Freezing of stool samples improves real-time PCR detection of *Entamoeba dispar* and *Entamoeba histolytica*.

Lieselotte Cnops*, Marjan Van Esbroeck

Department of Clinical Sciences, Institute of Tropical Medicine (ITM), Antwerp, Belgium

*Corresponding author:

Cnops L

Institute for Tropical Medicine
Kronenburgstraat 43/3
B-2000 Antwerpen
tel +32 3 2476436
fax +32 3 2476440

lcnops@itg.be
Summary

PCR as a tool for intestinal parasite diagnosis is expanding since differentiation between *E. histolytica* and *E. dispar* cysts is impossible with microscopy. Since pre-analytical factors influence DNA detection, we evaluated with real-time PCR the influence of storage time and temperature. We demonstrated an improved DNA detection in frozen stool samples.
Keywords: real-time PCR; *Entamoeba dispar/histolytica*; freezing; sample lysis; cyst.

Amoebiasis is caused by *Entamoeba histolytica* and responsible for a million cases of dysentery and liver abscess and 100,000 deaths each year (WHO, 1997). *E. histolytica* is morphologically indistinguishable from the nonpathogenic *E. dispar* although a separate species (Jackson, 1998). Differentiation between both species is important for accurate diagnosis of individuals at risk of invasive disease and to avoid unnecessary treatment of *E. dispar* infections (WHO, 1997). Microscopy remains often the first step for intestinal parasites diagnosis but nowadays, the interest in molecular techniques in clinical settings grows, especially because they can discriminate between morphologically identical *E. dispar* and *E. histolytica* cysts (Morgan and Thompson, 1998; Fotedar et al., 2007).

In this study, we used a real-time PCR adapted from Verweij et al. (2003) with *E. histolytica*/*E. dispar*-specific primers (250nM; Biolegio, the Netherlands) and FAM-labeled *E. dispar* and VIC-labeled *E. histolytica* MGB TaqMan probes (100nM; Applied Biosystems). Phocine Herpes Virus 1 primers (150nM) and a Cy5-probe (100nM) were used for internal control (IC) amplification. The 25µl reaction also contained 3.5mM MgCl$_2$, 12.5µl HotStarTaq mix (Qiagen Benelux), 0.1 mg/ml bovine serum albumin and 5µl DNA. The PCR was run on the Cepheid Smart Cycler II with a program of 1x 15 min at 95°C and 50x (5s at 95°C, 30s at 60°C and 30s at 72°C).

A well-validated PCR format is essential to guarantee high-quality results. The PCR was already carefully validated in endemic (Verweij et al., 2003; Kebede et al., 2004) and non-endemic settings (Verweij et al., 2004; Qvarnstrom et al., 2005; Vissers et al., 2006;
ten Hove et al., 2009). Since we utilized a slightly different set-up, we evaluated the PCR in our laboratory on 410 microscopic-analyzed samples according EN ISO norm 15189. Analytical specificity was assessed in 12 relevant non-target stool parasites which gave no PCR signal. Additionally, no DNA amplification was detected in eight negative controls derived from individuals with no known parasitic infection history. Diagnostic specificity and sensitivity of the PCR were respectively 97.83% and 93.41%. The reproducibility for 5 fractions of one freshly-stored sample revealed a mean Ct-value of 29.28 with a CV of 0.5% indicating a possible variation of 0.37 Ct-values between repeated extractions.

The increasing demand of molecular analyses on several sample types makes high quality DNA a necessity. It is therefore crucial to take pre-analytical factors and extraction methods into account, especially in case of stool specimens which are considered as the most complex samples for PCR analysis due to PCR inhibitors (Fotedar et al., 2007). Additionally, highly resistant cysts require intense lysis. Here, DNA was extracted with the QIAamp DNA stool kit (Qiagen Benelux) according to manufacturer’s guidelines with minor modifications.

For better DNA release, we tested a freezing step prior to DNA extraction. Stool samples were obtained from patients of the ITM outpatient clinic (Antwerp, Belgium). We selected 40 positive samples with enough material for various fractions. Thirty samples were divided in two fractions: a fresh sample that was stored for maximum one week at 4°C and a frozen sample that was stored at -20°C for medium (several days or weeks) or for long-term (months) storage times. Another ten samples were divided in four fractions.
to determine the effect of freezing temperature (4°C versus -20°C and -80°C) for short-term storage times (10 minutes, 4 hours and 24 hours). We detected 3 E. histolytica and 37 E. dispar specimens but no difference was seen between both species upon freezing. Figure 1 illustrates a higher median Ct-value in freshly analyzed specimens compared to their frozen counterparts while the median IC Ct-value in both groups was similar. Ct-values from frozen samples were significant different (p≤0.001) from those of freshly stored samples. In contrast, Ct-values of ICs were not significant different. Thus, improved parasite DNA detection was reflected by decreased Ct-values after freezing. The average improvement in all fractions was about three Ct-values (mean: 2.81; median: 3.08) which correlates to an 8-fold increase in analytical sensitivity.

The improved DNA detection for each sample was determined by subtracting the Ct-value derived from the fresh and corresponding frozen sample. The effect of storage time is represented in Table 1A. We demonstrated for 35/40 samples a better DNA amplification (△Ct >0). The best result noticed, was an improvement of 8.26 Ct-values after freezing for 3 days. Six of the seven samples with a minor decreased Ct-value (△Ct 0-1) were derived from the long-term storage category. Twenty samples were detected with a decrease of 2 to 6 cycles after freezing. The improved detection in the three categories was greater than could be expected from variations by repeated DNA extractions without freezing (see supra).

To evaluate the effect of storage temperature (Table 1B), the four fractions of the short-term category samples were simultaneous analyzed with real-time PCR. We found that incubation at -80°C and -20°C resulted in significant decreased Ct-values and thus better
DNA detection. Half of the samples that were stored at -80°C had a ΔCt of 4 to 5, while most samples stored at -20°C produced a decreased Ct-value between 2 and 4. Four aliquots demonstrated no better or inferior DNA detection after freezing. Storage at -20°C for 4h and 24h had the same positive effect as 10 minutes incubation at -80°C and no statistical difference was seen between those three conditions (Figure 2).

We thus demonstrated that the way of specimen storage can have a positive impact on the analytical sensitivity of the PCR. Without affecting the DNA quality, freezing improved the parasite detection, probably by releasing DNA from the highly resistant Entamoeba cysts that consist of a microfibrillar layer with crystalline sugars and lectins (Arroyo-Begovich et al., 1980). We advise laboratories to implement this very easy pre-analytic step for amoebiasis diagnosis but attention should be paid to all factors that influence PCR performance like transport and storage, extraction method, additives and specimen type.

We thank Kathy Demeulemeester, Kim Van Loon, Hilde Cox, Idzi Potters and Henk Vereecken for technical support.
References


Table 1: Improvement of DNA detection in terms of storage time (A) and storage temperature (B). The improvement is expressed as the difference in Ct-values (ΔCt) between fresh and frozen samples. No improvement was seen in the ΔCt<0 category. A decreased Ct-value between 2 and 4 indicates a 4-fold to 16-fold increased sensitivity while a ΔCt of 4 to 5 reflects a 16-fold to 32-fold increase in sensitivity. (A) All samples were stored at -20°C for short, medium or long-term storage conditions. (B) Short-term stored samples were frozen at -80°C or -20°C to determine the effect of storage temperature.

<table>
<thead>
<tr>
<th>A</th>
<th>△Ct category</th>
<th>0 to 1</th>
<th>1 to 2</th>
<th>2 to 3</th>
<th>3 to 4</th>
<th>4 to 5</th>
<th>5 to 6</th>
<th>6 to 7</th>
<th>&gt; 8</th>
<th>&lt; 0</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short (4h)</td>
<td></td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Medium (1d-1mth)</td>
<td></td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Long (≥1mth)</td>
<td></td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Storage temperature</th>
<th>△Ct category</th>
<th>0 to 1</th>
<th>1 to 2</th>
<th>2 to 3</th>
<th>3 to 4</th>
<th>4 to 5</th>
<th>5 to 6</th>
<th>6 to 7</th>
<th>&gt; 8</th>
<th>&lt; 0</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>-80°C (10 min)</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>-20°C (4h)</td>
<td></td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>-20°C (24h)</td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td></td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: A boxplot illustration of Ct-values of freshly-stored and frozen stool samples. The median Ct-value of the IC in both groups was similar (34.88 and 34.76 respectively). The Ct-values of freshly stored *Entamoeba dispar* or *histolytica* (Edh) specimens varied between 26.24 and 39.85 with a median of 29.63 and those of the corresponding frozen stool fractions between 22.22 and 39.05 with a median of 26.62.

Figure 2: Influence of the storage temperature on the Ct-value of stool samples. The average Ct-values of freshly stored fractions (4°C) and frozen fractions (-80°C for 10 min or -20°C for 4h or 24h). Statistical differences were indicated by ** (p<0.001) or * (p<0.01).