Combined nested PCR and High Resolution Melting for the screening of *Cryptosporidium parvum*

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Abstract— The aim of this study was focused on the capability of HRM (high resolution melting) analysis with the use of combined nested PCR for *Cryptosporidium* GP60 gene amplification in distinguishing positive, false-positive, negative and non-specific amplifications based on the melting curve structure and Tm difference. From 12 samples, successful amplification was seen on gel electrophoresis in 9 samples, one sample showed two bends and the other two samples were negative. Comparing result from electrophoresis with melt curve genotyping and Tm calling shown that all samples positive on gel electrophoresis had a same structure of the melting curve, with a temperature difference within 0.52 °C range (max = 86.74 °C; min = 86.22 °C, average 86.34°C). On the other hand, samples, where no amplification occurred, had different structures of melting curves with a different Tm (approx. ± 1 °C in comparison with an average of positive samples). Sample with double bands on gel electrophoresis showed two different melting domains with a temperature shift from average. We have concluded that the size of the GP60 final amplicon (450 base pairs), although larger than those commonly used for melting curve analysis, can be used as a screening tool for distinguishing samples positive for *Cryptosporidium parvum*.

Keywords*—Cryptosporidium*, diagnostic, genotypes, GP60, high resolution melting, screening

I. Introduction

Cryptosporidiosis is an infection caused by protozoan parasites from genus *Cryptosporidium*. *Cryptosporidium* spp. infect various vertebrate species, including humans [1-2]. Cryptosporidiosis can occur asymptomatically, or it can be manifested by profuse watery diarrhea, and can even cause death in immunocompromised individuals [2-7]. The infectious stages of *Cryptosporidium* spp., oocysts, are extremely resistant in the environment. Furthermore, oocysts are easily spread through water and are resistant to chlorination, which is a common method for drinking water treatment, aiding the transmission of the infection [8].

Various types of molecular diagnostic tools have been used for distinguishing *Cryptosporidium* genotypes/subtypes. Small sub-unit rRNA (SSU) gene-based tools are most frequently used in genotyping *Cryptosporidium* from animal, human and environmental samples. The use of SSU rRNA for genotyping is due to its multi-copy nature and also the presence of semi-conserved and hypervariable regions, facilitating the design of genus-specific primers [9]. Commonly used is the combined PCR-RFLP reaction using SspI and VspI restriction enzymes. Other markers, such as *Cryptosporidium* oocyst wall proteins (COWP), internal transcribed spacer region (ITS), thrombospondin-related adhesive protein (TRAP) or beta-tubulin are used, although less frequently [10]. Genotyping Cryptosporidium based on the GP60 gene coding the 60 kDa glycoprotein (GP60, also called GP40/15 is a popular method in subtyping *Cryprosporidium spp*.. GP60 subtyping is nowadays used for several species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. cuniculus*, *C. ubiquitum*, *C. erinacei* and other)[11]-[15]. High resolution melting analysis (HRM, HRMA) is a new method for rapid, high-output post-PCR analysis of genetic mutations or variations in nucleic acid sequences.

HRM can distinguish sequences by their nucleotide composition, length and the number of GC in the sequence [16]-[17]. HRM is, in comparison with standard PCR, extremely useful for its absence of post-amplification steps and the melting curves are obtained directly after amplification. Subsequently to amplification, the product is denaturized with increasing temperature, releasing the intercalating dye, resulting in the decrease of fluorescence. With the optimal setting of the reaction, HRM is sufficient enough to detect one nucleotide changes in otherwise identical nucleotide sequences [18]. For its simplicity, flexibility and high sensitivity, HRM analysis is becoming the method of choice for genetic variations screening. In real-time qPCR melting peaks are commonly known as low-resolution melting. The principle of HRM is equal to low resolution melting, except the temperature difference between every measured fluorescence. In HRM, the temperature gradient is reduced to intervals of 0.0008 to 0.2 °C. This enables more detailed melting profile analysis [19].

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The aim of this study was focused on the capability of HRM analysis with the use of combined nested PCR for GP60 gene amplification in distinguishing positive, false-positive, negative and non-specific amplifications based on the melting curve structure and Tm difference.

II. PROBLEM FORMULATION

Previous studies from our department shown GP60 primers used for genotyping have higher sensitivity and detection rate in amplifying the DNA of *Cryptosporidium* than primers targeting other regions (especially when host specific *Cryptosporidium* are expected in the sample, e.g. *C. parvum* and *C. hominis* in samples from humans) [20]. Also, these primers are more specific than primers amplifying the SSU sRNA region, which can produce non-specific amplifications or false-positive results, due to similarities in SSU sequences of *Cryptosporidium* and other pathogens (genus *Apicomplexa*, gregarines). Furthermore, one-step qPCR or HRM without the use of probes produced inconclusive results. Therefore, we have modified and combined classical nested PCR for the first reaction with high resolution melting for the second reaction using GP60 primers. We have chosen classic PCR mix for the first reaction due to a better capability of amplifying a product of a length of approx. 1,000 bp (base pairs) than commercial SYBR Green I mastermix. Our goal was to determine if HRM analysis is capable of successful amplification of the GP60 gene and to distinguish between positive, negative, false positive and non-specific amplifications based on melting curve genotyping analysis and Tm calling.

A. Genotyping C. parvum

Genotyping *C. parvum* based on the GP60 locus is a popular method in subtyping *Cryprosporidium* spp.. The GP60 gene sequence is similar to a microsatellite marker. It has tandem repeats of serine coding trinucleotides – TCA, TCT, and TCG (for *C. parvum* TCA and TCT) at the 5` end of the gene. In addition to variations in the number of repeats, differences in non-repetetive regions categorize *C. parvum* into several subtype families. GP60 subtype names start with the subtype family designation (i.e. IIa, IIb, IIc, IId etc. for *C. parvum*) followed by the number of repeats (TCA represented by letter A and TCT represented by letter T). For example, the name IIaA17G1 indicates that this subtype of *C. parvum* has 17 copies of TCA repeat and one copy of TCG. In *C. parvum* family IIa, a few subtypes have also a non-repetetive sequence ACATCA located downstream of the repeats [9]. Examples of *C. parvum* families are summarized in Table 1.

Species	Subtype	Dominant repeat	Other repeat
C. parvum	IIa	TCA, TCG	ACATCA
	IIb	TCA	
	IIc	TCA, TCG	

Table 1 examples of *C. parvum* families and their dominant repeats (Xiao, 2010, modified)

B. Samples

All 12 samples in this experiment were previously run on PCR with GP60 primers and subsequently compared with homologous sequences from GenBank. Genotypes were assessed using the rules of genotyping mentioned in point 2.1.1. Genotypes used in this experiment were *C. parvum* IIaA17G1R1 (n=5; accession no. EF576978.1), IIaA12G2R1 (n=1; EF576972.1), IIaA14G2R1 (n=1; EF576970.1), IIaA12G1R1 (n=1; JX575594.1), IIaA14G1R1 (n=1; KP997144.1), IIaA21G1R1 (n=2; JQ861957.1), IIaA16G1R1 (n=1; EF576969.1) [20]-[26]. Sample information is summarized in Table 2.

Sample	Source	Species and genotype
6t	calf	*C. parvum* IIaA17G1R1
8t	calf	*C. parvum* IIaA17G1R1
10t	calf	*C. parvum* IIaA17G1R1
BL1	human	*C. parvum* IIaA14G2R1
BL2	human	*C. parvum* IIaA12G1R1
BL7	human	*C. parvum* IIaA17G1R1
BL8	human	*C. parvum* IIaA14G1R1
IS1	human	*C. parvum* IIaA21G1R1
IS3	human	*C. parvum* IIaA21G1R1
IS4	human	*C. parvum* IIaA17G1R1
IS9	human	*C. parvum* IIaA16G1R1
3H	human	*C. parvum* IIaA12G2R1

Table 2 subtypes of *C. parvum* used in this experiment

C. DNA Isolation

Approximately 100 mg of feces was homogenized and the oocysts were disrupted at 6,500 rpm for 90 seconds with the addition of 0.5 mm glass beads, 1.0 mm zircon beads and 300 µL of lysis solution in Precellys 24 homogenizer (Bertin Technologies). DNA was extracted using the DNA Sorb-B nucleic acid extraction kit (AmpliSense, Russia). Isolated DNA was stored at -20 °C until the use in PCR.

D. Combined Nested PCR and High Resolution Melting

Classic PCR for GP60 amplification – first reaction

The total volume of the PCR reaction mixtures was 50 µL, with 5 µL of the DNA sample. Primers with a concentration of 0.2 µM and 5 U Taq DNA polymerase (FIREpol) were used. Outer primers GP60 F1/R1 were used (5`- ATG AGA TTG TCG CTC ATT ATC-3`/ 5`- TTA CAA CAC GAA TAA GGC TGC-3`), with a product length of 980-1,000 bp. PCR was run in thermocycler XP Thermal Cycler Blocks, with an incubation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 55 °C for 1 minute and elongation at 72 °C for 1 minute. A final

elongation step of 72 °C for 7 minutes was included to ensure the complete extension of the amplified products.

High resolution melting analysis – second reaction

5 µL of products from the first reaction were used as template DNA in 20 µL LightCycler FastStart DNA Master SYBR Green I mix (Roche) with inner primers GP60 F2/R2 (5`- GCC GTT CCA CTC AGA GGA AC-3`/5`- CCA CAT TAC AAA TGA AGT GCC GC-3`) with a product length of 450 bp [9]. A final concentration of 0.5 µM of primers and 3 mM of Mg^{2+} was used. PCR was run in LightCycler 480 II (Roche). Following cycling conditions were set: incubation at 95 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 20 seconds, annealing at 58 °C for 20 seconds and elongation at 72 °C for 1 minute with quantification. After amplification, a high resolution melting curve analysis was performed, from 40 °C to 95 °C with a slow ramp and continuous acquisition through the ramp (95 °C for 1 minute, 40 °C for 1 minute, 65 °C for 1 second and continuous acquisition at 95 °C)

E. Gel electrophoresis and melting curve analysis

Final products were evaluated by electrophoresis on 1.5 % agarose gel dyed with GoldView™ Nucleic acid gel stain (Solarbio) in TAE buffer. Samples were dyed with Red load™ PCR loading buffer (Top-Bio). Melt curve genotyping and Tm calling was calculated using LightCycler 480 Software (Roche).

F. DNA sequencing, sequence evaluation

Samples positive on gel electrophoresis were sent for DNA sequencing using Sanger`s method with 2nd forward primer. Obtained sequences were evaluated using ChromasPro DNA sequencing software (Technelysium Pty Ltd), compared with homologous sequences from GenBank using BLAST, compared with sequencing data from previous experiments using MultAlin (http://multalin.toulouse.inra.fr/multalin/) and aligned using BioEdit sequence alignment editor.

III. PROBLEM SOLUTION

From 12 samples, successful amplification was seen after gel electrophoresis in 9 samples (75 %), one sample shown two bends (8.3 %), and the other two samples were negative (16.6 %). Comparing result from electrophoresis with melt curve genotyping, and Tm calling in the analysis software shown that all samples positive on gel electrophoresis had a same structure of the melting curve, with a temperature difference within 0.52 °C range (max = 86.74 °C; min = 86.22 °C, average 86.34 °C). On the other hand, samples, where no amplification occurred, had different structures of melting curves with a different Tm (temperature difference approx. ± 1 °C in comparison with the average of positive samples). Sample with double bends on gel electrophoresis showed a larger temperature shift from average (-2.42 °C) and a different structure of the curve. Based on the differences in melting curves and Tm, it is possible to distinguish positive, negative and non-specific amplification using HRM analysis of the GP60 gene. Melting curve structures are represented by Fig.1, Fig. 2, and Fig. 3. Comparing sequencing results from positive samples from this study with homologous sequences from GenBank, and by comparing these sequences from sequences from the same samples run with GP60 primers obtained from our previous experiments in BioEdit, ChromasPro, and MultAlin, we have confirmed the presence of genotypes mentioned in section 2.1.2. Example of sequence comparison in ChromasPro is represented by Fig. 4. Tm calling, gel electrophoresis and sequencing results for all samples are summarized in Table 3.

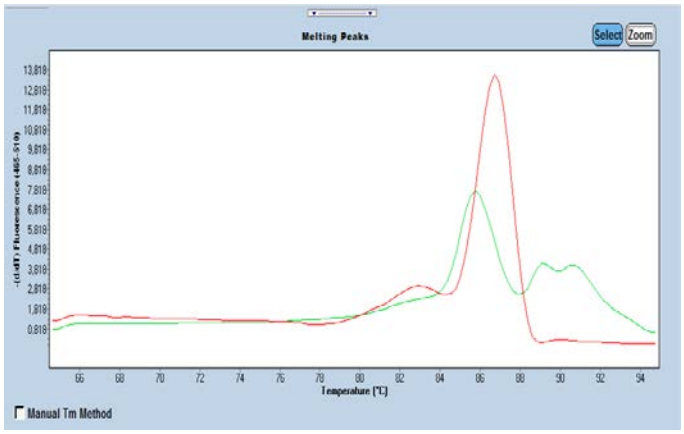

Fig. 1 comparison of melting peaks from samples positive and negative after gel electrophoresis (LightCycler 480 software) – red: positive sample (IS1, Tm = 86.74 °C); green: negative sample (BL1, Tm1 = 85.73 °C, Tm2 = 90.45 °C); negative sample shows a different melt curve structure with different melting temperature than sample positive on electrophoresis.

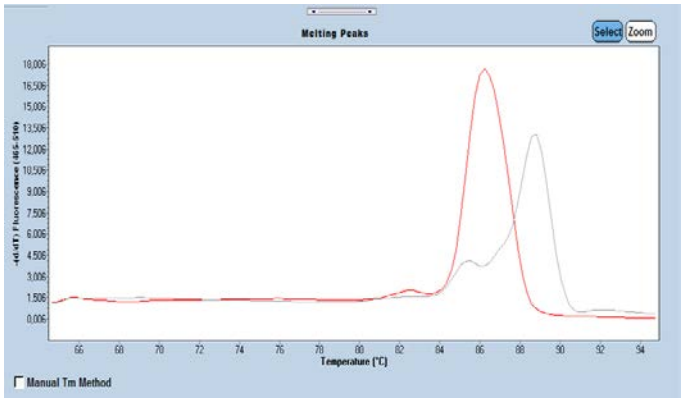

Fig. 2 comparison of melting peaks from sample positive on electrophoresis and sample with double bends (LightCycler 480 software) – red: positive sample (10t, Tm = 86.40 °C); grey: sample with double bends (IS4, Tm = 88.76); sample with two bends on electrophoresis shows a different structure of the melt curve and a temperature shift.

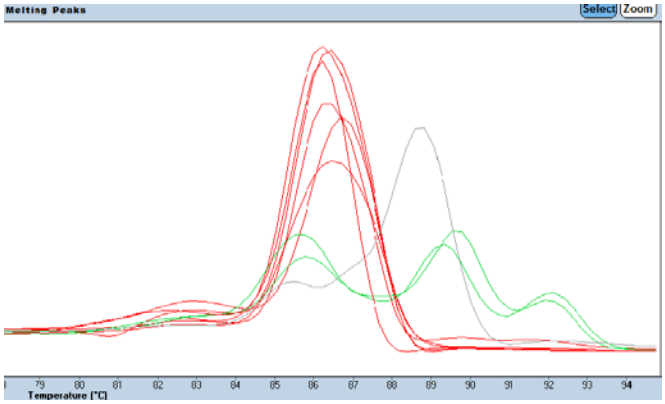

Fig. 3 melting curve structures for all samples: red - samples positive on gel electrophoresis (6t, 8t, 10t, 3H, BL2, BL7, BL8, IS1, IS9); green - samples negative on electrophoresis (BL1, IS3); grey-sample with double bends on electrophoresis (IS4); sample with double bends, and negative samples show different structures of melting peaks and a temperature shift from positive samples.

	Melting peaks (°C)		Gel	Sequencing
	Tm1	Tm2		
6t	86.46		+	IIaA17G1R1
8t	86.57		+	IIaA17G1R1
10t	86.40		+	IIaA17G1R1
3H	86.51			IIaA12G2R1
BL1	85.73	90.45		
BL2	86.51		+	IIaA12G1R1
BL7	86.57		+	IIaA17G1R1
BL8	86.26		+	IIaA14G1R1
IS1	86.74		+	IIaA21G1R1
IS3	85.65	89.68		
IS4	88.76		++	IIaA17G1R1
IS9	86.22		+	IIaA16G1R1

Table 3 Tm calling calculated using LightCycler 480 Software. Samples with two melting domains shown as negative on gel electrophoresis. Sequencing data confirmed genotypes mentioned in point 2.1.2.

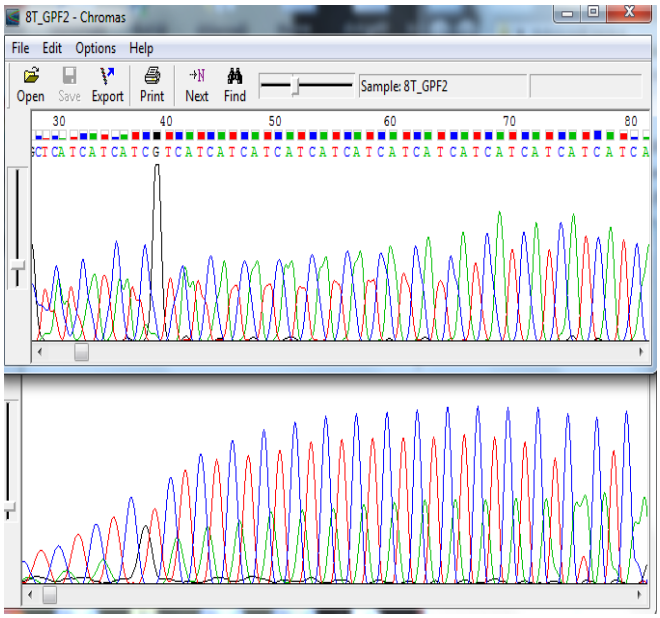

Fig. 4 comparison of positive samples 8t in ChromasPro program – upper: sequence from sample 8t in the experiment by Hatalová et al., 2017 [20]; lower: sequence from sample 8t in this experiment; as figure shows, the number and sequence of TCA and TCG repeats is identical.

PCR-based methods are the most sensitive techniques for detecting *Cryptosporidium* spp, although the ability to amplify the extracted DNA can be reduced by various inhibitors [10]. Primers amplifying the SSU region of rRNA show lower sensitivity, and in some cases, are capable of amplifying a different species, such as gregarines (unpublished results). Feng et al., (2007) confirmed in their study differences in the detection with SSU rRNA primers because of the different intensity of oocyst shedding [26]. The primers targeting the GP60 gene are used for genotyping various Cryptosporidium species [11-15], therefore, the primer targeting the GP60 region has to have a higher sensitivity and specificity than primers targeting the SSU rRNA region.

Different PCR protocols for detecting *Cryptosporidium* spp. are not capable of detection in all isolates, particularly the ones with a low number of oocysts. Also, the optimal PCR product length recommended for SYBR Green I reaction is 75-200 bp [27]. This can explain lower success rate of amplification in positive samples run on classic PCR in previous experiments. Long amplicons may produce more than one melting peak, because the amplicon can dissociate more than once, due to secondary structures with different melt temperatures (which occurred in sample IS4) [27].

To our knowledge, this is the first study using high resolution melting analysis in a combined nested PCR for the amplification of the GP60 gene of *Cryptoporidium*. Although with a relatively low number of samples, melting peak analysis, Tm calling, electrophoresis, and DNA sequencing provided relevant data for this topic to be a focus of further research and optimizing of the reaction, so that this combination can subsequently be applied for routine diagnostics of cryptosporidiosis in various samples.

IV. CONCLUSION

There are few studies about the use of qPCR and HRM analysis for *Cryptosporidium*. qPCR for the diagnostic of *Cryptosporidium* spp. usually requires probes to ensure higher specificity and species determination. In most cases, the target sequence is the SSU rRNA or ITS [28-29]. In the study by Hong et al. (2014) the target gene was SWI2/SNF ATPase [30].

In our study, we have used the GP60 gene as a marker, not only because it provides genotype data after DNA sequencing, we have also confirmed that GP60 primers have highest succession rate in amplifying target DNA. The length of the amplicon after 2nd reaction (~450bp) used in this study is larger than other commonly used for melting curve analysis. This fact reduces its mutation detection capability, not enabling it to distinguish between genotypes. Nevertheless, this study confirmed that even when using a larger amplicon in HRM analysis can prove useful in achieving specific amplification and differentiation among samples. Although this approach was used for the diagnosis of *C. parvum*, this specific primer can also be used for the diagnostics of *C. homins*. Furthermore, the use of GP60 primers provides immediate result about *Cryptosporidium* species and also genotypes, without the need of a prior reaction for species identification Also, GP60 genotyping is a popular method, and it serves for phylogenetic analyses in several *Cryptosporidium* species and can be applied for HRM analysis for these species as well. Samples positive on gel electrophoresis had an identical structure of the melting curve and Tm difference range was 0.52 °C. Non-specific amplifications had a different melting curve structure, with a wider temperature shift.

Optimizing the reaction is still needed, but we will focus our research to further improve this assay, with the goal of constructing a standard curve, when afterward PCR products can be sent directly for genotyping (for which the GP60 region is used), simply by comparing melting curves and Tm shifts, without the use of electrophoresis. The biggest advantage of this particular primer is, that after sequencing, we are capable of distinguishing *C. parvum*, *C. hominis*, but also their genotypes and subtypes, which can be used further for phylogenetic analyses.

ETHICAL APPROVAL

Approval for the study was obtained from the Ethics Committee of the Faculty of Medicine at P. J. Šafárik University in Košice (No. 104/2011).Study was performed in accordance with the ethical standards as laid down in the Declaration of Helsinki of 1975 and revised in 2008. Participation in the study was voluntary and anonymous and informed consent was obtained prior to the medical examination.

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