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CHEMICAL COMPONENTS OF POLYMERASE CHAIN REACTION IN 18S rRNA FOR DETECTION OF *Cryptosporidium* FROM RIVER WATER SAMPLES

(Komponen Kimia Tindak Balas Berantai Polimerase di dalam 18S rRNA untuk Pengesanan *Cryptosporidium* dari Sampel Air Sungai)

Mohd Aiman Barudin[1], Mohammad Lokman Md Isa[2,3], Afzan Mat Yusof[2,3]*

[1]*Department of Biomedical Science, Kulliyyah of Allied Health Sciences*
[2]*Department of Basic Medical Sciences, Kulliyyah of Nursing*
[3]*Integrated Cellular and Molecular Biology Cluster (iMolec)*
International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.

**Corresponding author: afzan@iium.edu.my*

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Abstract

The gene of 18S ribosomal RNA or 18S rRNA is the universal gene function as a general genetic marker for species identification of microorganisms including parasites. *Cryptosporidium* has distinct 18S rRNA genes along different species within the same genus. In this study, polymerase chain reaction or PCR was used to study chemical components of PCR setup in amplification of 18S rRNA gene of this parasite. *Cryptosporidium* was collected from river water samples and its presence was confirmed using specific immunofluorescence detection of this parasite. Isolated water containing *Cryptosporidium* was then subjected to genomic DNA extraction before PCR step. The chemical components of PCR consisting of $MgCI_2$, deoxynucleotide triphosphate (DNTPs), Polymerases, free DNase-water, universal primers and PCR buffer were studied in different volume and concentration. Each chemical component of PCR was optimized differently in yielding the same final volume of 20 µL per each reaction. The value range of chemical components of PCR consisted of $MgCI_2$ (0.1 µM-0.5 µM), dNTPs (50-250 mM), free DNase water (5-10 µL), polymerases (0.2-0.5 U) and universal primers (2-20 µM). The result indicated that 0.2 µM of $MgCI_2$, 100 mM of dNTPs, less than 10 µL of free DNase water, 0.5 U of polymerases and 10 mM of universal primers were the best combination to get better result for molecular identification of 18S rRNA *Cryptosporidium*. As a conclusion, accurate and proper concentration or volume of each PCR chemical components is essential for molecular identification of 18S rRNA *Cryptosporidium* gene. In future studies, study on gradient temperature parameters of PCR run can be included to study the chemical nature of amplified genes either in denaturation, annealing or extension steps.

Keywords: chemical, *Cryptosporidium*, polymerase chain reaction, 18S ribosomal RNA gene

Abstrak

Gen 18S ribosomal RNA adalah gen universal yang berfungsi sebagai penanda genetik umum untuk pengenalpastian spesies mikroorganisma termasuk parasit. *Cryptosporidium* mempunyai gen 18S rRNA yang berlainan daripada spesies berbeza dalam genus yang sama. Dalam kajian ini, tindak balas berantai polymerase atau PCR digunakan untuk mengkaji komponen kimia susun atur PCR dalam amplifikasi gen 18S rRNA bagi parasit ini. *Cryptosporidium* telah diambil dari sampel air sungai dan disahkan kehadirannya menggunakan pengesanan immunopendaflour terhadap parasit ini. Sampel air yang diambil mengandungi *Cryptosporidium* yang kemudiannya diteruskan untuk pengekstrakan genomik DNA sebelum peringkat PCR. Komponen kimia PCR yang terdiri daripada $MgCI_2$, deoksinukleotida trifosfat (dNTPs), polimerase, air yang bebas DNase, primer umum dan larutan penimbal PCR telah dikaji dalam isipadu dan kepekatan yang berbeza. Setiap komponen kimia PCR dioptimakan secara

berbeza dalam menghasilkan isipadu akhir 20 uL bagi setiap tindak balas. Nilai julat komponen kimia PCR terdiri daripada $MgCI_2$ (0.1 µM-0.5 µM), dNTPs (50-250 mM), air bebas DNase (5-10 µL), polimerase (0.2-0.5 U) dan primer primer umum (2-20 µM). Keputusan menunjukkan bahawa 0.2 µM $MgCI_2$, 100 mM dNTPs, air bebas DNase yang kurang daripada 10 µL, 0.5 U polimerase dan 10 mM primer umum adalah kombinasi terbaik untuk mendapatkan dapatan yang lebih baik bagi pengenalpastian molekul *Cryptosporidium* 18S rRNA. Kesimpulannya, kepekatan atau isipadu yang tepat dan sesuai bagi setiap komponen kimia PCR adalah penting untuk pengenalpastian molekul gen *Cryptosporidium* 18S rRNA. Bagi kajian masa depan, kajian ke atas kecerunan parameter suhu untuk menjalani PCR boleh dimasukkan untuk mengkaji sifat semulajadi kimia pada gen yang diamplifikasi untuk peringkat-peringkat penyahaslian, pelekatan atau penyambungan.

Kata kunci: kimia, *Cryptosporidium*, tindak balas rantai polimerase, gen 18S ribosomal RNA

Introduction

Cryptosporidiosis is prevalent in developing countries, perhaps because the high contamination from environmental and hygiene conditions [1]. In contrast, the cryptosporidiosis transmission in developed countries is possibly not similar as compared to developing countries. *Cryptosporidium* oocysts are frequently found in contaminating surface waters because of anthroponotic activity, agricultural practices, sewage effluent, native animal or livestock fecal contamination as well as extremely resistant to chlorine and monochloramine for water disinfection [2]. In addition to being resistant to commonly used disinfectant chemicals, it is commonly considered that oocysts can persist for several months or more in the aquatic environment [3]. Other than that, the relatively low infectious dose of *Cryptosporidium* at least 10 oocysts can pose a challenge to water utilities for provision of purified drinking water [4]. To date, this parasitic occurrence has been reported in different geographical location of river [5]. In fact, 11.5% of Malaysian river water samples were reported to get contaminated with *Cryptosporidium* oocysts [6]. Recently, two cases have been reported on *Cryptosporidium* occurrence in Kuantan, Pahang [7,8].

Meanwhile, amplification of 18S rRNA gene loci is been the most widely accepted method to provide information on species identification [9]. An optimized method of PCR for *Cryptosporidium* oocysts is essential in detection of single loci with specific primers. DNA extraction for this parasite turns to be effective once disruption of the robust oocyst wall takes place for releasing sporozoite nuclei. Meanwhile detachment of DNA and histone protein is important for primer to have accessibility to anneal and thus, facilitating the amplification of specific DNA sequence using PCR [10]. It is a challenge for diagnosing *Cryptosporidium* to the species/genotype level due to the lower occurrence and the rich presence of PCR inhibitors from environmental water samples. It is essential for the public and environmental health assessment for identifying the contamination sources of *Cryptosporidium* oocysts [11]. In addition, optimization of chemical constituents of PCR reagents is also important to get correct amplicon of PCR upon the completion of PCR with the specific primer on annealing specific gene sequences [12]. All chemical reagents like dNTPs, $MgCI_2$, primers, polymerases and water have their own limitation either low or high proportion could lead to successfulness of PCR [13]. Manipulation of chemical nature of those chemical reagents can be useful to set up a better reaction mix of PCR prior to run the reaction [14]. Thus, in this study, chemical reagent constituents of PCR are optimized accordingly to result in successfulness of PCR on amplification of *Cryptosporidium* 18S rRNA gene.

Materials and Methods

Collection of river water samples

This study was carried out in Kuantan and Balok, Pahang. The upstream, midstream and downstream area in both Kuantan River and Balok River were selected as the sampling sites. Kuantan River runs from Lembing River through Kuantan City where this city is located near the mouth of Kuantan River before this river flows out to the South China Sea. Meanwhile, Balok River is located close to industrial area and residential area in Gebeng area and Balok city, respectively.

Water processing

United States Environmental Protection Agency 1623.1 method was used to detect *Cryptosporidium* from river water samples [15]. River water samples were processed to be concentrated using continuous flow centrifuge (CFC Express System) (Scientific Methods Inc, Granger, Indiana, USA) prior to immunomagnetic separation using Dynabeads® GC-Combo (Dynal, Cat. no. 730.02, Oslo, Norway).

Fluorescein isothiocyanate staining
Purified oocysts of river water samples were stained using a fluorescein isothiocyanate labelled monoclonal antibody kit specific to *Cryptosporidium* oocysts (Cellabs Pty Ltd., cat. no. KR2111A6, Brookvale, Australia) and then were examined using fluorescence microscope under 400x magnification.

DNA extraction from water samples
DNA of *Cryptosporidium* oocysts from river water sample was extracted by using QIAamp DNA Mini Kit (QIAGEN, Germany) with minor modifications.

Optimization of chemical components in PCR reaction mixture
Each chemical component of PCR was optimized differently in yielding the same final volume of 20 µL per each reaction. The value range of chemical components of PCR consists of $MgCI_2$ (0.1 µM-0.5 µM), dNTPs (50-250 mM), free DNase water (5-10 µL), polymerases (0.2-0.5 U) and universal primers (2-20 µM). The results of several PCR reactions in different volume of PCR chemical components were run based on nested PCR.

Identification of 18S rRNA sequence
Nested PCR of 18S rRNA was carried out for *Cryptosporidium* genotyping [16]. Purified commercially genomic DNA of *C. parvum* was set to be a positive control. Distilled water was used as a negative control for nested PCR. Agarose solution (1.2%) was prepared with 1 µL GelRed. The gel was electrophoresed at 100 V for 40 min and then visualized using GelDoc Ez System (Bio-Rad).

Results and Discussion

Out of six river water samples, five were detected with *Cryptosporidium*. The findings were confirmed by using 18S rRNA-based PCR method. The result of secondary PCR products of 18S rRNA gene showed that only one sample from upstream point of Kuantan River did not appear a single band on the 1.2% agarose gel. In this study, the positive results of genotyping approach showed that only five water samples out of six samples from two rivers contained DNA of *Cryptosporidium* oocysts. A previous study had revealed distribution of *Cryptosporidium* species in river water samples of Kuantan River and Balok River, especially at downstream point [7, 8]. Each chemical component of PCR was optimized differently in yielding the same final volume of 20 µL per each reaction. The value range of chemical components of PCR consisted of $MgCI_2$ (0.1-0.5 µM), dNTPs (50-250 mM), free DNase water (5-10 µL), polymerases (0.2-0.5 U) and universal primers (0.1-0.5 µM). The result indicated that 2 mM of $MgCI_2$, 100 mM of DNTPs, less than 10 µL of free DNase water, 0.5 U of polymerases and 10 mM of universal primers were the best combination to get acceptable results for molecular identification of 18S rRNA *Cryptosporidium*. To understand the function of chemical reagents used in this study, it is critical to optimize and troubleshoot the optimal PCR conditions at a time in obtaining the desired PCR product of *Cryptosporidium* 18S rRNA gene (Table 1). However, unbalanced composition of PCR chemical reagents may cause non-specific amplicons and reduce PCR specificity [17].

Table 1. Different volumes of PCR chemical components in nested PCR

$MgCI_2$ (µM)	dNTPs (mM)	Free-DNaseWater (µL)	DNA Polymerases (U)	Universal Primers (µM)
0.1	50	5	0.2	2
0.2	100	6	0.3	8
0.3	150	7	0.4	12
0.4	200	8	0.45	16
0.5	250	9	0.5	20

In this study, Taq DNA polymerase was used as it is suitable for obtaining desired amplicon products and more efficient as a hot start PCR [18]. Normally, the molar concentration of polymerase is comparatively low as compared to other chemical reaction components as recommended volume supplied is 1-2.5 Units per 100 µL reaction volume. Reduced concentration of polymerases ensure specificity while higher counterpart causing nonspecific amplicons [19]. DNA polymerases are favored in PCR process with a cofactor like magnesium ion [20]. They are easily manipulated resulting in the stringency of PCR as its ionic concentration is critical. In fact, it is also determined by EDTA in PCR buffer and deoxynucleotide 5'-triphosphates (dNTPs). In principle of PCR chemistry, dNTP-Mg^{2+} soluble complexes tend to interact with the DNA template backbone thereby activating DNA polymerase function (Figure 1). Soluble complexes are recognized as a substrate for DNA polymerase to act on [21]. However, increased magnesium ion can reduce polymerase specificity, stabilize the duplex strand of DNA template or fidelity of Taq DNA polymerase, stabilize the duplex strand of DNA template and spurious incorrect primer annealing thereby resulting in undesired or non-specific amplicons [22]. In contrast, insufficient concentration of Mg^{2+} can cause no amplicons or yields are likely to be poor [23]. $MgCI_2$ was optimized in this study because the reaction cannot proceed without the sufficient concentration of Mg^{2+} ion. The optimized concentration is additional option from the recommended protocol because the nature of DNA template can be affected by PCR inhibitors from environmental factors [24]. In such case, it is crucial to increase the concentration of magnesium ion for PCR stringency and specificity.

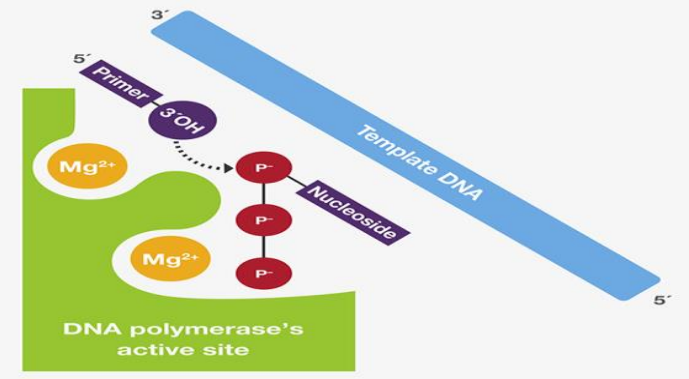

Figure 1. Illustration of polymerase chain reaction on DNA molecules along with other PCR chemical components involved in the PCR reaction

On the other hand, four dNTPs should be at the appropriate equivalent concentrations of 50 µM even though the reaction commonly can compromise between 20 and 200 µM [25]. In our study, dNTPs were optimized accordingly from the lowest to highest concentration to find an optimal concentration that is suitable for PCR reaction. Reduced dNTPs concentrations can increase PCR specificity while higher one can work oppositely [26]. The desired length of PCR products is not long, below 500 bp and thus, higher dNTP concentration is not required. In our practice, optimization of each dNTP is essential for working dNTPs solution to reach the optimal balance in final reaction coupled with other PCR reagents. It is important that four dNTP's concentrations should be used equivalently for minimizing misincorporation errors [27]. One of the main components to be discussed is universal primers. Its concentration should be optimal at 0.1-0.5 µM. In our study, primer was optimized for both concentrations and final volume in 20 µL PCR reaction mix. Higher concentration of universal primers can result in template mis priming at non-specific sites and thus, leading to accumulated non-specific products formation [28]. However, the primers should be prepared at slightly higher concentration than optimal to avoid of its exhaustion that possibly compromise the desired amplicon yield upon PCR completion [29]. Finally, free-DNase water is generally the easiest component for optimization since it is essentially important to be included in PCR reaction mix for avoiding any residual enzymatic activity such as DNase that can degrade DNA template during PCR reaction until its completion [30]. However, the volume of water should not higher than half of PCR mix volume. In our study, the volume of PCR mix was set to be at 20 µl per reaction only. Thus, it is only tolerated for manipulation below than 10 µL to avoid diluting other essential chemical components of PCR such as DNA polymerase, dNTPs, MgCI2 and universal

primers as primers were already diluted from its working concentration of primer stock. Equal concentration of dNTPs with the excessive volume of water in PCR reaction mix indirectly can affect the formation of desired amplicon upon PCR completion [31].

Conclusion

Accurate and proper concentration or volume to each PCR chemical components is essential for molecular identification of 18S rRNA *Cryptosporidium* gene. These optimized PCR chemical components can be applied to other water samples infected with *Cryptosporidium* for species identification. In future studies, study on gradient of temperature parameters of PCR run can be included to study the chemical nature of amplified genes either in denaturation, annealing or extension steps.

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