy	NA	Equal	276	Quantitative monitoring of levels in rats
	Tissue	LightCycler FRET HP	*dhfr*	Expmt1	Stains, microscopy	NR	Equal	250	Quantitative; research in rats
	BAL fluid	ABI 7700 TaqMan	mt LSU	LD	Stains, microscopy	Greater	Equal	308	Quantitative
	BAL fluid, oral washes, sputum	LightCycler FRET HP	*msg*	LD	Stains, microscopy	Equal	Equal	128, 251	Quantitative
Pneumocystis	BAL fluid	LightCycler SYBR	mt *SSU*	LD	Stains, microscopy	NR	Equal	204	Quantitative
	Nasopharyngeal aspirate	ABI 7700 TaqMan	mt *LSU*	LD	Stains, microscopy	NR	Equal	497	Comparison with nested PCR, noninvasive
	Plasmid	ABI 7700 SYBR	*dhps*	LD	Conventional PCR, sequencing	NA	Faster	334	Genotyping of *dhps* gene
	Oral wash	LightCycler FRET HP	*msg*	HB	Stains, microscopy	Lower	Equal	249	Quantitative, noninvasive
	Sputum, bronchial wash	LightCycler SYBR	5S rRNA	LD	Stains, microscopy	NR	Equal	360	Comparison with conventional PCR
Stachybotrys	Culture	ABI 7700 TaqMan	18S rRNA	LD	Culture, microscopy	NA	Faster	85	Quantitative

[a] Abbreviations: ASR, analyte-specific reagent; LD, laboratory developed; NA, not applicable; NR, not reported; cyto., cytochrome; Expmtl, experimental; HP, hybridization probe; Mito. or mt, mitochondrial; BAL, bronchoalveolar lavage; HB, home brew; ITS, internal transcribed spacer; LSU, large subunit; SSU, small subunit.

spacer 1 (ITS1) and ITS2 regions flanking the 18S, 5.8S, and 28S rRNA genes and four sets of sequence-specific FRET hybridization probes. The assay was 100% sensitive and specific for 62 blood culture isolates containing yeasts compared with culture and identification using phenotypic and biochemical methods.

Maaroufi et al. (292) developed a TaqMan-based real-time PCR assay for detection of *Candida* spp. from blood samples that featured a *Candida* genus-specific probe and a *Candida albicans* species-specific probe. One-hundred twenty-two blood samples from 61 patients with clinically proven or suspected systemic *Candida* infections were evaluated using the assay and the sensitivity and specificity for *Candida albicans* detection was reported as 100 and 97%, respectively. The *Candida* genus-specific probe cross-reacted with a number of other fungal organisms and the sensitivity and specificity of the genus-specific probe was reported to be 100 and 72%, respectively.

Two real-time PCR methods have been described to detect point mutations in the *erg11* gene that are associated with fluconazole resistance in *Candida* spp. (256, 284).

Pneumocystis jiroveci

Laboratory detection of *Pneumocystis jiroveci (*formerly *Pneumocystis carinii* f. sp. *hominis*) has traditionally been achieved by examination of a fluorescent smear or through the use of a direct fluorescent antibody. The smear is relatively quick and inexpensive but has the disadvantages of being insensitive and highly dependent upon reader expertise. The direct fluorescent antibody test has been problematic of late due to the discontinuation of control materials by kit manufacturers and the lack of readily available laboratory developed controls at many institutions due to a decline in the number of *Pneumocystis jiroveci*-infected patients in this era of highly active antiretroviral therapy.

Pneumocystis jiroveci detection is likely to be one of the few instances in which real-time PCR is slower than the conventional method. Performing both a fluorescent stain and reading the slide takes approximately 30 min while the extraction and real-time PCR assay has an analytical turnaround time of approximately 3 h. However, the enhanced sensitivity and objective nature of the real-time PCR assay make this method more appealing than direct staining.

The role of quantitative PCR for *Pneumocystis jiroveci* has received attention since *Pneumocystis jiroveci* can colonize healthy individuals (neonates, pregnant woman, etc.). Larsen et al. (251) describe a quantitative, touch-down, real-time PCR assay for the diagnosis of *Pneumocystis carinii* pneumonia (PCP). The authors examined lower respiratory tract and oral washes from PCP and non-PCP patients, targeting the major surface glycoprotein (*msg*) gene of *P. jiroveci*. They found that lower respiratory tract samples from the PCP and non-PCP patients contained a median of 938 (range, 2.4 to 1,040,000) and 2.6 (range, 0.3 to 248) copies of *msg* per tube, respectively. Similarly, the oral washes from PCP and non-PCP patients contained a median of 49 (range, 2.1 to 2,595) and 6.5 (range, 2.2 to 10) copies per tube, respectively. The authors suggest that applying a cutoff value of 10 target copies per reaction reduces the number of false-positive results. However, use of this cutoff value also raised the number of false negative results. See Table 22 for a literature review.

PARASITES

Plasmodium spp.

Among parasitic infections, real-time PCR has been applied most vigorously in the diagnosis of malaria. Conventional diagnosis is based on microscopic examination of peripheral blood smears, and accuracy is dependent upon the training and experience of the preparers and readers of the slides. Expertise is often lacking. On the other hand, microscopy is inexpensive, does not require complex equipment, and is relatively rapid. Although the startup costs for real-time PCR are high, the test reagents are inexpensive, interpretative subjectivity is eliminated, and no special expertise is required by technologists. This methodology may not be usable in remote field areas of countries where malaria is endemic, where electricity would not be available, but could be valuable in regional clinics for rapid detection of *Plasmodium*, and importantly for proper treatment, accurate determination of the infecting species. The latter is especially critical since *Plasmodium falciparum* infection has a significant mortality rate and treatment is different than for other species. Resistance to antimalarials is also a problem and real-time PCR has the potential to rapidly detect the resistance genes, although this application has not yet been developed.

The RealArt Malaria LC PCR assay (Artus GmbH, Hamburg, Germany) is a commercially available kit which was developed for use on the LightCycler (Roche Diagnostics). It targets the 140-bp region of the 18S rRNA genes of the four species of *Plasmodium* which infect humans. This assay detects the presence of *Plasmodium* in blood but does not determine which species is present, which is a significant limitation. In a study of 259 travelers to areas where malaria is endemic, the Artus kit was 99.5% sensitive and 100% specific in the detection of *Plasmodium* compared to a nested PCR method (124). A limited evaluation of the quantitation of parasitemia was performed but there was only a low to moderate correlation with gene copy number and microscopic determinations.

Laboratory-developed assays have also been developed for the LightCycler and FRET technology using either SYBR green (R. U. Manson, K. A. Mangold, R. B. Thomason, Jr., E. Koay, L. R. Peterson, and K. Kaul, Program Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. P-414, 2003) or LC Red 640 as the acceptor dye. The latter method, in conjunction with melting curve analysis, was used to evaluate blood transported on IsoCode Stix from patients from Gabon and Thailand suspected of having malaria (A. Muyombwe, I. Lundgren, L. M. Sloan, J. E. Rosenblatt, P.G. Kremsner, S. Borrmann, and S. Issifou, Program Abstr. 52nd Am. Soc. Trop. Med. Hygiene, abstr. 744, 2003; J. E. Rosenblatt, A. Muyombwe, L. M. Sloan, P. Petmitr, and S. Looareesuman, Program Abstr. 11th Int. Cong. Infect. Dis., abstr 14.006, 2004). In general, this method was equivalent to conventional microscopy in the detection and identification of species of *Plasmodium* present. Other real-time PCR laboratory developed assays also targeting the 18S rRNA gene have been developed using fluorescence-based 5′ nuclease TaqMan

technology (Roche Molecular Diagnostics) and either the iCycler (Bio-Rad Labs) (255) or the ABI 7700 (Applied Biosystems) (372). Again, detection of *Plasmodium* by these methods compared well with microscopy, but they do not allow identification of the particular species present in a single test format as can be accomplished by melting curve analysis using the LightCycler.

Babesia spp.

A laboratory-developed real-time PCR assay using FRET technology and LC-Red 640 dye has been developed for use with the LightCycler in the detection of *Babesia microti* in blood. This assay is a modification of a conventional PCR assay for *Babesia microti* developed in our laboratories at the Mayo Clinic (237). In studies of patients on Block Island, R.I., PCR was more sensitive and at least as specific as blood smear and hamster inoculation for the diagnosis of acute babesiosis. PCR may be particularly useful during acute infection before serology becomes positive or when blood smears are negative or intraerythrocytic forms are difficult to differentiate from *Plasmodium*. Epidemiology may help in this determination, but occasionally confounding circumstances (such as prior travel to areas where it is endemic or blood transfusion) may be present. PCR may also be helpful in the recognition of coinfection with other tick-transmitted organisms, such as *Ehrlichia* and *Borrelia* spp.

Trypanosoma spp.

Although no real-time PCR assay has yet been developed for the detection of *Trypanosoma* spp. in human blood, an investigational FRET/LightCycler method has been used to detect *Trypanosoma cruzi* in experimentally infected mouse tissues (86). SYBR green and primers targeting a kintoplast minicircle sequence or a 195-bp satellite DNA sequence were used. The assay was used to quantitate the parasite burden during acute and chronic phases of infection and the analytical sensitivity was determined to be 0.1 to 0.01 parasite equivalents. These workers hope to apply this technology to diagnosis of *Trypanosoma cruzi* in tissues of infected humans, which would be very useful since there is no specific method for identifying these organisms by tissue microscopy. Conventional PCR has been used to identify *Trypanosoma cruzi* in the blood of patients with Chagas' disease (48, 177). Future development of real-time PCR methods will be a welcome advance since *Trypanosoma cruzi* are difficult to detect in blood smears and serology may not be readily available or be positive in acute infections. Quantitation of parasitemia will also be useful in following response to antitrypanosomal therapy.

Leishmania spp.

An investigational assay using FRET, SYBR green, and the LightCycler has been used to detect and differentiate cultured strains of Old World *Leishmania* spp. (*Leishmania major*, *Leishmania donovani*, *Leishmania tropica*, and *Leishmania infantum*) (340). Primers were chosen to amplify a 120-bp conserved region of kinetoplast DNA minicircles and the detection limit was 0.1 to 1.0 parasite per reaction. The authors, however, cautioned that because kinetoplast DNA has a high degree of polymorphism, appropriate internal biprobes or alternative gene targets will have to be identified for this method to find practical diagnostic use.

In fact, Schulz and colleagues (441) designed primers for amplification of an 18S rRNA leishmanial segment for detection and differentiation of species of *Leishmania* using cultured parasites and blood, bone marrow, and tissues from infected patients. Their laboratory-developed assay used FRET and LC Red 640 dye and the LightCycler with melting curve analysis of amplicons. Parasites were detected in 12 clinical samples and the analytical sensitivity (94 parasites per ml of blood) was within a range which would facilitate the diagnosis of visceral leishmaniasis from peripheral blood. This assay allows discrimination of three clinically relevant *Leishmania* groups (*Leishmania donovani* complex, *Leishmania braziliensis* complex, and others).

Bossolasco et al. also used an 18S rRNA target with ABI Prism technology to develop a real-time PCR assay for monitoring HIV-infected patients with visceral leishmaniasis (40). They detected decreasing levels of *Leishmania* DNA in the peripheral blood of patients after treatment with liposomal amphotericin B. Moreover, elevated parasite levels were detected in patients who relapsed following discontinuation of therapy.

Toxoplasma spp.

Several laboratories have developed real-time PCR assays for the detection of *Toxoplasma gondii* in blood, serum, CSF, and amniotic fluid (82, 312; L. M. Sloan, P. S. Mitchell, R. Patel, and J. E. Rosenblatt, Program Abstr. 101st Annu. Meet. Am. Soc. Microbiol., abstr. C-312, 2001). Each of these used the B1 gene of *Toxoplasma gondii* as a target and the LightCycler with FRET technology. Two studies found real-time PCR to be equivalent to PCR-ELISA with CSF and amniotic fluid (L. M. Sloan, P. S. Mitchell, R. Patel, and J. E. Rosenblatt, abstr. C-312) or serum, buffy coat, and CSF in a stem cell transplant patient with CNS toxoplasmosis (312). In the latter case, detection was earlier and more persistent in buffy coats, suggesting that this is the optimum type of blood specimen for real-time PCR.

Another study used the assay in serum for diagnosis and follow-up of four stem cell transplant patients (82). They compared their results with a conventional PCR but also were able to quantitate parasitemia using real-time PCR by correlating extracted DNA assay crossing points with corresponding tachyzoite counts of cultured *Toxoplasma gondii*. They were able to correlate low parasitemias with clinical improvement following treatment in three patients and increasing parasite counts in one patient who developed CNS toxoplasmosis. These studies illustrate the special utility of real-time PCR in the diagnosis of toxoplasmosis in immunosuppressed patients and pregnant women or neonates. This is an important advance since alternative diagnostic methods such as culture or serology are not routinely available or difficult to interpret in these types of patients.

TABLE 23. Real-time PCR assays for parasites

Species	Specimen	Technology	Target[a]	Status[b]	Conventional method	Sensitivity vs. conventional method	Turnaround time vs. conventional method	Reference(s)[c]	Comments[d]
Plasmodium	Blood[e]	LightCycler FRET	18s rRNA	ASR, HB	Microscopy	Equal	Equal	124, A, B, C	ASR does not identify species
	Blood	iCycler TaqMan	18s rRNA	HB	Microscopy	Equal	Equal	255	Does not identify species in one test
	Blood	ABI 7700 TaqMan	18s rRNA	HB	Microscopy	Equal	Equal	372	
Babesia	Blood	LightCycler FRET		HB	Microscopy, serology	Greater, greater	Equal, faster	237 (modified)	Serology total AB
Trypanosoma	Tissue	LightCycler FRET	kDNA	Expmtl	Microscopy	Greater	Faster	86	Research in mice
Leishmania	Cultures	LightCycler FRET	kDNA	Expmtl	Microscopy	Greater	Faster	340	Identify cultured parasites
	Blood, bone marrow, tissue	LightCycler FRET	18S rRNA	HB	Microscopy culture	Greater	Faster	441	Identifies by MCA
	Blood	ABI Prism TaqMan	18S rRNA	HB	Culture	Greater	Faster	40	Quantitation of parasitemia
Toxoplasma	Blood, serum, CSF, amniotic fluid	LightCycler FRET	B1 gene	HB	Culture, serology	Equal, greater	Faster, faster	82, 312, D	Good for acute toxoplasmosis
Trichomonas	Urine	Light-Cycler FRET	β-Tubulin gene	HB	Microscopy, culture	Greater, equal	Equal, faster	165	Urine vs. vaginal swabs a question
Giardia, Entamoeba, Cryptosporidium	Cultures, cysts, stool	iCycler TaqMan	SSU rRNA	HB	Microscopy, antigen detection	Greater, equal	Equal, equal	34, 516, 517	Multiplex assay, limited data
Entamoeba	Stool	LightCycler FRET	rRNA	HB	Microscopy, antigen detection	Greater, equal	Equal, equal	34	Separates *E. dispar* and *E. histolytica*
Encephalitozoon	Stool	LightCycler FRET	16S rRNA	HB	Microscopy (cannot identify species)	Greater	Equal	548	Identifies by MCA
Enterocytozoon	Stool	LightCycler FRET	16S rRNA	HB	Microscopy (cannot identify species)	Greater	Equal	E	Identifies *E. bieneusi* by MCA
	Stool	ABI Prism 7700 TaqMan	SSU rRNA	HB	Microscopy (cannot identify species)	Greater	Slowe	311	Quantitates *E. bieneusi*

[a] kDNA, kinetoplast DNA; SSU, small subunit.
[b] HB, home brew; Expmtl, experimental.
[c] A, R. U. Manson, K. A. Mangold, R. B. Thomason, Jr., E. Koay, L. R. Peterson, and K. Kaul, Program Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother, abstr. P-414, 2003; B, A. Muyombwe, I. Lundgren, L. M. Sloan, J. E. Rosenblatt, P. G. Kremsner, S. Borrmann, and S. Issifou, Program Abstr. 52nd Meet. Am. Soc. Trop. Med. Hyg., abstr. 744, 2003; C, J. E. Rosenblatt, A. Muyombwe, L. M. Sloan, P. Petmitr, and S. Looareesuman, Program Abstr. 11th Int. Cong. Infect. Dis., abstr. 14.006, 2004; D, L. M. Sloan, P. S. Mitchell, R. Patel, and J. E. Rosenblatt, Program Abstr. 101st Annu. Meet. Am. Soc. Microbiol., abstr. C-312, 2001; E, N. L. Wengenack, D. M. Wolk, S. K. Schneider, L. M. Sloan, S. P. Buckwalter, and J. E. Rosenblatt, Program Abstr. 103rd Annu. Meet. Am. Soc. Microbiol., abstr. C-283, 2003.
[d] MCA, melting curve analysis.
[e] Whole blood or spotted on filter paper or IsoCode Stix.

Trichomonas spp.

Real-time PCR was used to detect *Trichomonas vaginalis* in the urine of sexually active high school students (165). The nucleic acid target was a 112 bp segment of the β-tubulin gene and FRET/LightCycler technology was employed. The assay consistently detected one to four *Trichomonas vaginalis* per PCR run and approached the sensitivity (97.8%) and specificity (97.4%) of a TaqMan-based PCR using vaginal swabs (202). It was not directly compared to culture either of urine or vaginal swabs which would have added important information since culture is generally considered the gold standard. The commercial availability of this laboratory developed assay would be significant since culture of vaginal samples is considered to be too complex and time consuming for routine use and microscopy is insensitive.

Cryptosporidium, *Entamoeba*, and *Giardia* spp.

A number of real-time PCR assays have been developed for the detection of protozoan pathogens in stools (34, 311, 516, 517, 547; N. L. Wengenack, D. M. Wolk, S. K. Schneider, L. M. Sloan, S. P. Buckwalter, and J. E. Rosenblatt, Program Abstr. 103rd Annu. Meet. Am. Soc. Microbiol., abstr. C-283, 2003). Verweij et al. initially described a specific assay for a 62-bp fragment of the small-subunit rRNA of *Giardia lamblia* using TaqMan probes and the iCycler real-time detection system (Bio-Rad). This assay was as sensitive as an antigen detection method (ELISA, Alexon-Trend) and more sensitive than microscopy of stool concentrates (517). Subsequently, the same laboratory described a multiplex real-time PCR assay for the simultaneous detection of *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium parvum* (516). The target for *Entamoeba histolytica* was a 172 bp fragment of small-subunit rRNA (differentiates from *Entamoeba dispar*) and that for *Cryptosporidium parvum* was a 138-bp fragment inside the *Cryptosporidium parvum*-specific 452-bp fragment. TaqMan probes were used with the iCycler technology. The assay was performed on species-specific DNA controls of cultures of *Entamoeba histolytica* and isolated cysts of *Giardia lamblia* and *Cryptosporidium parvum* and patient specimens were analyzed by microscopy and/or antigen detection tests. The multiplex assay was described as being 100% sensitive and specific, but only 20 positives for each organism were examined and antigen tests were not performed for *Cryptosporidium parvum* and *Entamoeba histolytica* and *Entamoeba dispar* (these two *Entamoeba* spp. cannot be distinguished by microscopy).

Another real-time PCR assay for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* has been described which used FRET technology with LC Red 640 dye and the LightCycler (34). In this study, the target was a 310-bp fragment from the rRNA amoeba episome. Sensitivity was evaluated by spiking normal stools with cultured trophozoites of each organism and the detection limits were 0.1 cell per gram of stool. The primers for each organism were specific and did not amplify the other amoeba. Assay results were compared with microscopy and culture of specimens from several hundred patients from Vietnam and South Africa. PCR was more sensitive than the other methods and was 100% specific compared to culture and subsequent isoenzyme analysis for differentiation of *Entamoeba histolytica* and *Entamoeba dispar*. The assay was not compared to antigen detection assays which also differentiate these amoeba and therefore their relative efficiencies in diagnosing amoebiasis could not be determined.

Microsporidia are difficult to detect in stools because of their small size, somewhat nonspecific staining characteristics, and lack of experience of most diagnostic laboratories in identifying this infrequently recognized protozoan. A real-time PCR method has been described for the detection of *Encephalitozoon intestinalis* in stools (547). Primers were designed to amplify a 268-bp region of the 16S rRNA gene of *Encephalitozoon* spp. (*Encephalitozoon intestinalis*, *Encephalitozoon hellem and Encephalitozoon cuniculi*). FRET/LT Red 640 dye technology was used with the LightCycler and melting curve analysis was performed to determine species identification. The assay was evaluated by spiking normal stools with various dilutions of *Encephalitozoon* spores and comparing PCR results with microscopy using trichrome blue stain. Real-time PCR was significantly more sensitive than microscopy and the three *Encephalitozoon* species were accurately differentiated, which cannot be accomplished by microscopy.

Subsequently, the same laboratory has described a similar LightCycler assay for the detection of the microsporidium which is most frequently associated with intestinal infection, *Encephalitozoon bieneusi* (N. L. Wengenack, D. M. Wolk, S. K. Schneider, L. M. Sloan, S. P. Buckwalter and J. E. Rosenblatt, abstr. C-283). Using spiked stools and five *Encephalitozoon bieneusi* clinical specimens, the method was shown to detect as few as 1 to 10 targets per PCR run and *Encephalitozoon bieneusi* could be accurately identified by melting curve analysis. Menotti et al. used a real-time PCR assay to quantitatively follow *Encephalitozoon bieneusi* infection in immunosuppressed patients who were being treated with fumagillin (311). They amplified a 102-bp fragment of the small-subunit rRNA gene using a TaqMan probe with the ABI Prism 7700 sequence detection system and compared PCR results with microscopy using Uvitex 2B and trichrome blue stains. They correlated microscopic counts with PCR copy numbers derived from dilutions of plasmid controls and determined that real-time PCR performed better than did semiquantitative counts by microscopy of parasite burden in response to therapy.

See Table 23 for a literature review.

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