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Abstract

Over the past 15 years, there has been a dramatic evolution in molecular approaches to study parasites and parasitic diseases. Many of these advancements have been brought about through the development of new applications of the polymerase chain reaction (PCR). Enhancements in sensitivity that can be achieved using PCR now permit scientists to investigate changes at the level of a single cell, far below what is often needed for parasite-derived applications. PCR has had a substantial impact on advances made in the areas of parasite systematics and epidemiology, immunology and host–parasite interactions, recombinant DNA vaccine development and most recently, the analysis of whole genomes either through directly sequencing the DNA, the analysis of expressed sequence tags (ESTs) or through the rapidly growing field of functional genomics. This paper, however, focuses on the application of PCR methodology to parasite detection and differentiation, and the diagnosis of disease. Specific attention is given to advances provided by multiplex PCR, fluorescence-based "real-time" PCR, and the utilization of PCR as a quantitative technique. Published by Elsevier Science B.V.

Keywords: Diagnosis; Differentiation; Multiplex; Parasite; PCR; Real-time; RT-PCR; Quantitation

1. Introduction

Differentiating and detecting parasites, and diagnosing parasitic infections have been performed by numerous methods, ranging from the more steadfast morphological and biological techniques to using state-of-the-art biochemical, immunological and molecular assays. Not only have molecular methods, in many cases, enhanced the sensitivity and specificity of the detection process, but they have reduced much of the subjectivity inherent in interpreting morphological and biological data. Furthermore, except for rare instances

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such as in chromatin diminution which has been observed within the first few somatic cell divisions in some parasitic nematodes (Boveri, 1887), the content and integrity of parasite DNA is essentially invariant throughout its many life-cycle stages and does not succumb to short term environmental stress factors that can alter transcriptional and post-transcriptional events. Thus, molecular-based identification methods that focus on the parasite genome are generally not limited to any particular developmental stage.

Direct testing for the presence of the parasite through molecular or biochemical assays can avoid many of the ambiguities associated with indirect detection methods. One biochemical assay that has proven to be an invaluable tool in this regard is comparative isoenzyme analysis where putative homologous proteins that show differences in amino acid composition routinely display variations in electrophoretic mobility. Because results from protein-based methods can be influenced by environmental factors that affect gene transcription, translational or post-translational events, information on the normal level of variation within a particular set of allozymes and within a specific parasite group is a requisite to interpret isoenzyme data and ascertaining whether observed differences have diagnostic value.

Restriction fragment length polymorphism (RFLP) of total genomic DNA was among the first molecular techniques used for parasite differentiation. Repetitive DNA fragments generated from restriction enzyme-digested DNA and separated by agarose gel electrophoresis were visualized in earlier years by direct examination of ethidium bromide stained gels. Later, Southern blotting was used to enhance sensitivity using highly abundant radiolabeled probes such as ribosomal RNA (rRNA), cloned ribosomal DNA (rDNA) fragments or undefined repetitive DNA fragments. However, the use of repetitive DNA probes for parasite identification and differentiation has declined in recent years and has been replaced by the safety, enhanced sensitivity and specificity of the polymerase chain reaction (PCR). PCR-based technologies such as PCR-RFLP and random amplified polymorphic DNA (RAPD) have been used extensively for parasite identification and differentiation whereas the advantages of single-sequence conformational polymorphism (SSCP) (Gasser and Monti, 1997; Gasser et al., 1998), multiplex PCR (Zarlenga et al., 1999, 2001), and real-time fluorescence-based PCR (Costa et al., 2000; Jauregui et al., 2001) are just now being realized.

Amidst the plethora of advantages that PCR technology presents to the field of parasite detection and differentiation, there are some difficulties. Clearly, as with any technology that boasts the level of sensitivity that can be achieved by PCR, cross-contamination and false-positive signals become a major concern, especially when such a technique is used as a defining method. A second problem unique to PCR is that the enzymes can be sensitive to inhibitors found in blood, in by-products of blood-derived DNA isolations, and in other bodily fluids. Thus, imaginative approaches must be used to abrogate inhibitor effects.

Enzymatic amplification using species or genus specific sequences has long been applied to single parasite identification (for review see McKeand, 1998; Gasser, 1999); however, the methodology is now available to develop PCR tests that simultaneously identify more than one parasite group, where several specific primer sets are combined into a single PCR assay, i.e. multiplex PCR. Such a test can substantially simplify analyses of mixed parasite populations. In addition, sensitive, fluorescence-based “real-time” PCR techniques that combine both PCR and fragment analysis are being used to both identify parasites and parasitic diseases as well as quantitate parasite levels in biological samples. Heightened

interest in these methodologies has prompted a review of their application and potential for future use in parasite research.

2. Traditional approaches to PCR-based parasite identification

Conventional PCR requires information on the target molecule to design sequence specific primers. Though numerous genes have been targeted for diagnostic potential, the ideal sequences are often mid to highly repetitive in nature and possess a high level of sequence congruence between multiple copies. Ribosomal DNA repeat sequences have been used extensively for this purpose given their abundance within all genomes. When possible, however, focusing on rRNA through a cDNA intermediate can have a distinct advantage because normally, there is up to 50 times more RNA than DNA in a typical cell, of which 90–95% is rRNA. With the discovery of thermostable, RNA-dependent-DNA polymerases, the use of RNA targets has become a viable approach to further increasing sensitivity.

Welsh and McClelland (1990) and Williams et al. (1990) described a unique approach to produce genetic or diagnostic markers in the absence of any specific sequence information. This method, which utilizes a single, randomly designed PCR primer, usually 10–12 bp in length and greater than 50% GC, is targeted primarily to abundant sequences within the genome, and usually generates a population of amplification products referred to as RAPD that can be characteristic of a specific organism. MacPherson and Gajadhar (1994) successfully used this technology to identify primers that specifically amplified *Sarcocystis cruzi* DNA. In this study, no cross-reactivity was observed with other closely related coccidian species or host DNA, permitting them to use a single amplified product as a *S. cruzi* hybridization probe. Bandi et al. (1993, 1995) as well as others (Arribas et al., 1994; Dupouy-Camet et al., 1994) used RAPD banding patterns to differentiate individual larva of *Trichinella* and generate similarity indices from which phenetic relationships could be inferred. Shianna et al. (1998) developed RAPD primers to assess genetic variability in *Cryptosporidium parvum* isolates obtained from discrete geographical localities. In a modification of RAPD technology, Wu et al. (2000) cloned putative species-specific RAPD fragments from which they generated specific and more sensitive primers to differentiate *Cryptosporidium* species. They used this same approach to delineate species and genotypes of *Toxocara* (Wu et al., 1997) and *Trichinella* (Wu et al., 1998). Employing a slightly different strategy, Tsuji et al. (1997) applied RAPD technology to differentiate chicken *Eimeria* species by first amplifying the small subunit ribosomal DNA (ssrDNA) from each species using a single set of specific and highly conserved primers. Following the first round of PCR, a nested PCR was performed with 10 different RAPD primers to finally discriminate between the *Eimeria* species. However, for such a technique to be useful, the level of sequence variability between species must be relatively high.

With all the benefits of RAPD as a quick, simple and sensitive technique, there remain distinct caveats to this methodology. Pozio et al. (1999) clearly demonstrated that when working with *Trichinella* larvae, the PCR profile and therefore the reliability of the technique can be significantly affected by small differences in the integrity of the target DNA where bias towards amplifying smaller fragments obviously occurs with partially degraded DNA. As a result, extensive controls are necessary to utilize RAPD technology as a universal

diagnostic method. Amplification bias is not only a function of DNA integrity between samples, but results also from minor differences in the efficiency of the PCR equipment and enzymology (MacPherson et al., 1993; Muralidharan and Wakeland, 1993; Ellsworth et al., 1993; Schierwater and Ender, 1993). Thus, transferring RAPD technology between labs can be problematic. Secondly, because primer binding can occur with any DNA at the lower annealing temperatures required by RAPD technology, diagnosis of tissue or blood-borne parasitic diseases often requires a pre-selection step. Finally, it is important to understand that RAPD is, in effect, a form of competitive PCR and thus is prone to signal artifacts resulting from the competition of PCR fragments for reaction ingredients, and to inefficiencies in primer binding during the earlier rounds of the amplification program. This is likely the biggest contributing factor to many of the inconsistencies of the technique. As a result, other techniques such as amplified restriction fragment polymorphism (AFLP) (Vos et al., 1995) have emerged that involve restriction enzyme digestion of the DNA followed by attaching specific primer binding sites to the termini to allow more stringent PCR conditions to be used. Blears et al. (2000) demonstrated the utility of this technique to generate DNA fingerprints of *Cryptosporidium* isolates. This technology, however, has been more valuable in fine mapping of genomic sequences and in parasite genetics similar to the way that microsatellites, transposon-associated markers and expressed sequence tags (ESTs) have been used.

3. Alternatives to conventional PCR

3.1. Multiplex PCR

Although RAPD is laden with technical difficulties, it has the distinct advantage of not requiring specific sequence information for amplification. A second and important advantage is that, classic RAPD technology most often compares PCR banding profiles rather than relying on the appearance or absence of a single parasite-specific PCR product generated from a parasite-specific primer set. Thus, RAPD is capable of differentiating between numerous parasite groups using a single reaction. Though PCR is amenable to laboratory studies, making the transition to clinical applications will require the best characteristics of both RAPD and primer-specific PCR methodologies**.** In this regard, one would like to have a single PCR test that is highly specific, capable of differentiating several parasite species or genera simultaneously, and capable of reducing the plethora of additional positive and/or negative control reactions required by conventional PCR diagnostic methods.

A simple approach to reducing the need for PCR controls is exemplified in the identification of PCR primer sets for delineating *Taenia saginata* from *Taenia asiatica* (Zarlenga et al., 1991), or *Haemonchus contortus* from *Haemonchus placei* (Zarlenga et al., 1994). In both instances, morphological distinction between these parasite species can be difficult especially when the sample numbers are low because of the overlapping nature of their morphological characters. To resolve these issues, single, highly specific PCR primer sets were identified for each genera which, when used to amplify unknown DNA, produce a PCR banding pattern unique to each species. The unique profiles resulted from a combination of sequence length differences within their respective regions of the rDNA repeat,

and the existence of multiple rDNA repeating units. Thus, the absence of a PCR signal can be interpreted as a failed reaction obviating the need for extensive positive controls. The tests described here, however, are best utilized at the level of a single worm because when analyzed by gel electrophoresis, partially overlapping PCR fragment profiles will not permit differentiation of DNA from mixed populations. Bowles and McManus (1994) accomplished this for *Taenia* species using PCR-RFLP of mitochondrial DNA sequences.

A second approach to obviate the need for additional control reactions is the use of multiplex PCR. This form of PCR involves the simultaneous amplification of more than one target gene per reaction by mixing multiple primer pairs with different specificities. One can include among the specific diagnostic primer sets, a cross-specific primer pair that will generate a PCR product for all parasites to be differentiated, and in this way create an internal control for the integrity of the reaction. This was performed on a more simplistic level in the detection of *Schistosoma mansoni* in snails using a mitochondrial DNA minisatellite repeat sequence as the specific primer target and a second primer pair complementary to the ssrDNA of the snail to serve as an internal control (Jannotti-Passos et al., 1997). The ssrDNA primers also generated a different sized fragment in the presence of trematode DNA and assisted in differentiating *S. mansoni* from other trematodes infecting the snails.

On a larger scale, a multiplex PCR was designed to differentiate all eight currently recognized species and genotypes of *Trichinella* (Zarlenga et al., 1999). In this assay, one primer pair generated against expansion segment V sequences of the large subunit rDNA (lsrDNA), functioned both as a diagnostic reagent as well as an internal control in that PCR products were produced from all *Trichinella* genotypes among which several were uniquely sized. The balance of the diagnostic primer pairs were designed to amplify various regions of the internal transcribed spacers 1 (ITS1) and 2 (ITS2); highly variable regions of the rDNA repeat that have received substantial attention for both parasite diagnosis and phylogenetic applications. This assay results in unequivocal identification when performed on individual larvae, and its specificity has been verified further through several epidemiological studies (personal communications from Drs. E. Pozio and C.M.O. Kapel). However, on rare occasions where hosts are infected with more than one genotype of *Trichinella*, analysis of population-derived genomic DNA can lead to ambiguous results.

In some cases, sufficient anecdotal evidence may preclude the need for extensive internal controls as in the multiplex PCR test for delineating morphologically indistinguishable eggs from common bovine gastrointestinal nematodes (Zarlenga et al., 2001). In this assay, sequence data from internal and external (ETS) transcribed spacers of the rDNA repeats as well as the 3′-end of the ssrDNA and 5′-end of the lsrDNA were used to generate five primer sets. Each primer set produces a unique electrophoretic DNA banding pattern characterized by a single DNA fragment for *Ostertagia ostertagi* (257 bp), *H. placei* (176 bp), *Oesophagostomum radiatum* (329 bp), *Trichostrongylus colubriformis* (243 bp) and *Cooperia oncophora* (151 bp) (Fig. 1). Such a test is likely to be more representative of transmission potentials of infected animals than reliance on culture-derived data, and may eventually replace the more labor intensive and conventional culturing techniques for these nematodes.

To enhance sensitivity, multiplex PCR can be coupled with a secondary detection method as in the assay for detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* by Figueroa et al. (1993b). This multiplex PCR utilized species-specific primers during the PCR step followed by blotting the gel separated PCR products and hybridizing the blot to

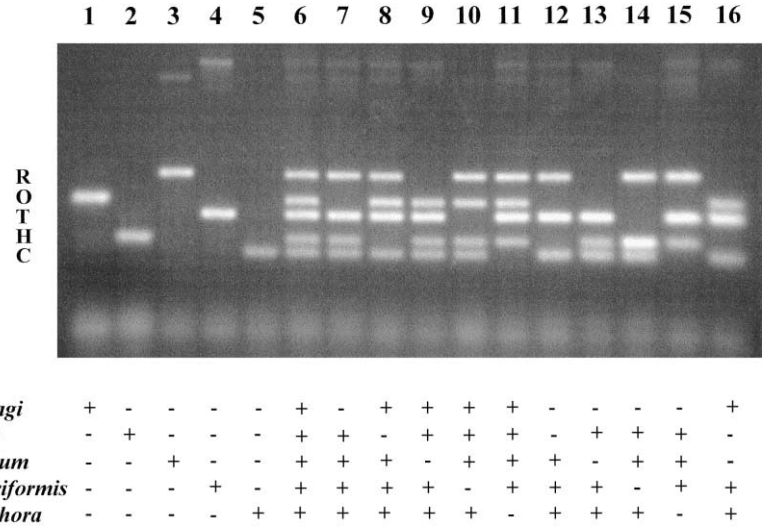

Fig. 1. Multiplex PCR of gastrointestinal nematode genomic DNA. Adult parasite-derived genomic DNAs from *O. radiatum* (R), *O. ostertagi* (O), *T. colubriformis* (T), *H. placei* (H) and *C. oncophora* (C) were mixed in equal quantities (20 pg/parasite sample) in the combinations as indicated in this figure by "+". PCR was performed in a 50 μl reaction which contained all five primer sets, each of which is specific for one of the five species indicated above. Primers of the multiplex PCR primer mix are defined in Zarlenga et al. (2001). PCR products (10 μl) were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. As demonstrated in the figure, even in the presence of all five primer sets, positive PCR signals were only observed in those samples where the homologous genomic DNA was included in the PCR reaction. This figure was reprinted from Zarlenga et al. (2001).

internally generated digoxigenin labeled probes. Though laborious, the test was effective in epidemiological studies in Mexico where greater than 60% infection rates were observed for all three bovine hemoparasites (Figueroa et al., 1993a). Later work suggested, however, that the assay was not as sensitive as the direct examination of blood smears (Figueroa et al., 1996).

A similar approach used PCR amplification followed by DNA hybridization to chemiluminescent probes in the development of a multiplex PCR assay to detect *C. parvum* and *Giardia lamblia* in water samples (Rochelle et al., 1997), though maximum sensitivity could only be achieved after two rounds of amplification. This test involved the amplification of two unrelated genes, a 256 bp portion of the ssrDNA sequence from *C. parvum* and a 163 bp region from the heat shock protein gene from *G. lamblia,* whose copy numbers are likely not equivalent between the genomes of the respective parasites. The lack of congruence between the copy numbers of these genes in conjunction with the potential for amplification bias that can occur in multiplex PCR can unknowingly reduce the sensitivity of such an assay for the parasite harboring the gene with fewer copy numbers. Thus, care must be taken in developing tests of this nature to insure that sensitivity is not inadvertently compromised by the choice of target sequences. Patel et al. (1999) also developed a multiplex test for *Cryptosporidia* species using a two gene system; however in this case, amplification of *C. parvum* or *C. wrairi* DNA resulted in similar sized PCR fragments that were collectively differentiated from *C. muris* and *C. baileyi* using ssrDNA primers. Further delineation of *C. parvum* from *C. wrairi* required PCR/RFLP methodology using primers derived from the gene encoding a *Cryptosporidium* outer wall protein.

Other multiplex PCR tests have been developed for differentiating New World *Leishmania* complexes (Harris et al., 1998; Belli et al., 1998) using the spliced leader RNA (mini-exon) gene repeats; for delineating *T. saginata*, *Taenia solium* and *Echinococcus granulosis* infections (Gonzalez et al., 2000) using amplified repetitive DNA sequences; and for identifying *Dirofilaria immitis* and *Wuchereria bancrofti* with theoretical detection levels as low as 0.1 L3 larva (Nicolas and Scoles, 1997). In an effort to further enhance sensitivity, Evangelopoulos et al. (2000) developed a nested multiplex PCR to detect *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples**.** In this study, the investigators targeted the ssrDNA using a single set of primers that amplify both species, followed by species-specific nested primers during the second round PCR. Surprisingly, though detection levels were enhanced for both species, they found fivefold differences in the levels of sensitivity when DNA from both parasites was present. This is likely the result of unforeseen amplification bias related to the fragment size differential of the amplified products. Thus, multiplex PCR can pose significant problems with false-positive signals and sensitivity issues during the development stage of the assay; however, the potential for simplifying the identification process down to a single, reproducible PCR reaction can outweigh many of the technical barriers one often encounters along the way.

3.2. Real-time PCR

Since the first dedicated thermal cycling instrument was produced in 1987, molecular techniques have promised to revolutionize the detection of pathogens in clinical specimens. Where initially, the use of PCR was restricted to molecular biology laboratories because

of the cost and labor involved with each assay, efforts have been made to market more user-friendly systems for clinical facilities. Today, simultaneous amplification of the target sequence and analysis of the products within a single instrument is made possible by incorporating fluorescent probes or dyes into the PCR reaction mix to obviate the need for subsequent gel electrophoresis. The use of an optical device in the instrument, such as a CCD camera, allows continuous monitoring of emissions from the fluorescent reagent, hence a "real time" display of results. The ultimate goal of this technology is to enable large numbers of samples to be tested at once, and with minimal manual input to keep labor and material costs down. While this has been accomplished to a degree in a variety of automated platforms, and has been made available in the form of prepackaged assays for the detection of blood-borne pathogens such as the human immunodeficiency and hepatitis C viruses, molecular-based clinical assays for the detection of veterinary pathogens are lacking. Nonetheless, the potential application to veterinary parasitology warrants a discussion of the technology.

Fluorescence-based PCR assays can generally be approached in two ways. The first utilizes standard enzymology in the presence of two sequence-specific primers and a relatively inexpensive dye such as SYBR Green^{TM} or ethidium bromide. Because intercalating dyes fluoresce poorly when not bound to double-stranded DNA, fluorescence intensity becomes proportional to the amount of double-stranded PCR product synthesized and will therefore increase with each successive round of amplification. SYBR Green^{TM}-based real-time PCR has been used for quantitating *Borrelia burgdorferi* organisms in a mouse model for Lyme disease (Morrison et al., 1999), and in an improved diagnostic assay for ehrlichiosis (Edelman and Dumler, 1996). The drawbacks of this approach are that non-specific amplification products cannot be differentiated from target products and are detected as a positive signal by the software. This requires meticulous attention to primer design in order to eliminate primer–dimer formation. Alternatively, setting the instrumentation to monitor fluorescence intensities at elevated temperatures where small, non-specific PCR products are destabilized can abrogate the effects of non-specific signals in some cases. In general, common intercalating dyes such as SYBR Green^{TM} and ethidium bromide can have an inhibitory effect on the PCR enzymology unless Mg^{2+} concentrations are experimentally optimized for each new primer set (Nath et al., 2000).

Accordingly, many investigators rely on the second method of real-time PCR which utilizes gene-specific oligonucleotide probes labeled with a fluorescent dye to mediate product detection during PCR. Once a sequence has been identified that is specific for a given organism, a "probe" is synthesized containing 5′- and 3′-termini covalently modified with reporter (fluorophor) and quencher (6-carboxy-tetramethyl-rhodamine) dyes, respectively, that hybridizes to the unique sequence during the primer extension step of the PCR. When the probe is intact, suppression of fluorescence results through fluorescence energy transfer between the two attached dyes. During primer elongation, the reporter group on the hybridized probe only, is cleaved by the 5′ nuclease activity of *Taq* polymerase (Heid et al., 1996; Holland et al., 1991) thereby permitting its fluorescence. As with using intercalating dyes, fluorescence intensity is directly related to the amount of PCR product to which the probe can hybridize. Real-time PCR has been used to develop diagnostic probes for *Toxoplasma gondii* (Lin et al., 2000; Costa et al., 2000), where one assay had a dynamic range of $0.75–0.75 \times 10^6$ parasites per PCR reaction and was useful in monitoring reactivation of

T. gondii in immunosuppressed patients (Lin et al., 2000). A similar technology was used to develop a real-time PCR test for swine toxoplasmosis using a probe derived from the rDNA repeat (Jauregui et al., 2001). The test showed no cross-reactivity with DNA from *Neospora caninum* or *Hammondia hammondi* and could be adapted to diagnose tissue cysts of *T. gondii*. Fluorescent probes have also been designed for diagnosing *Erhlichia* species in cattle, dogs and horses (Pusterla et al., 1999, 2000). Work is currently underway to develop a similar diagnostic test for *Trypanosoma cruzi*-based upon the work of Britto et al. (1999). Finally, a real-time PCR assay is being developed and optimized for identifying *C. parvum* isolates in fecal samples based upon ssrDNA sequences (J. Higgins, unpublished data). At present, a correlation has been observed between parasite loads and signal intensities, though sensitivity levels are low due to the presence of fecal inhibitors (Fig. 2). Real-time PCR has rapidly expanded to enable the study of transcriptional changes in animal cytokine genes (Leutenegger et al., 1999a,b; Collins et al., 1999) and cell signaling molecules which will be instrumental in delineating host immune responses to parasitic infections.

Other forms of fluorescent probes referred to as molecular beacons (Tyagi et al., 1998; Vogelstein and Kinzler, 1999) and scorpions (Whitcombe et al., 1999) rely upon conformational changes in the probe from a stem-loop (hairpin) structure to a linearized sequence when hybridized to the target region on the DNA strand. When hybridized to DNA, the reporter and quencher dyes become sufficiently separated from one another to eliminate the energy transfer effects of the quencher and therefore enhance the fluorescence characteristics of the reporter. Molecular beacons can be used in real-time thermal cyclers and, in some instances, offer the added advantage of fluorescing brightly enough to be observed under UV illumination with the unaided eye should sufficient probe be available. Scorpions obviate the need for internal probes used in conventional real-time PCR by incorporating a fluorophor directly into one of the PCR primers. Beacons are amenable to labeling with several different dyes making them suitable for use in one-tube multiplex PCR assays. Recently, beacons have been adapted for use on an experimental, submicrometer-sized DNA biosensor (Liu et al., 2000), a development which may lead to affordable, portable PCR-based veterinary diagnostic devices for applications in the field.

3.3. PCR as a quantitative technique

The sensitivity of PCR has permitted the adaptation of this technology to areas of research other than the diagnosis of disease. Quantitating pathogen levels within samples as well as studying changes in gene transcription both in the parasite and in host tissues responding to the infection, are just two examples of how PCR can enhance studies of altered microenvironments. Quantitative PCR is rooted in the principle that the amount of final PCR product can be used to deduce either the starting number of target molecules in a given sample (quantitative PCR) or the relative starting levels of target molecules among a number of samples (relative PCR). If the target molecule is parasite-derived, one can develop PCR assays to ascertain parasite levels in infected hosts. Likewise, one can study perturbations in transcription levels of host-derived target genes, such as those associated with the immune system, to assist in understanding the host response to the infection process.

Caution must be exercised when utilizing this technology specifically as it relates to normalization of amplification inconsistencies between samples. In this regard, it is

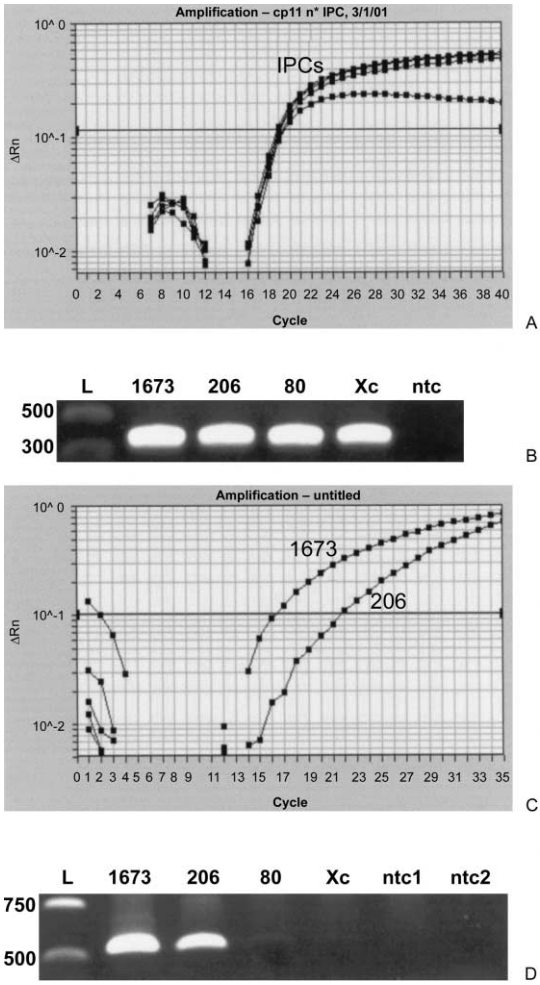

Fig. 2. Demonstration of the TaqMan™ real-time PCR assay on *C. parvum* oocyst DNA. Panel A. DNA was extracted from various quantities of *C. parvum* oocysts and then spiked with plasmid DNA designed for use as an internal positive control (IPC). Real-time PCR was performed using two external primers and a fluorescent probe specific to the IPC to determine if the feces-derived *C. parvum* DNA contained contaminants that would inhibit the PCR reaction. The graph shows the positive amplification plots for the four genomic DNA samples (1673, 206 and 80 oocysts and the extraction control) with the cycle number plotted on the *x*-axis and the normalized fluorescence plotted on the *y*-axis. Panel B. PCR products derived from the TaqMan™ assay in Panel A were also separated on an agarose gel then stained with ethidium bromide. Numbers above each lane refer to the quantities of *C. parvum* oocyst DNA equivalents used for each reaction. Xc is the extraction control, ntc the no template control and L the DNA molecular weight standards. Results demonstrate that the oocyst DNA was essentially free of inhibitory substances. Panel C. A *C. parvum*-specific TaqMan™ assay was performed for the Cp11 gene. Only DNA extracted from 1673 and 206 oocysts was positive, i.e. the amplification plots were above the threshold line at $\Delta Rn = 10^{-1}$ after 40 cycles. Panel D. PCR products derived from the TaqMan™ assay in Panel C were also separated on an agarose gel then stained with ethidium bromide. Numbers above each lane refer to the quantities of *C. parvum* oocyst DNA equivalents used for each reaction. Xc is the extraction control, ntc the no template control and L the DNA molecular weight standards. Results indicate that only DNA extracted from 1673 and 206 oocysts is detectable by PCR, and that 80 oocysts is below the level of detection using the present assay.

commonplace to include an exogenous template in the PCR reaction that competes with the target molecule for reagents and therefore functions as an internal control for PCR integrity; thus the origin of the term "competitive PCR", or "competitive RT-PCR" if the amplified product is derived from a cDNA intermediate. Competitor molecules are designed to have identical primer binding sites such that signal intensities derived from simultaneously amplified competitor and target molecules can be compared between samples to obtain absolute or relative values of initial target molecules present.

Recently, competitive PCR has been shown to be useful in quantitating parasite levels within infected hosts. Liddell et al. (1999) demonstrated that by synthesizing an internal standard to an *N. caninum*-specific, Nc5 repetitive genomic sequence, they were able to determine the relative parasite burdens in a mouse model in the hopes of developing a test to evaluate the efficacy of vaccine candidates against *N. caninum*. A similar, semi-quantitative test was devised to study the kinetics of the *T. gondii* infection (Luo et al., 1997) and for measuring the efficiency of vaccine candidates and antimicrobial agents against *T. gondii* infections using a competitor synthesized from the B1 gene (Kirisits et al., 2000). By spiking samples with known amounts of competitor, Chung et al. (1999) applied standard curve methodology in conjunction with competitive PCR to quantitate *C. parvum* levels in municipal water samples. One example exists where competitive PCR was used to quantitate *Ostertagia* levels in mixed populations of cattle nematode eggs (Zarlenga et al., 1998) in the absence of an exogenous control. In this test, a single primer pair generated a PCR fragment for *O. ostertagi* that was distinct from the PCR product generated from most other major parasite nematode species that infect cattle. The inherent competition between these populations of parasite DNAs was sufficient to derive the relative contribution of eggs from *Ostertagia* in the entire egg mixture using standard curve methodology and the ratio of signal intensities from the two distinct PCR signals. A plethora of assays have been developed to quantitate human parasitic infections as well, including but not limited to *T. gondii* levels in infected AIDS patients (Bretagne et al., 1995) and chronic *T. cruzi* infections using a kinetoplast minicircle DNA competitor (Centurion-Lara et al., 1994).

PCR as a quantitative tool has been used extensively to study perturbations in the host immune response to parasitic infections. In large animal systems, this has been particularly beneficial because of the dearth of specific immune reagents available for directly assessing levels of secreted cytokines. In these analyses, competitor molecules were generated for each unique cytokine or were produced in the form of a polycompetitor (Ready et al., 1996) prior to studying transcriptional changes in the gene. The major concern using this methodology is equating changes in gene transcription to the secretion of a biologically functional protein. Interleukin-15 is one such cytokine that appears not to follow this scenario (Bramford et al., 1996); however, in general, changes in mRNA levels have been used to identify cytokine profiles associated with the infection and have been used to design more specific analyses to correlate gene expression with the biology of the response.

When working with large animals where the size of experimental groups are kept to a minimum, the importance of enumerating the molecules of a specific mRNA by quantitative competitive RT-PCR is substantially overshadowed by the high variability in immune responses between animals. This results primarily from non-homogeneous environmental and host genetic factors. Therefore, defining relative changes in mRNA levels (relative competitive PCR) between control and treatment groups can be of equal importance in assessing

the effects of external stress factors both peripherally and locally within the host. Relative competitive RT-PCR has been used to study changes mRNA transcription of bovine IL-2, IL-4, IL-10 and IFN-γ in both peripheral and localized responses to primary infections of *O. ostertagi*, using RNA-derived from abomasal lymph nodes (Canals et al., 1997) and lamina propria lymphocytes (Almeria et al., 1997), respectively. Results were consistent with a Th1/Th2 type profile that is less restricted than observed for murine models, whereby elevations in mRNA levels were demonstrated in both IFN-γ and IL-4. A similar approach was used to assess localized cytokine responses, i.e. IL-10, IL-12, IL-15 and IFN-γ, in cattle harboring primary infections of *C. parvum* where consistent increases were observed in transcription levels of both IL-12 and IFN-γ mRNA, whereas a decrease was seen in IL-15 (Canals et al., 1998). Recently, evidence for a Th1-type response was further verified at the mucosal interface using competitive RT-PCR to study changes in TNF-α, IFN-γ, IL-10 and iNOS (Wyatt et al., 2001).

Real-time PCR can enhance the reproducibility of assigning relative values and therefore has been used to measure levels of expression of immunologically important genes. Analysis of fluorescence data by a computer workstation takes only a few minutes, and the investigator can use internal controls and/or standards via multiplex PCR to increase inter-assay reproducibility. Real-time assays have been developed for a number of animal cytokine genes. Leutenegger et al. (1999a) examined the expression of feline IL-4, IL-10, IL-12 p35, IL-12 p40, IFN-γ and IL-16 in peripheral blood mononuclear cells (PBMC) and frozen cell pellets from domestic cats. The assay detected an elevation in IFN-γ expression in stimulated vs. unstimulated cells, with greater sensitivity provided by a two-step RT-PCR reaction as opposed to single tube. These same authors investigated real-time quantitation for bovine cytokines also, to study gene transcription in cells recovered from milk (Leutenegger et al., 2000). They demonstrated the ability of their assays to detect TNF-α, GM-CSF, IL-12 p40 and IFN-γ in all healthy, mid-lactating animals examined, whereas detectable transcription levels of IL-6 could only be detected in two animals. Unfortunately, the detection limits for IL-2 were below the level of assay sensitivity. Although real-time PCR assays developed by Leutenegger et al. (1999a, 2000) and Collins et al. (1999) to examine feline and bovine cytokine expression were not used in parasite research, one can clearly see the benefits of using these reagents to study host responses to various parasitic infections such as *Toxoplasma* in cats or gastrointestinal nematodes in cattle. Leutenegger et al. (1999b) also recently developed real-time assays to study changes in equine cytokines. In this research, mRNA extracted from equine undecalcified bone sections, fixed in paraformaldehyde and embedded in a hydrophobic acrylic resin was examined. The authors were able to detect the expression of a common PCR housekeeping gene, GAPDH, as well as various equine cytokines genes suggesting that nucleic acids in archived specimens may be suitable for use in quantitative molecular assays if properly fixed. These reagents may 1 day assist studies on *Sarcocystis neurona*, the causative agent of equine protozoal myeloencephalitis in horses.

3.4. Future directions

Applications of PCR to answer questions associated with veterinary parasite research are still evolving, though the process appears to have taken cues from technological advances driven by research on humans and on mouse models. Nonetheless, as state-of-the-art

techniques such as the analysis of EST libraries, the generation of single nucleotide polymorphisms (SNPs), laser capture microdissection, and the construction of PCR-based cDNA libraries from single cells, are combined with current advances in microarray and microchip technology for large-scale gene analysis, scientists are able to ask highly targeted questions yet derive their answers from a more holistic assessment of the genetics of the parasite and/or the host. Specifically, these technologies are leading the search for host genotypic markers for disease susceptibility and resistance, identifying host production traits, and for developing reproducible, DNA-based diagnostic tests. However, as with adapting any new technology to mainstream veterinary applications, certain hurdles will have to be overcome. The most obvious of these relate to control reagent and instrumentation costs, optimizing sample preservation and processing, and training laboratory personnel in state-of-the-art molecular techniques, data interpretation and troubleshooting. Nonetheless, utilization of these technologies in veterinary parasitology once again may take its cues from human research where eventually, one can look for more sophisticated tests to be performed in fully integrated, diagnostic laboratories rather than in the veterinarian's office.

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