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Clinical Microbiology Diagnostics

Application of Real-time PCR

Since the advent of the polymerase chain reaction (PCR) nearly a quarter century ago, PCR has become a benchmark tool in clinical diagnostics, offering the advantages of high sensitivity and specificity of nucleic acid detection of pathogens. Over the last decade, fluorescent detection of PCR products during the ongoing amplification process, termed real-time PCR, has emerged as a mature technology with distinct improvements over standard PCR approaches.

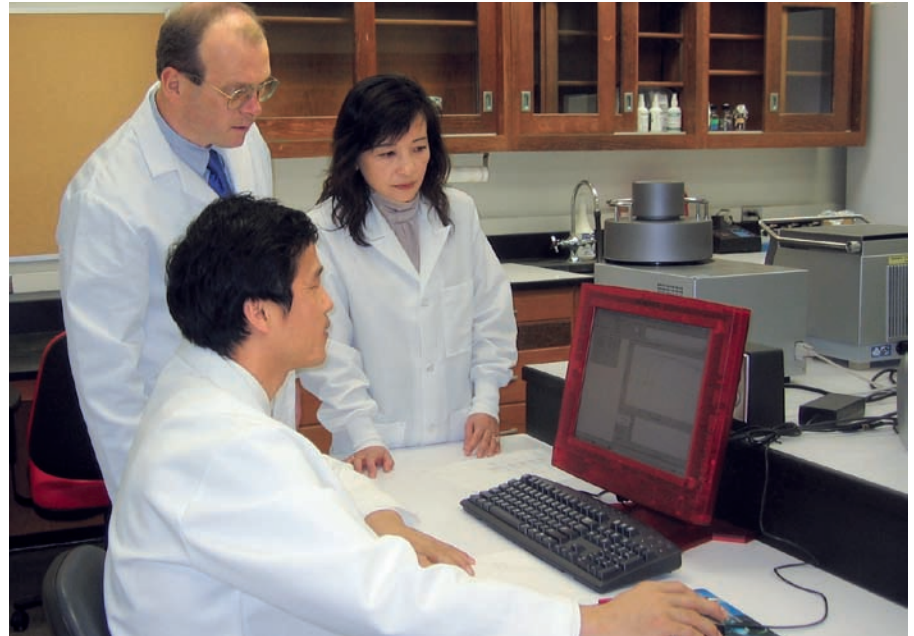

Fig. 1: Members of the Molecular Diagnostics Laboratory at Auburn University evaluate the results of a newly developed real-time RT-PCR for detection and quantification of the *gag* gene of all known strains of feline immunodeficiency virus. Front to back: Chengming Wang, research fellow; Prof. Bernhard Kaltenboeck, group leader; Dongya Gao, research assistant.

In particular, the rapid single-tube assay format offers numerous advantages for clinical microbiology, such as turn around time from sample receipt to result in as few as four hours, avoidance of product carry-over contamination, and differentiation of amplified sequences with hybridization probes. To fully exploit the diagnostic potential of real-time PCR, it is important to consider critical limiting factors in specimen collection, isolation of specimen nucleic acids for amplification, and in design of effective PCR methods [1].

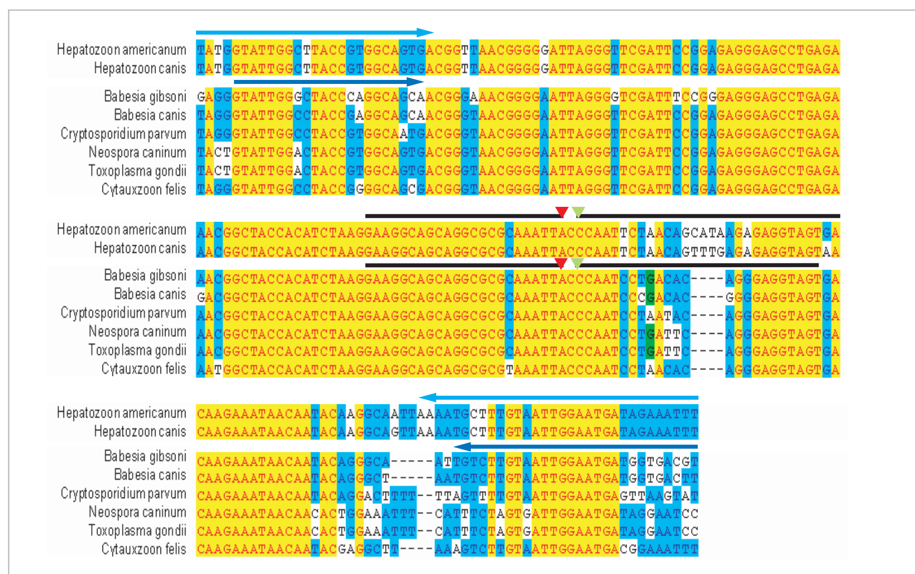

Fig. 2: Fluorescence resonance energy transfer (FRET) PCR approach for amplification of the 18S rRNA target gene of protozoal parasites. The alignment shows conserved and divergent sequence regions of protozoal parasites. Primers are shown as lines with arrows (*Hepatozoon americanum* or *H. canis*: light blue; *Babesia gibsoni* or *B. canis*: dark blue). Probes are shown as black lines, with the green triangle indicating the green fluorescent energy donor dye, and the red triangle indicating the energy acceptor dye that emits red fluorescence after stimulation of the donor dye by blue light. The high specificity is ensured by highly specific primers, and FRET probe technology, in which energy transfer from the donor to the acceptor dye occurs only after simultaneous attachment of both probes. The result is emission of red fluorescent light.

Real-time PCR and Traditional Gel-based PCR

Detection and quantification of target sequences by real-time PCR is based on the measurement of a fluorescent signal in each amplification cycle. This fluorescent signal is generated by specific interaction of fluorescent dyes in various formats with the amplification product. Quantitative information about the amplification process is obtained by plotting fluorescence intensity versus cycle number [4]. This is in contrast to the traditional gel-based PCR approach in which only qualitative information is obtained by detecting the presence or absence of a specific double-stranded DNA product by gel electrophoresis at the end of PCR amplification. Direct product monitoring in real-time PCR has profoundly reduced processing time by eliminating the need for post-amplification handling of the samples. This has also minimized the potential for sample contamination with amplification products (product carry-over) from gels for PCR analysis.

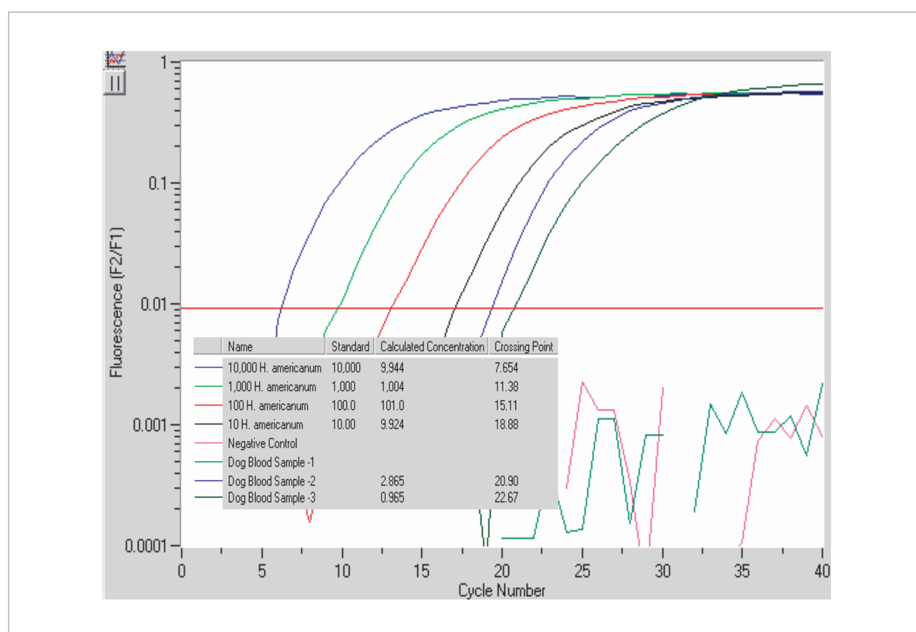

Fig. 3: Robust and sensitive real-time PCR methodology for reliable quantification of *Hepatozoon americanum*. Three aliquots of nucleic acids extracted from EDTA blood of a dog chronically infected with low numbers of *Hepatozoon americanum* are examined by amplification of *Hepatozoon* spp. 18S rRNA gene target sequences. The cycle number at which the fluorescent signal reaches the threshold level is negatively correlated to the logarithm of target copies in the sample. Standards from 10,000 to 10 copies of the *Hepatozoon* spp. 18S rRNA gene target are used to compute a linear regression between target copy number and threshold cycle for quantification of unknowns. The robustness and sensitivity of this assay is indicated by the fact that sample aliquots with 0.965 or 2.865 target copies (presumably containing 1 and 3 target copies, respectively) display as efficient amplification as the standards with high copy numbers. In contrast, a sample aliquot without *Hepatozoon americanum* target DNA shows only a background signal below the detection threshold, just as the negative control sample does.

An ideal clinical diagnostic method should offer rapid turn around time coupled with a high number of tests. When standard gel-based end-point PCR was used, only 20 (3.6%) of the results of 554 samples for detection of influenza virus and piconavirus were available within three days, with most results reported within 14 days [2]. In a typical real-time PCR setup, a single laboratory technician can process 48 samples in a morning, and obtain diagnostic PCR results in an afternoon. Therefore, four technicians using real-time PCR methodology can report the results of 554 samples within three days. After a complete re-organization to real-time PCR assays, the Molecular Diagnostics Laboratory at Auburn University (fig. 1) has been able to report all diagnostic results within 24 hours. This has had a profound impact on the prognosis of patients, because clinicians can immediately apply the diagnostic results to modify the therapy of a patient rather than obtain retrospective validation (or rejection) of clinical diagnosis and therapy.

Execution of Diagnostic Real-time PCR

Nucleic acids used for PCR analysis include total nucleic acids, purified DNA, total RNA, and poly(A)$^+$ RNA. Preservation of target nucleic acids in specimens and efficient extraction of nucleic acids without retention of low-copy targets are crucial steps to ensure high sensitivity of real-time PCR [1]. These steps require effective interaction between clinician and laboratory.

Real-time PCR uses numerous signal detection formats that are all based on emission of fluorescent light following specific interaction of detector molecule with the amplification product. These formats include nucleic acid-binding dyes, dual-labeled hybridization probes, molecular beacons, FRET probes and labeled primers. The initial step in development of a real-time PCR assays identifies conserved regions of the target sequence (fig. 2). Software is available for design of primers and probes. A well-designed and reproducible real-time PCR assay has robust performance and reliably amplifies single template copies (fig. 3). Assay specificity is determined by product sequencing and, for FRET probe assays, by “melting curve” analysis of the hybridization temperature of the probes. Mutations in the target sequence change the melting point and therefore detect genetic polymorphisms that allow differentiation between strains of a pathogen (fig. 4).

Application of Real-time PCR in Clinical Microbiology

A significant number of real-time PCR assays have been developed and applied for detection, quantification, and differentiation of human and animal viruses, bacteria, and parasites [1, 3, 4, 6], and for detection of genes that cause drug re-

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sistance of clinical isolates of pathogens [7]. Real-time PCR has ascertained the diagnosis of many previously underdiagnosed diseases caused by microorganisms that are difficult to cultivate. It helps to identify co-infections in epidemiological studies rapidly, discriminates a multiplicity of pathogen genotypes within a single reaction vessel, gives accurate quantitative results of infection load and its change after therapy, and demonstrates the epidemiological links between unique pathogen sequences and clinical signs of disease. The short time for real-time PCR diagnosis and the information about pathogen load and genetic properties impacts clinical medicine by insuring better management of patients, reducing health care costs, and slowing the problem of drug resistance.

The application of real-time PCR is currently in a phase of exponential growth, similar to the use of gel-based PCR a decade ago, and real-time PCR is rapidly becoming the "gold standard" for nucleic acid sequence detection and quantification [4, 5]. Real-time PCR has many unique advantages such as i) ease and speed of assay execution for large batches of samples; ii) highest sensitivity; iii) wide dynamic range; iv) highest specificity approaching 100% in well designed assays with hybridization probes; and v) differentiation of detected nucleic acid sequences. These advantages will virtually guarantee that real-time PCR will replace many diagnostic assays in the clinical microbiological laboratory, and that new assays will be developed that would not have been possible without real-time PCR. Current trends suggest that real-time PCR will become within the next decade the dominant method in genetics-based diagnostics.

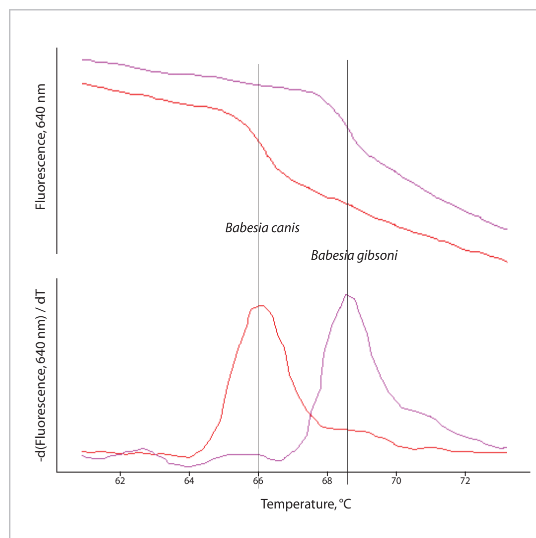

Fig. 4: Melting point determination of the amplification products of *B. gibsoni* and *B. canis*. Determination of genetic polymorphisms of the amplified pathogen sequences allows typing of pathogens or detection of mutations associated with genetic diseases. The *B. gibsoni/canis* real-time PCR assay amplifies the target sequence of both species of these protozoa that parasitize red blood cells of dogs, but the sequences to which the green fluorescent donor probe attaches differ by 2 bases between *B. gibsoni* and *B. canis*. These differences result in a melting temperature (T_m) of 68.6°C for *B. gibsoni* and of 66.0°C for *B. canis*, allowing clear differentiation of the two species by melting curve analysis in the same PCR.

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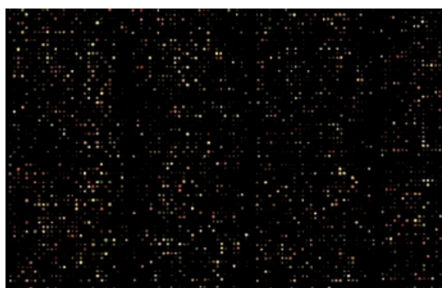

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