

## Research Article

## Potential Transmission of *Cryptosporidium* sp. in Ciliwung River Water, Jakarta

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### Abstract

*Cryptosporidium* sp. is an intracellular protozoa parasite that causes diarrhea. Contaminated water supply with *Cryptosporidium* oocyst may cause diarrhea outbreak. Inadequate wastewater treatment facilities in Jakarta become the main cause of water contamination, even in the river that was designated for the clean water reservoir. This study aims to evaluate the *Cryptosporidium* sp. existence in Ciliwung river water. This study used seven samples of Ciliwung river water and was conducted in Parasitology Laboratory, Faculty of Medicine Universitas Indonesia in 2018. Microscopic examination using modified Ziehl-Neelsen (mZN), Auramine Phenol staining (AF), Immunofluorescent Assay (IFA) and Polymerase Chain Reaction (PCR) was performed to detect *Cryptosporidium* oocyst. Oocyst enumeration was done by the IFA method. Assessment of oocyst viability was performed by the addition of 4'-6-diamidino-2-phenylindole (DAPI) solution to the IFA method. The results showed that *Cryptosporidium* oocyst found upstream and downstream of Ciliwung river water. Contamination of *Cryptosporidium* oocysts shows higher contamination in the downstream river. Assessment of the viability of *Cryptosporidium* sp. oocyst showed that *Cryptosporidium* oocysts in the downstream Ciliwung river water are viable, suggesting that oocysts are infectious. It can be concluded that upstream and downstream of Ciliwung river water are contaminated with *Cryptosporidium* sp. and potential as a source of cryptosporidiosis infection.

**Keywords:** *Cryptosporidium*, river water, Immunofluorescent assay.

## Potensi Transmisi *Cryptosporidium* sp di Sungai Ciliwung Jakarta

### Abstrak

*Cryptosporidium* sp. adalah protozoa intraseluler penyebab diare. Sumber air yang terkontaminasi oocista *Cryptosporidium* dapat menyebabkan wabah diare. Minimnya fasilitas pengolahan air limbah di Jakarta mengakibatkan pencemaran yang terjadi di badan-badan air, bahkan badan sungai yang diperuntukkan sebagai bahan baku air bersih telah tercemar. Tujuan penelitian ini adalah untuk mengevaluasi keberadaan *Cryptosporidium* sp. pada air sungai Ciliwung. Penelitian dilakukan di Laboratorium Parasitologi Fakultas Kedokteran Universitas Indonesia pada tahun 2018. Pada penelitian ini digunakan 7 sampel air sungai Ciliwung. Pemeriksaan dilakukan dengan metode mikroskopis pewarnaan modifikasi Ziehl Neelsen (mZN), auramin fenol (AF), Immunofluorescent Assay (IFA) dan metode molekuler Polymerase Chain Reaction (PCR) untuk deteksi oocista *Cryptosporidium*. Enumerasi oocista dilakukan dengan metode IFA. Penilaian terhadap viabilitas oocista, dilakukan dengan penambahan larutan 4'-6-diamidino-2-phenylindole (DAPI) pada metode IFA. Hasil penelitian menunjukkan bahwa oocista *Cryptosporidium* sp. ditemukan pada aliran sungai Ciliwung bagian hulu dan hilir. Kontaminasi oocista *Cryptosporidium* menunjukkan kontaminasi lebih tinggi pada bagian hilir. Penilaian viabilitas oocista *Cryptosporidium* sp. menunjukkan oocista *Cryptosporidium* pada air sungai Ciliwung bagian hilir bersifat viabel, menunjukkan bahwa oocista bersifat infeksius. Kesimpulan yang diperoleh adalah air sungai Ciliwung bagian hilir dan hulu terkontaminasi oleh *Cryptosporidium* sp. dan berpotensi sebagai sumber infeksi kriptosporidiosis.

**Kata kunci :** *Cryptosporidium*, air sungai, Immunofluorescent assay.

## Introduction

*Cryptosporidium sp.* is an intracellular protozoan parasite found worldwide and capable of infecting various vertebrate hosts, including reptiles, birds, fish, amphibians, and mammals.<sup>1</sup> *Cryptosporidium* has been the leading cause of opportunistic infections among HIV/AIDS patients. Diseases caused by this protozoa called cryptosporidiosis.<sup>2,3</sup> Clinically, cryptosporidiosis in humans shows mild gastrointestinal symptoms or acute self-limiting diarrhea in immunocompetent individuals, however, in the immunocompromised individual, it will lead to serious problems such as chronic diarrhea followed by extreme cramp, weight loss, anorexia, malaise, and death.<sup>4,5</sup>

*Cryptosporidium sp.* infection occurs when ingesting *Cryptosporidium sp.* oocyst either directly through contact with infected animals/humans as well as from food/water contaminated *Cryptosporidium sp.*<sup>4,6</sup> Several risk factors could lead to infection such as minimum clean water supply, poor sanitation, animals around the house, a house that is closely located to farm and river, flood, climate change, and malnutrition.<sup>7</sup>

Cryptosporidiosis diagnosis usually performed by *Cryptosporidium sp.* oocyst detection in the fecal sample. Fecal samples are mainly examined microscopically with several staining methods such as auramine phenol (AF), Ziehl-Neelsen (ZN), and Immunofluorescent Assay (IFA).<sup>8</sup> IFA and ZN could be used to detect oocyst in the water-related sample. Apart from that, several research and diagnostic references nowadays using a molecular method such as polymerase chain reaction (PCR) for 18S rRNA. The benefits of using this method can identify *Cryptosporidium sp.* parasites on the species level, as well as determining its potential as the source of infection.<sup>9,10</sup>

Cryptosporidiosis case was often reported along with water-borne outbreak.<sup>5</sup> The first reported water-borne cryptosporidiosis was in 1984 in Braun Station, Texas, confirmed by a serological and fecal test.<sup>11</sup> In 1993 there was the biggest water-borne *Cryptosporidium sp.* outbreak in Milwaukee with more than 400.000 victims.<sup>11</sup> Meanwhile, in Indonesia *Cryptosporidium sp.* was positively identified on the water in public bath facilities in East and West Lombok.<sup>13</sup> These studies have shown that *Cryptosporidium* transmission could occur through water, and it will lead to a severe threat of human health.

The water pollution problem in Indonesia's cities, including Jakarta, has shown some severe symptoms. The inadequate wastewater management facility has led to pollution on water sources, including the river that was purposed for a drinking water source. River water pollution itself can be caused by human, animal, or plant organic waste which was dump over to the river. A study performed by Hendrawan confirmed that 83% of river water in Jakarta was in the poor category, according to the value of water quality index.<sup>14</sup>

Water-borne diseases are caused by water contamination such as a virus, protozoa, or chemical agent. In addition, the major risk factor for water-borne diseases caused by water microbe is human or animal waste. These contaminants can directly be transmitted to a human if the polluted water is used as drinking water, recreation, or other household needs. WHO has decided the pathogen that can cause the water-borne disease, one of which is *Cryptosporidium*, and the normal index for the microbiology parameter is zero or none per water volume.<sup>15</sup> However, there are many challenges in enhancing water quality, protecting it from *Cryptosporidium* oocyst contamination. Several factors that can lead to *Cryptosporidium* oocyst water contamination are fecal contamination in the area where human and animal life. *Cryptosporidium sp.* oocysts are immune to disinfection (including chlorination); and low infection doses of *Cryptosporidium hominis* and *Cryptosporidium parvum*, where only 9-10 oocysts can cause diseases. Thus, the *Cryptosporidium* oocyst existence has to be detected.<sup>16</sup> The objective of this study is to evaluate the *Cryptosporidium sp.* existence, viability in Ciliwung river water and examine the level of its contamination level.

## Method

### Characteristics of The Sample

This research was a cross-sectional study that conducted from January until May 2018 in Laboratory of Parasitology, Faculty of Medicine, Universitas Indonesia. The sample used in this study is the Ciliwung river water. The sample is taken from Ciliwung river water (upstream) and Ciliwung river water (downstream) (Table 1.). The sample collection was under collaboration with PT Palyja and Faculty of Engineering Universitas Indonesia. The river water collected from each collecting point was 10 L.

**Table 1. Characteristics of The Sample**

Code	Water Sample Origin
P0a	Ciliwung river water (downstream)
P1a	Ciliwung river water (downstream)
P1b	Ciliwung river water (downstream)
W1a	Ciliwung river water (upstream)
W1b	Ciliwung river water (upstream)
W2a	Ciliwung river water (upstream)
W2b	Ciliwung river water (upstream)

### Preanalytical Water Sample

Collected river water samples were then stored at room temperature for 24 hours to allow sedimentation to occur. Next, the water samples were pumped, and the 500 mL residual filtrate water was kept. The remaining water was then centrifuged in 3750 rpm for 20 minutes. The supernatant was discarded, and 50 mL of filtrate was kept. The remaining filtrate was transferred to a sterile 50 mL tube and then centrifuged in 3750 rpm for 10 minutes. The supernatant was discarded, and 1 mL of filtrate was kept for further analysis.<sup>17</sup>

### *Cryptosporidium* Oocyst Detection in Water Sample

*Cryptosporidium* oocyst detection with the modified Ziehl-Neelsen method (mZN). The filtrate was pipetted for 10 µL and smeared on a microscope slide. The samples were fixated on methanol absolute. Cold strong Carbol fuchsin 3% solution was poured on the slides, then washed under running water, followed by pouring of the methanol-HCl solution on the glass. The last step was the Malachite green 0.4% solution was poured on the glass. The oocyst appeared as a red, round, 4-6µm size substance, with a pale green background. The level and proportion of oocyst was varied among the samples, and in this step, the existence of sporozoite was also examined.

The filtrate was pipetted for 20 µL and smeared on a microscope slide. The samples were fixated with methanol for 10 minutes. Auramine-phenol, 3% solution, was then pipetted on the glass, followed by the addition of methanol-HCl 3% solution. The slides were washed, and the potassium permanganate 0.1% solution was pipetted onto the glass. The oocyst appeared as a round or ring-shaped 4-6 µm size substance with green to the yellow fluorescent characteristic and dark background.<sup>15</sup>

The *Cryptosporidium* oocyst detection with Immunofluorescent Assay (IFA) method was performed with immunofluorescent instrument Crypto/Giardia Cel (Cellabs, Pte Australia), and the procedure was following its manufacture standard. Fluorescent-labeled mouse monoclonal antibody reagent will specifically bind to *Cryptosporidium* oocysts on the specimen. The tagged oocyst showed green fluorescent with a distinct morphology. *Cryptosporidium* oocyst viability was assayed with the addition of 50 µL DAPI (stock solution concentration of 5 mg/mL) in PBS (1:5000) on the microscope slides.<sup>8</sup>

### Results

The success of microscopic technique was evaluated by using a positive control derived from a fecal sample of cryptosporidiosis patient in Parasitology Department, Faculty of Medicine Universitas Indonesia. The results were pronounced positive when the samples reveal the *Cryptosporidium* sp. oocyst by using modified acid-fast staining (Ziehl Neelsen), auramine phenol staining, or immunofluorescent assay. Table 2 shows the comparison of those three-staining observed from the Ciliwung river samples. There are only 4 samples positives from Auramine-phenol staining and five samples positives from the immunofluorescent assay.

**Table 2. Comparison of Three Examination Methods *Cryptosporidium* sp. Diagnosis**

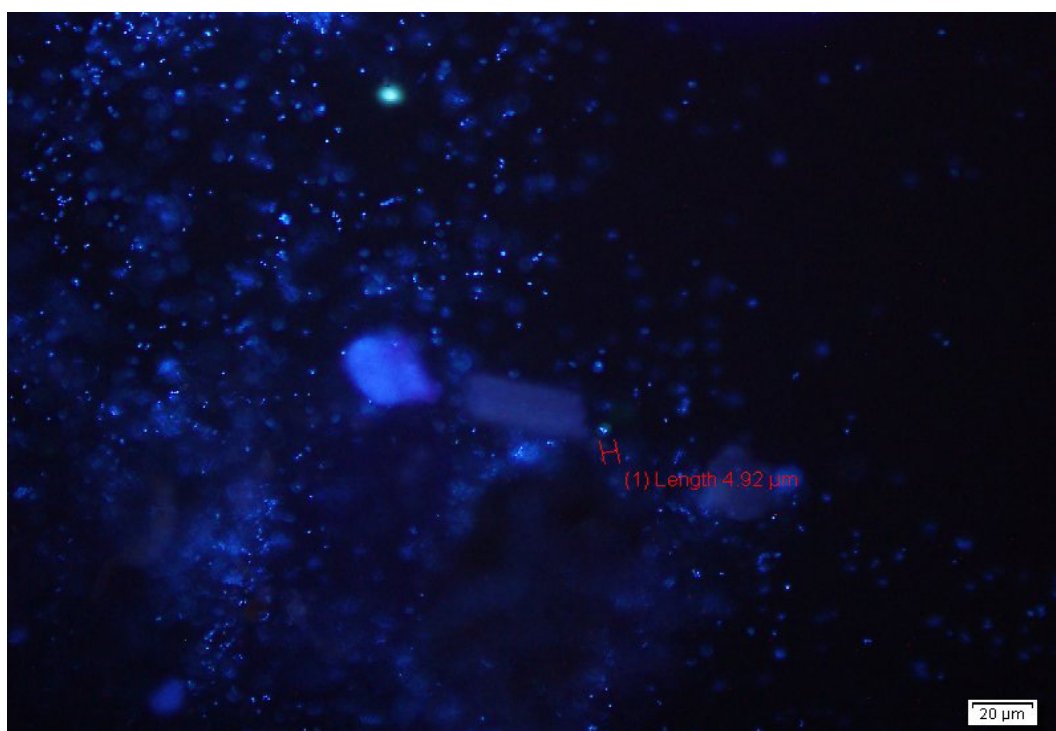
Sample Code	Method		
	mZN	AF	IFA
P0a	-	+	+
P1a	-	+	+
P1b	-	+	+
W1a	-	-	-
W1b	-	-	-
W2a	-	+	+
W2b	-	-	+
Positive Amount	0	4	5

The enumeration was assessed on positive samples detected on the IFA method, and oocyst examination was performed on the high-power field (10x100 magnification). The number of oocysts was shown more than 1 x 10<sup>3</sup> in samples P0a, P1a and P1b (Table 3.).

**Table 3. Enumeration of *Cryptosporidium sp.* Oocyst on Water Samples**

Sample Code	Oocyst Enumeration/L
P0a	$6,0 \times 10^3$
P1a	$1,5 \times 10^3$
P1b	$4,0 \times 10^3$
W2a	$1,0 \times 10^3$
W2b	$1,0 \times 10^3$

*Cryptosporidium sp.* oocyst viability assessment was performed based on the existence of sporozoite. Oocyst viability assessment was carried out by adding DAPI to five positive samples with IFA (P0a, P1a, P1b, W2a, and W2b). The results of this examination showed that 4 samples (P1a, P1b, W2a, and W2b) showed negative DAPI results because no sporozoites were found in the oocyst. Positive DAPI was only found in P0a samples (Figure 1), which were obtained by oocysts containing sporozoites.



**Figure 1. *Cryptosporidium sp.* Oocysts. with The IFA Method (DAPI filter) on Water Sample P0a (400x Magnification)**

**Discussion**

In Indonesia, especially Jakarta, pollution occurs in bodies of water, even river that is destined as raw drinking water. Contamination of water may be caused by viruses, bacteria, protozoa, or chemicals. Pollution or contamination can cause disease (waterborne disease) for humans. WHO has established several microbial pathogens that cause waterborne disease, one of which is *Cryptosporidium* protozoa.<sup>15</sup> *Cryptosporidium* is a pathogenic protozoan cause of diarrhea. In immunocompetent individuals, diarrhea is usually self-limiting, but for immunocompromised individuals, it can lead to chronic diarrhea resulting in death. This study aims to evaluate the protozoa parasite *Cryptosporidium sp.* on Ciliwung river water.

The sample used in this study is seven samples of Ciliwung river water, which is the source of raw water. The inspection of the preparation is done throughout the field of view for each method. This is done because of the small probability of obtaining oocysts in samples from the environment compared with fecal samples from immunocompromised individuals.

There is no positive samples found on detection with mZN staining from the seven samples examined. This is thought to be due to the very small number of oocysts or below the limits of mZN staining detection. Based on the sensitivity test of microscopic examination on water samples that have been done in this study (data not attached), the analysis with mZN staining has a detection limit starting from the concentration of  $10^4$  oocysts



per liter of water. In addition, staining with the ZN method has a deficiency that not all oocysts absorb the dye stuff well, so not all oocysts are colored and this technique has a low sensitivity of 37-90%.<sup>18</sup>

There are four positive samples in detection with AF staining from seven samples examined. In this study, AF staining has better sensitivity than mZN staining because it can provide positive results that not obtained on mZN staining. This is in accordance with previous research by Khurana et al<sup>18</sup> which reported the AF sensitivity could reach 100%. Excess AF staining, among others, this coloration can color the outer wall of the oocyst and the internal structure of oocyst. In addition, the phenol in AF staining accelerates the penetration of AF through the oocyst wall. Detection with AF method also has advantages that can be done at low magnification (400x).

Detection by IFA examination has five positive samples. The results of this study are similar to those of Chalmers et al., who reported that immunofluorescence screening was more sensitive than AF staining.<sup>8</sup> In this study, the immunofluorescence method was considered more sensitive because it could produce the highest positive results and detect oocysts even at low concentrations in the sample. Therefore, IFA examinations can be an alternative to rapid screening of large sample quantities.

The addition of DAPI on examination by the IFA method in the preparation is to detect the presence or absence of sporozoite nucleus in oocysts to assess the viability of detected oocysts. The viable oocysts are those containing sporozoites.<sup>19</sup> The result showed that *Cryptosporidium* oocysts in the downstream Ciliwung river water are viable, suggesting that oocysts are infectious. In this study, IFA methods have higher sensitivity than mZN and AF. In addition, the use of monoclonal antibodies to the IFA method reduces doubt on detection.

The transmission of *Cryptosporidium* in the environment is usually not separated from the role of water. River water in Jakarta has a dual function that is for the necessities of life and as a place of disposal of waste materials so that contamination can happen. WHO drinking water standards is that drinking water should be free of microbial pathogens that cause waterborne disease, one of which is the *Cryptosporidium* protozoa parasite. Normal values for WHO-based microbiological parameters are zero or none per volume of water.<sup>15</sup> In this study, enumerated results show that the downstream of Ciliwung river water has severe contamination by *Cryptosporidium* oocyst with concentration

>1000 oocyst/L. Contaminated river water with *Cryptosporidium* oocyst is a risk factor and potential as a source of cryptosporidiosis infection. In addition, previous studies have shown that some *Cryptosporidium* isolates have low dose infections; 9-10 oocysts already can cause disease.<sup>20</sup>

However, the results of viability detection with DAPI showed that most of the obtained oocysts did not contain sporozoite nuclei. IFA method used antibodies that bind to the oocysts wall so that the IFA positive results can be derived from antibodies that bind the oocysts walls that do not contain sporozoites.

### Conclusion

The results showed that *Cryptosporidium* oocyst found upstream and downstream of Ciliwung river water. Contamination of *Cryptosporidium* oocysts shows higher contamination in the downstream river. Assessment of the viability of *Cryptosporidium* sp. oocyst showed that *Cryptosporidium* oocysts in the downstream Ciliwung river water are viable, suggesting that oocysts are infectious.

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