Detection of *Cryptosporidium sp* infection by PCR and modified acid fast staining from potassium dichromate preserved stool

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Abstrak

Tujuan *Untuk mengetahui frekuensi infeksi Cryptosporidium sp pada anak bawah tiga tahun (batita) dengan deteksi gen 18S rRNA dari tinja yang sudah dipreservasi lama dan membandingkannya dengan modifikasi metode tahan asam (MTA) dari tinja hasil konsentrasi.*

Metode *Sejumlah 188 feses anak batita yang telah tersimpan selama 13 bulan di 4°C, dikonsentrasikan dengan teknik air eter, selanjutnya dibuat sediaan, dipulas dengan pewarnaan MTA; sisa konsentrat diekstraksi DNA dengan teknik kejut panas dingin dan penambahan proteinase K, lalu dilakukan PCR langsung terhadap gen 18S rRNA.*

Hasil *Proporsi sampel positif Cryptosporidium adalah 34.6% dengan PCR gen 18s rRNA dan 4.8% dengan pulasan MTA dari tinja konsentrasi. Secara statistik perbedaan kedua hasil tersebut bermakna.*

Kesimpulan *Frekuensi infeksi Cryptosporidium sp di batita tinggi sekali dan penyimpanan tinja dalam larutan kalium dikromat selama 13 bulan, tampaknya tidak mempengaruhi hasil PCR. Tingginya frekuensi infeksi Cryptosporidium di populasi itu menunjukkan tingginya transmisi di daerah tersebut sehingga berpotensi menular ke kelompok yang rentan misalnya imunokompromais.* ***(Med J Indones 2009; 18: 149-54)***

Abstract

Aim To identify the frequency of *Cryptosporidium* infection in children below 3 years old by examining concentrated long term preserved stool using PCR detection of 18S rRNA gene and compared with modified acid fast staining technique.

Methods Hundred eighty eight stools from children ≤ 3 years old were stored for 13 months in 2.5% K2Cr2O7 solution at 4^0C. *Cryptosporidium* oocysts were isolated by water-ether concentration technique. The concentrates were smeared onto object glass and stained with modified acid fast staining, and the rest of the concentrates were DNA extracted by freezing and thawing cycles and proteinase K digestion, then direct PCR was done to detect 18S rRNA gene.

Result The proportion of positive stools for *Cryptosporidium sp* by acid fast staining from concentrated stools and 18S rRNA PCR were 4.8% and 34.6% respectively, which showed statistically significant difference.

Conclusion The frequency of *Cryptosporidium* infection among children ≤ 3 years old was very high and stool storage in K2Cr2O7 for 13 months did not affect the PCR result. High prevalence of *Cryptosporidium* infection indicated high transmission in that area and the potential to be transmitted to other individuals such as the immunocompromised. ***(Med J Indones 2009; 18: 149-54)***

Key words: *18S rRNA, cryptosporidiosis*

DNA can be isolated from any biological specimens; the most often widely used specimen is blood and hair because they are easily available. The DNA will be used to identify an organism by PCR, which is a method firstly introduced by Mullis in 1985. This method was developed further by the Department of Human Genetics, Cetus Corporation, California for the amplification of β-globin human gene to diagnose prenatal genetic disorder such as sickle cell anemia.[1]

The rRNA is present in ribosomes of all organisms, the pro and eucaryote, which consist of small and large subunits. The 18S rRNA is present in the small ribosome subunit in the cytosol of eucaryotes.[2] In medicine, rRNA is the target of antibiotics, while in evolution, rRNA can be used in the taxonomy of an organism, to calculate the distance of relationship between one organism to another, and to calculate species divergence.[3] Identification of 18S rRNA gene was used to study a number of eukaryotes such as plants, animals and protozoa as well as *Cryptosporidium*.[4]

Cryptosporidium sp. is an intestinal coccidian protozoa, which infects animal as well as human and causes

diarrhoea in immunocompetent and immunocompromised; with severe clinical manifestation in immunocompromised. *Cryptosporidium* is transmitted through fecal contamination of food or drinking water. Animals that are most frequently infected by *Cryptosporidium sp* are mammalian, poultry, reptile and fish. Distribution of *Cryptosporidium* varies considerably, depending on the geography and host's type. *Cryptosporidium* infection is cosmopolitant. Low economy status, poor sanitation and water treatment will result in high prevalence of infection and diarrhoea epidemic. Infection mostly happen in children less than two years old and immunocompromised individuals.[4]

Cryptosporidium prevalence varies in different groups/ population. Kurniawan et al[5] reported that 11.9% of HIV patients (n= 318) with chronic diarrhoea in Jakarta were positive for *Cryptosporidium sp* oocysts, while in Medan General hospital only 2.9% of children with diarrhoea (n= 172) were postive.[6] In West Africa the prevalence was 7.7% in children of less than 3 years old,[7] whereas in Iran the prevalence was 25.6 and 3.7% in adults with and without diarrhoea respectively.[8] All the data were obtained by performing modified acid fast staining (AFS), the most common method used in nearly every laboratory in developing countries.[9]

The use of AFS method is relatively time consuming and needs skilled technician due to the very small oocyst (size: 4-6 µm),[4] which is sometimes difficult to differentiate from the fungal spores of the same size and stained red too. In order to solve the problem, better techniques with higher sensitivity and specificity is necessary such as the PCR.

DNA isolation from faecal specimen is not as simple as those from blood; this is due to the presence of inhibitors in stool that can interfere with the PCR reaction. Apart from that, target gene, type of preservative solution and duration of storage determine the success of the test.

During a field study, a lot of specimens were collected, which were not possible to be examined immediately. Therefore, they were stored in a preservative solution such as formaldehyde or potassium dichromate until the test was performed. Johnson et al[10] recommended 2.5% potassium dichromate as the best preservative without affecting PCR result. Oocyst viability can stay up to 18 months without degradation of its quality.

There are several target genes for the diagnosis of *Cryptosporidium sp*. such as *Cryptosporidium* oocyst wall protein (COWP),[11] 18S rRNA and some other genomes; the 18s rRNA gene is the most specific gene with the highest sensitivity and able to detect up to one oocyst.[12]

Cryptosporidiosis thus represents a global public health problem that affects mainly children and the immunocompromised, and reliable detection methods are needed in order to identify the real prevalence, source of infection and transmission. Soetomenggolo et al[13] found a 2.1% prevalence of *Cryptosporidium* among children below 3 years old that lived in the flooded area at Ciliwung riverside in East Jakarta, based on the modified AFS method from unconcentrated stools. This prevalence is much lower than reports from other developing countries with similar situation, culture and geography. This study aimed to reveal the frequency of *Cryptosporidium* infection among children below 3 years old, who lived at Ciliwung riverside in East Jakarta by PCR detection of 18S rRNA gene from long term preserved stool, and to compare the PCR method with modified AFS method on concentrated stools.

METHODS

This was a descriptive, cross sectional study. The number of samples (188) was determined by statictical calculation using two proportional sampling test.[14] The study was carried out at the Department of Parasitology, Faculty of Medicine, University of Indonesia.

Samples

The 188 stools were randomly selected from 486 stools from previous study which were collected from children ≤ 3 years old who lived at the Ciliwung riverside, and preserved in 2.5% potassium dichromate,[13] and stored at 4°C for 13 months. We used the modified AFS method and PCR to detect cryptosporidiosis on concentrated stools. The stages that was performed to detect 18S rRNA gene by PCR were stool concentration to isolate the oocysts, DNA extraction and PCR amplification.

Stool concentration

Water ether stool concentration was performed following the Smith method.[12] About 200 µl of stool was mixed with 700 µl of water in a 1.5 ml centrifuge tube, and vortexed for 30 seconds. Diethyl ether was added as much as 400 µl, shook and spinned for 60 seconds at 13,000g. The supernatant was discarded and 200 µl sediment was left. The sediment was washed 3 times with water and last sediment left was 50 µl. Further 100 µl of lysis buffer was added, vortexed for 10 seconds, and the stool concentrate was processed for staining, or stored at 4°C for the next procedure. By this method, the oocysts in the stool were concentrated.

Modified AFS

Ten µl of the stool concentrate was smeared onto object glass, dried at room temperature and fixed with

methanol for 3 minutes, then stained with 3% carbol fuchsin for 15 minutes. After washing with water, the slide was dipped into acid methanol for 10 seconds, then washed and counter stained with 0.4% malachite green for 30 seconds, dried and read under light microscope at 400–1,000 x magnification, to look for cryptosporidium oocyst.

DNA extraction from stool concentrate[15]

The rest of the stool concentrate was processed further for *cryptosporidium* oocyst DNA extraction by performing 15 cycles of freezing and thawing at one minute each in liquid nitrogen and 65^{0}C waterbath. Particulate matter was removed by vortexing and centrifugation every five cycles. After this process, 2 µl of 10% proteinase K solution was added and incubated for 3 hours at 55^{0}C then transferred to 90^{0}C for 20 minutes and cooled on ice for one minute. The samples were then centrifuged for 5 minutes at 13,000 x g. Oocyst DNA containing supernatants were recovered and measured for DNA concentration and purity following Sambrook et al technique,[1] then stored at -20°C until it was used for PCR amplification.[15]

PCR amplification and gel analysis of PCR products [16]

Amplification of *Cryptosporidium* DNA was directed against 18S rRNA gene. Optimation of DNA template volume and the PCR reaction were done using positive control, *Cryptosporidium* DNA supplied by SPDL, UK and *Cryptosporidium* DNA isolated from HIV individuals positive for cryptosporidiosis. One µl of the oocyst DNA was used as amplification template in 25 µl reaction mixture containing 10 x Taq buffer, 2mM dNTP, 4mg/ml BSA, 20% Tween-20, 25mM $MgCl_2$, Taq polymerase (Qiagen), and 5'AAGCTCGTAGTTGGATTTCTG 3' and 5' TAAGGTGCTGAAGGAGTAAGG 3' primers.

Reaction mixtures were subjected to 39 cycles that consisted of 30 seconds denaturation at 94^{0}C, 30 seconds annealing at 55°C, and then 4 seconds elongation at 72°C. Each amplification run included a negative control (PCR water) and a positive control. The PCR products were analyzed on horizontal 1.2% agarose gels in TBE 0.5 x buffer pH 8.0; positive result showed the 435 bp band.

Statistical analysis

Statistical analysis was performed using SPSS version 12.0 software (SPSS Inc., Chicago, IL, USA). Difference between the result obtained by PCR and modified AFS from concentrated stool was tested using Mc Nemar bivariate analysis. Statistical analysis differs significantly if the *P* is <0.05.

RESULTS

Stool concentration and modified AFS

Modified AFS on 188 stool concentrates showed that nine samples (4.8%) were positive for *Cryptosporidium* oocysts (table 1). The oocyst were round, 4-5 µm in size and stained red with green background (Figure 1).

Table 1 : Proportion of *Cryptosporidium* positive samples by PCR and modified AFS from concentrated stools.

Methods	Total	
(n= 188)	positive	negative
PCR	65 (34.6%)	123(65.4)
Modified AFS concentrated stool	9 (4.8%)	179 (95.2%)

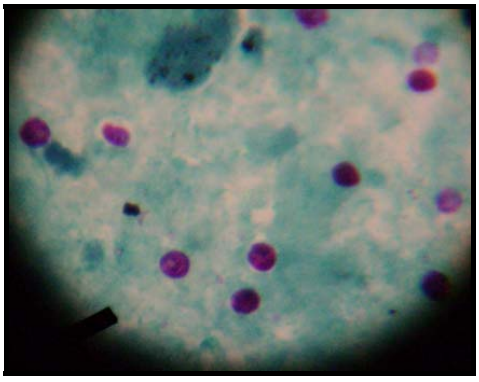

Figure 1. Cryptosporidium oocysts in stool concentrate, stained with modified acid fast staining (10 x 40)

DNA extraction from stool concentrate

The rest of concentrate was extracted for its oocyst DNA. Determination of DNA concentration by spectrophotometer showed high concentration of DNA isolated from the 188 stools. The average amount of isolated DNA was 83.288 µg with average purity index of 1.09.

PCR Amplification of 18S rRNA Gene

Optimation essay to determine the volume of template, which gave the best result showed that 1/10 dilution was the optimum volume of DNA template (Figure 2). From the 188 DNA samples that were analyzed by direct PCR against 18S rRNA gene, 65 samples were positive (34.6%), while the same samples that were examined by AFS showed only nine (4.8%) positive for

Cryptosporidium oocysts. Statistical analyses showed significant difference between the result obtained by PCR and microscopic examination by AFS from concentrated stools (Mc Nemar, *P*= 0.000).

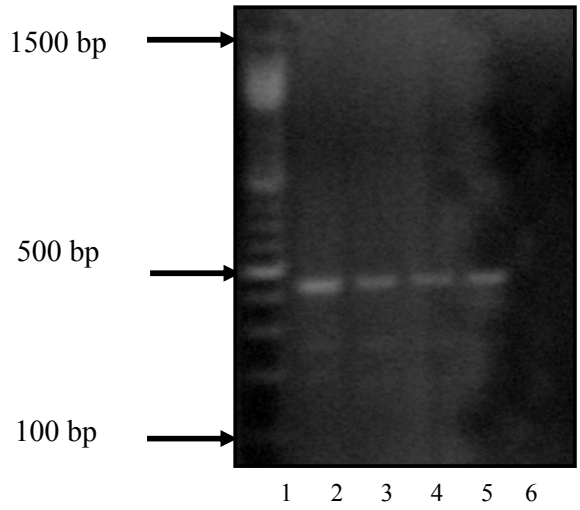

Figure 2. Results of PCR analysis on positive and negative control

Lane 1 = 100 bp DNA marker (Promega)
Lane 2 = 1st positive control (*Cryptosporidium* DNA, SPDL, UK)
Lane 3 = 2nd positive control (1:9 dilution) (*Cryptosporidium* DNA, FKUI)
Lane 4 = 2nd positive control (1:5 dilution) (*Cryptosporidium* DNA, FKUI)
Lane 5 = 2nd positive control (1:1 dilution) (*Cryptosporidium* DNA, FKUI)
Lane 6 = negative control

DISCUSSIONS

Cryptosporidium sp. oocysts can be detected in stool, sputum and biopsy tissue. It is commonly found in stool because the habitat is in the gastrointestinal tract; thus intestinal cryptosporidiosis is a common manifestation.

Johnson et al,[10] reported that the duration of storage and type of preservative solution had an influence on the PCR result. *Cryptosporidium* DNA was still detected when the stool was stored in 2.5% potassium dichromate solution up to 6 months, in contrast to preservation in 10% formaldehyde. Formaldehyde will harden the oocysts, and make it difficult to be broken and to isolate the sporozoites DNA. The dichromate ion is stable in acid environment (pH= 7) and can conserve the oocyst and its DNA.[17] Chan-Gu et al,[17] in his study reported that *C. baileyi* oocysts were still alive after 18 month storage in 2.5% potassium dichromate solution.

In this study, the samples had been stored for 13 months in 2.5% potassium dichromate solution. In positive samples, modified AFS of concentrated stool still showed a high number of oocysts upon clear background; a condition that made it easier to identify the oocysts and to differentiate them from fungal spores. The stools were concentrated in order to increase the sensitivity of the test, because concentration allowed more volume of stool to be examined.

The concentration technique in this study was the water-ether method, which is recommended as the best technique to recover the oocyst (46-75%) in comparison to the other two techniques, the sucrose density (24-65%) and zinc sulphate flotation technique (22-41%).[12]

Cryptosporidium sp. DNA can be detected after going through three stages: stool concentration to isolate the oocyst, DNA extraction, and DNA amplification and analysis. At oocyst isolation stage, extra washing with demineralized water during concentration technique was necessary in order to remove the preservative solution.[1,4]

During DNA extraction, firstly the oocysts were broken to release the sporozoites. This was done by performing heat and cold shock method, through heating at 65^0C and chilling in liquid nitrogen. The isolated DNA was then measured for its purity by comparing the absorbance of DNA and protein. It was found that the average purity index in this study was 1.09 that suggested low purity; however, the DNA was still able to be detected. Sambrook et al,[1] mentioned that it is not necessary to purify the DNA that is used as a template for PCR, because the primers were very specific.

The average concentration of isolated DNA in this study was 83.288 µg; this fact revealed that there was enough template for PCR, which basically needs 0.1–1 µg per reaction.[1]

Apart from the factors mentioned above, the primers, polymerase and the presence of any inhibitor may have an influence on the PCR result. Stool contains more inhibitors such as bilirubin, bile salt and polysaccharide compared to other specimen or environment. Those inhibitors may interfere with the polymerase; thus to neutralize the inhibitors, bovine serum albumin (BSA) 4mg/ml[18] was added. The reaction misture should be mixed well with the DNA template to avoid the formation of sodium dodecyl sulphate (SDS) crystal in the lysis buffer, which may interfere the action of polymerase. There was also addition of 20% Tween-20 solution to neutralize the SDS.[1,12] The method and reaction mixture to isolate the DNA from stool needs special precaution to minimize the effect of inhibitors that are present in stool. The PCR is a very sensitive and

specific detection technique, which resulted in much higher proportion of *Cryptosporidium* positive samples in comparison to the standard technique, which is used in the health laboratories, the acid fast staining.

This study showed that *Cryptosporidium* DNA could still be detected in stool that was preserved in 2.5% $K_2Cr_2O_7$ for 13 months, and also in negative samples (no oocyst found) by modified AFS. Our result suggests $K_2Cr_2O_7$ that is a good preservative for *Cryptosporidium*. The negative results by modified AFS could be due to either very few oocysts that were present in the stools, or there was no excreted oocyst at all. Negative result by modified AFS does not exclude any *Cryptosporidium* infection, because AFS cannot detect the thin wall oocysts, which are not excreted in the stool, but continue to infect other enterocytes in the host intestines (autoinfection).

Further, the actual prevalence of *Cryptosporidium* infection among children below 3 years old is high (34.6%, n= 188), but most are silent infection, without obvious clinical manifestation, the diarrhoea.[13] Therfore, they can be regarded as carriers with a possibility to spread the infection through improper personal hygiene or bad sanitation to other individuals, in particular the immunocompromised such as those with HIV infection. Finally, the amount of *Cryptosporidium* DNA that was isolated from the preserved stool was more than enough to give a positive result, even using a direct method.

The use of PCR to detect *Cryptosporidium* infection will be very useful when dealing with a lot of specimens (such as in a survey) or in cases where oocysts are very few, or for environmental samples such as surface and river water, while the AFS that is less sensitive is better used for public service in hospitals and health laboratories, where the number of specimens are few. Sensitivity can be increased by stool concentration and repeated slide examination before a negative result is stated.[19]

The high proportion of *Cryptosporidium* infection in the area and its severe manifestation in immunocompromised individuals necessitate a further study to reveal the transmission route and species of Cryptosporidium.

In conclusion, the frequency of *Cryptosporidium* infection among children ≤ 3 years old was very high and stool storage in $K_2Cr_2O_7$ for 13 months did not affect the PCR result. High prevalence of *Cryptosporidium* infection indicated high transmission in that area and the potential to be transmitted to other individuals such as the immunocompromised.

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