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Quantification of In Vitro and In Vivo *Cryptosporidium parvum* Infection by Using Real-Time PCR

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Established methods for quantifying experimental *Cryptosporidium* infection are highly variable and subjective. We describe a new technique using quantitative real-time PCR (qPCR) that can be used to measure in vitro and in vivo laboratory infections with *Cryptosporidium*. We show for the first time that qPCR permits absolute quantification of the parasite while simultaneously controlling for the amount of host tissue and correlates significantly with established methods of quantification in in vitro and in vivo laboratory models of infection.

Cryptosporidium spp. are waterborne protozoan parasites that cause a range of diseases in humans, including self-limited diarrhea in immunocompetent hosts, persistent diarrhea in children living in developing countries, and severe infections in individuals with compromised immune systems. A massive waterborne outbreak in 1993 in Milwaukee, Wisconsin, resulted in over 400,000 human cases of cryptosporidiosis, and the parasite continues to cause sporadic water- and food-borne outbreaks in the United States (17, 31). The environmental form of the parasite, the oocyst, is resistant to chlorination, and effective therapies for human infection are limited. The Centers for Disease Control and the National Institutes of Health, acknowledging the potential risk of intentional dissemination, included the parasite on their priority lists of bioterrorism agents (24).

Research on the pathogenesis of *C. parvum* has been hampered by several factors, including inadequate animal models, inability to propagate the parasite in vitro, and imprecise methods for quantification of infection. Laboratory models of *C. parvum* infection have a critical role in advancing our understanding of many different aspects of the basic biology of the parasite and are also essential for the testing of potential interventions. Accurate, objective, and reproducible methods of quantifying *C. parvum* infection are necessary for these studies. Established methods of quantification of experimental *C. parvum* infection, such as counting intracellular forms of the parasite on hematoxylin-eosin-stained histopathologic specimens (7, 8), counting shed oocysts in stool (13), and enumerating intracellular stages by immunofluorescence assays (IFA) in in vitro samples, are imprecise, time-consuming, and subjective (25).

The use of molecular techniques, PCR in particular, has significantly advanced our understanding of *C. parvum* epidemiology, taxonomy, and biology (18, 20, 26, 27) and may have applications in the clinical setting (21). Semiquantitative PCR and quantitative real-time PCR (qPCR) have been used to detect *C. parvum* in environmental (9, 10) as well as fecal (30) samples. Either DNA or RNA that has been reverse transcribed into cDNA can be quantified using this technique. Recently, this technique has also been applied to measurement of in vitro laboratory infections (3, 6, 11, 14, 15). Using qPCR analysis, we developed an objective and reproducible technique that can be used to quantify experimental *C. parvum* infections in in vitro and in vivo models. We demonstrate for the first time that qPCR analysis positively correlates with established techniques for the measurement of in vivo and in vitro experimental *C. parvum* infections.

qPCR correlates significantly with the IFA for the measurement of experimental *Cryptosporidium* infection in vitro. We used an established technique, IFA, for quantifying in vitro infection (12) and compared the results with those provided by qPCR. The statistical methods used to compare these and all subsequent techniques were as follows: after normal distribution was demonstrated by use of the Kolgorov-Smirnov test, linear regression was then used to correlate qPCR results with the dose of parasite used (in vitro) as well as with results obtained with a "gold standard" method of quantification, such as IFA or the enumeration of the parasite in histological sections (in vitro and in vivo). All in vitro analyses were performed in duplicate or in triplicate. The relationships were analyzed by plotting least-squares linear regression lines and computing coefficients of determination (r^2) and corresponding P values. When data were not normally distributed, the Spearman rank correlation was used, and a two-tailed test of significance was used to determine P values. All statistical analyses were performed with SPSS 10.0 (Chicago, IL).

Two intestinal epithelial cell lines which support *C. parvum* infection in vitro, one of murine (16) and the other of human (12) origin, were used. The murine m-IC_{cl2} cell line was derived from the small intestine of a transgenic L-PK-tag 1 mouse carrying the simian virus 40 large T antigen under the control of the 5′ regulatory sequence of the L-type pyruvate kinase promoter (2). The human intestinal-like $Caco_{2A}$ cell

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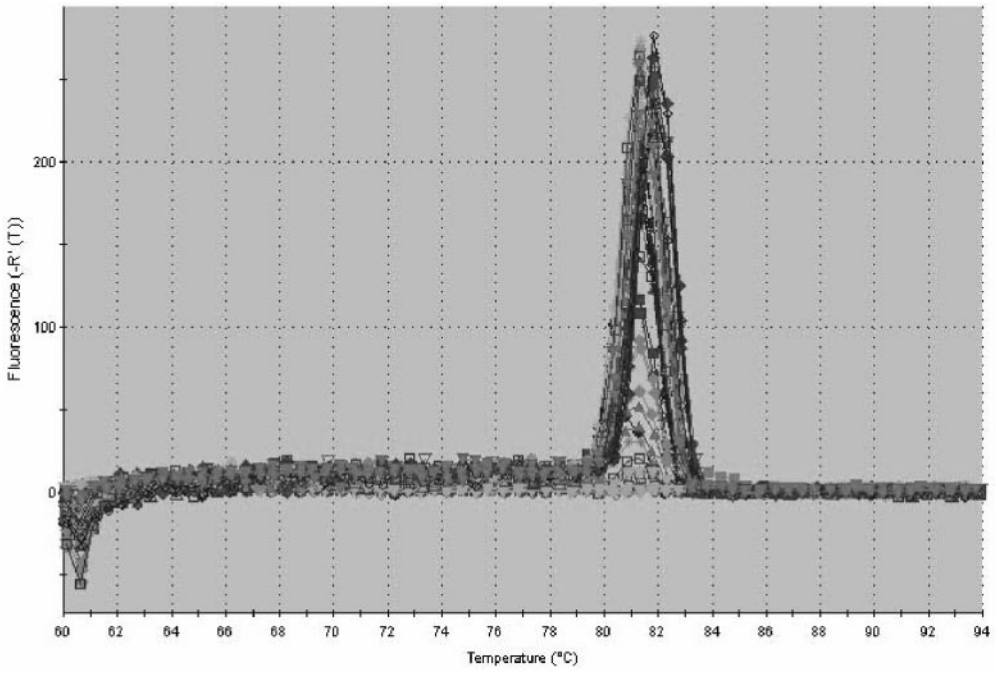

FIG. 1. Melting-curve analysis of gp40 primers for qPCR performed using SYBR green.

line was originally obtained by the GRASP digestive disease center from H. Buller (Academic Medical Center, Amsterdam, The Netherlands) (28). Collagen-coated glass chamber slides (BD Falcon, Bedford, MA) were seeded with 4×10^4 cells/well, and 24-well plates (Costar; Corning, NY) were seeded with 2×10^5 cells/well, which were grown to confluence under previously defined conditions (2, 28). Confluent cells grown on glass slides or cultured plates were then infected with various doses of *C. parvum* oocysts (IOWA isolate; Bunch Grass Farms, Deary, ID) for 24 h as described previously (12). All experiments were performed in triplicate except where stated otherwise.

IFA was performed as described previously (12). Twenty-four hours after infection, the cells were washed with complete medium and then fixed with methanol for 30 min at room temperature. After nonspecific binding was blocked with 5% normal goat serum in phosphate-buffered saline (PBS), intracellular stages of the parasite were detected with the monoclonal antibody 4E9, which is specific for a carbohydrate epitope on the sporozoite antigens gp40 and gp900 (4). After being washed with PBS, cells were incubated with Alexafluor 488-conjugated goat anti-rabbit immunoglobulin M and counterstained with 4′,6′-di-amidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Slides were examined by fluorescence microscopy, and parasitic stages were counted in a blinded manner in 20 high-powered fields and expressed in terms of the number of parasites per high-powered field. Using IFA, we found that murine m-IC_{cl2} cells contained fewer parasites than human $Caco_{2A}$ cells after infection with equivalent doses of *C. parvum*, which is in agreement with a previous report (see Fig. 2) (16).

We then used qPCR to measure *C. parvum* infection from cells infected in parallel to those in which infection was quantified by IFA. Total RNA was isolated with an RNeasy kit (QIAGEN, Valencia, CA). After DNase (Ambion, Austin, TX) treatment, a reverse transcription reaction was performed using Stratascript (Stratagene, La Jolla, CA). Total DNA was extracted from intestinal tissue by using a GNOME DNA kit, (Qbiogene, Irvine, CA). qPCR was performed by using primers designed with Primer Express (Applied Biosystems, Foster City, CA) to amplify a conserved region of the gp15 portion of the *Cpgp40/15* gene (4, 27). The sequences of these primers were as follows: forward, 5′-TCA TTT GTA ATG TGG TTC GGA GAA-3′, and reverse, 5′-AGG GTA AAG GCA AAC AAA TCG A-3′. Primers designed to amplify the murine housekeeping genes encoding nidogen (22) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (23) and the human gene encoding β-actin (1) were used as the internal standard as previously described. qPCR was performed using an ABI Prism 7700 thermocycler (Applied Biosystems) with the following conditions: 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s. The *Cpgp40/15* primers produced a single PCR product when used to measure *C. parvum* in in vitro and in vivo samples, thus demonstrating specificity for the target gene (Fig. 1). For each sample, infection was quantified by dividing the number of copies of *Cpgp40/15* by the number of copies of the respective endogenous control as described previously (22). We found that in comparison with qPCR performed using cDNA, qPCR performed using DNA could not reliably distinguish between viable and heat-inactivated parasites in vitro (data not shown). Therefore, qPCR using cDNA was used for all subsequent in vitro experiments. Linear regression was then used to correlate the results of qPCR with the dose of parasite used to infect the intestinal epithelial cells. We found that the values obtained by qPCR positively correlated with the dose of parasite used to

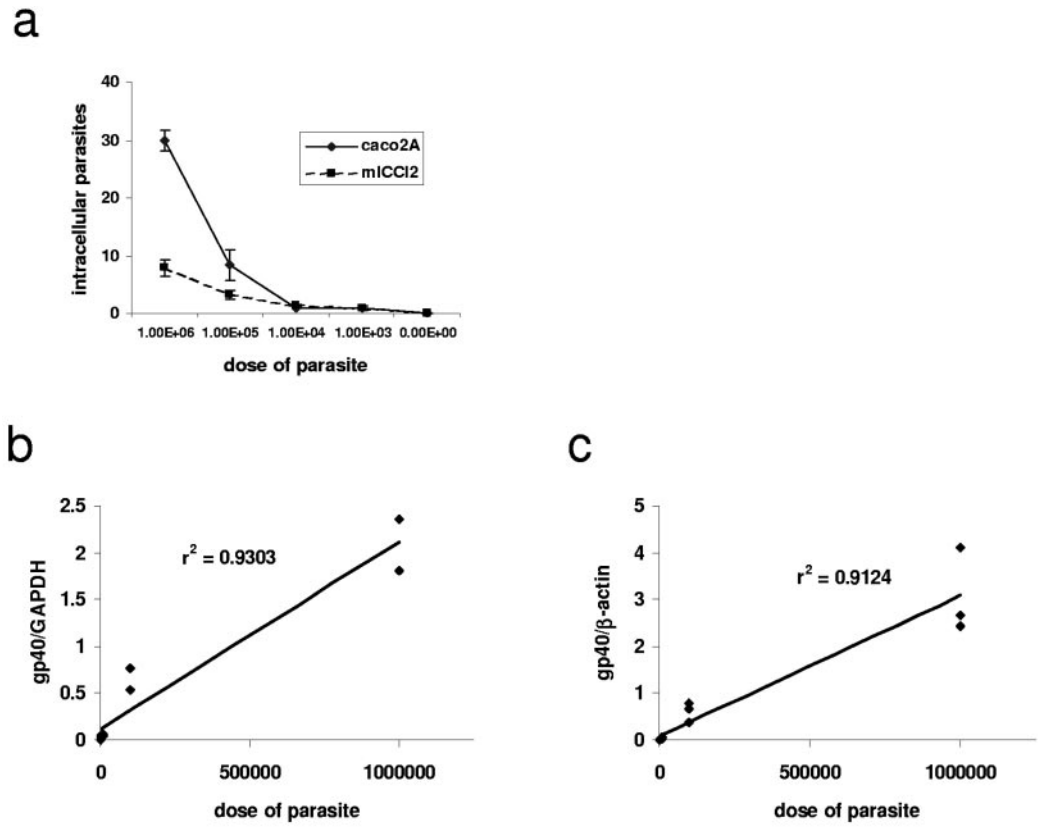

FIG. 2. (a) IFA used to quantify *C. parvum* infection in $Caco_{2A}$ and m-IC_{cl2} cells. (b and c) Linear regression analysis comparing qPCR result (*y* axis) and parasite inoculum (*x* axis) after a 24-hour infection in m-IC_{cl2} (b) and $Caco_{2A}$ (c) cells. The parasite inoculum ranged from 103 to 106/ml, and these data are representative of two independent experiments performed in triplicate (for each experiment, $n = 15$).

infect both the m-IC_{cl2} ($r^2 = 0.930$; $P < 0.0001$) and the $Caco_{2A}$ ($r^2 = 0.912$; $P < 0.0001$) cells over a fourfold log range (Fig. 2). Again, we used linear regression to correlate the results of qPCR and IFA. We found that qPCR positively correlated with the established technique of IFA for measurement of infection in both m-IC_{cl2} ($r^2 = 0.959$; $P < 0.0001$) and $Caco_{2A}$ ($r^2 = 0.853$; $P < 0.0001$) cells (Fig. 3). Therefore, qPCR correlates well with IFA when used to measure *C. parvum* in vitro infection over a wide range of parasite doses.

qPCR correlates significantly with histological measurement of experimental *Cryptosporidium* infection in vivo. *C. parvum*-infected intestinal samples from a previously published

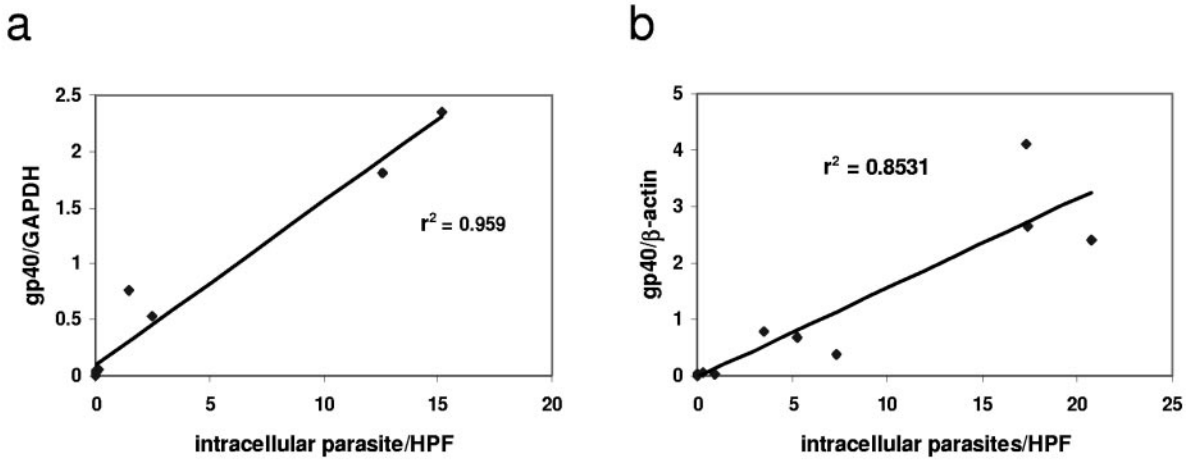

FIG. 3. Linear regression analysis comparing qPCR result (*y* axis) and IFA (*x* axis) to quantify parasites after a 24-hour infection in m-ICcl2 (a) and $Caco_{2A}$ (b) cells. The parasite inoculum ranged from 103 to 106/ml, and these data are representative of two independent experiments performed in triplicate (panel a; $n = 15$) or in duplicate (panel b; $n = 10$). HPF, high-powered field.

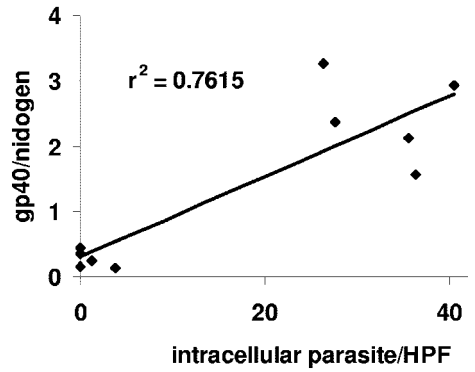

FIG. 4. Linear regression analysis comparing qPCR result (*y* axis) and histological measurement (*x* axis) to quantify parasites after a 24-hour infection in mice. The data are representative of two independent experiments ($n = 10$). HPF, high-powered field.

study were used to compare techniques for quantifying infection (19). For histological analysis, portions of the terminal ilea of 10 *C. parvum*-infected mice were removed and flushed with PBS prior to either fixation in 10% buffered formalin or freezing in liquid nitrogen. After paraffin embedding, sections of the intestine were stained with hematoxylin-eosin. The parasite burden for each animal was determined by counting the mean number of intracellular forms of the parasite within a 10-by-10 grid counted in five separate high-powered fields within each sample (7). *C. parvum* infection was also quantified in parallel by qPCR performed using DNA and extracted from a segment of the terminal ilea adjacent to that used for histological analysis. qPCR was performed as described above for the in vitro experiments, and the parasite burden was expressed as the number of copies of *Cpgp40/15* divided by the number of copies of the endogenous control, the gene encoding nidogen. Comparison of qPCR with histological analysis using linear regression demonstrated a statistically significant positive correlation ($r^2 = 0.761$ and $P = 0.001$) (Fig. 4). qPCR was performed in parallel on cDNA obtained after reverse transcription of RNA extracted from adjacent segments of terminal ileum. We found that the results obtained by use of cDNA correlated poorly with histological analyses and therefore did not use them for further analysis (data not shown).

In summary, we found that qPCR compared favorably with other established methods of quantification used in experimental infections with *C. parvum*. Unlike other microbial organisms that can readily be propagated in cell-free medium in vitro and are thus more easily quantified, the parasite *C. parvum* has traditionally required direct visualization for its enumeration. However, techniques such as IFA and histological measurements are labor-intensive and prone to interobserver variability. The development of objective and less time-consuming assays to measure infection is therefore desirable. Enzyme-linked immunosorbent assay-based assays are an objective and reliable alternative and have been used successfully for in vitro (29) and in vivo (5) infections. However, these assays have several drawbacks, including an inability to absolutely quantify the parasite and a reliance on oocyst shedding, which may be temporally dissociated from the actual infection in in vivo experiments. While there have been several other recent reports demonstrating that qPCR can be used to quantify in vitro *C. parvum* infection (3, 6, 14, 15), qPCR was not validated by comparison with gold standard techniques. Additionally, only one of these studies used host genes to correct for the amount of sample when quantifying *C. parvum* in vitro (3). Importantly, we demonstrate that qPCR can be used to measure parasite burden in an in vivo model of *C. parvum* infection and have correlated this technique with histological quantification. To our knowledge there has been only one previous study in which qPCR was used to detect, but not quantify, *C. parvum* in a tissue sample (11).

In this report we show that qPCR can be used to quantify parasites in in vitro and in vivo laboratory models of *C. parvum* infection. Objective and reproducible assays for the measurement of experimental infection in animals are particularly relevant for studies of the host immune responses to *C. parvum*. In these circumstances, targeted gene deletions or neutralization of target proteins affecting the immune response of the host may result in slight but significant differences in parasite burden which might not be detectable by use of more-subjective methods of quantification. Our technique permits absolute quantification of parasites while simultaneously controlling for the amount of host tissue and thus may be useful in such future studies.

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