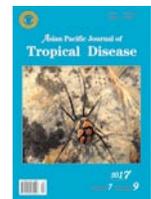


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First molecular characterization of *Cryptosporidium* from three different points of two main rivers in Kuantan, Malaysia using 18S rRNA gene nested PCR

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ABSTRACT

Objective: To identify 18S ribosomal RNA (18S rRNA) partial sequences from three different points, namely, downstream, midstream and upstream of two major rivers in Kuantan, Pahang, Malaysia.

Methods: In this study, six river water samples were collected from three different points of both Kuantan River and Balok River. Each water sample was processed and concentrated using continuous flow centrifuge machine and purified using immunomagnetic separation technique. Nested PCR was applied to detect 18S rRNA sequence in those samples after DNA extraction. Genotyping and phylogenetic analysis were carried out based on the gene of 18S rRNA sequence of *Cryptosporidium*.

Results: A total of five samples from six different points of both Kuantan River and Balok River were contaminated with *Cryptosporidium*. Only river water sample from the upstream point of Kuantan River was negative for *Cryptosporidium*. In this study, the sequencing results of five samples from both Kuantan River and Balok River belonged to *Cryptosporidium parvum*.

Conclusions: This is the first study of *Cryptosporidium parvum* genotyping in both Kuantan River and Balok River by using molecular approach nested PCR on 18S rRNA gene.

1. Introduction

Cryptosporidium is known to be one of the etiological agents of waterborne outbreaks worldwide. *Cryptosporidium parvum* (*C. parvum*) and *Cryptosporidium hominis* are two most common genotypes and species found in surface and waste waters[1]. However, other species of *Cryptosporidium* were also reported to be found in water samples[2]. Waterborne infection caused by this parasite is highly stable in the environmental water,

resistant to disinfectants and can survive for up to 1 year in colder temperature[3]. The infectious dose for *Cryptosporidium* oocysts is relatively low for only 10 oocysts[4].

Sources of water contamination can be traced using genotyping tools thereby providing the information of source tracking for human-type and animal-type pathogenic genotypes of *Cryptosporidium*[4]. In fact, genotyping techniques can differentiate *Cryptosporidium* species between human and other hosts due to host specificity[5]. However, the precise identification such as genotyping and phylogenetic analysis are essential to evaluate the risk of cryptosporidium infection either human or animal of origin[6]. In addition, some species or genotypes of *Cryptosporidium* have to be specific for host preference while others are opposite[5]. *C. parvum* could represent as a zoonotic potential source of transmission[5]. So far, the occurrence of *Cryptosporidium* oocysts in many water sources like river water has been reported to happen from different geographical settings, but not all genotypes of *Cryptosporidium*

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can be determined in river water samples, which is truly health-threatening to human[7].

In Malaysia, 11.5% of river water samples were reported to get contaminated with *Cryptosporidium* oocysts[8]. However, there is paucity of information regarding to *Cryptosporidium* identified from river especially in Kuantan, Pahang. A recent study conducted in Kuantan, Pahang reported higher occurrence of *Cryptosporidium* in Kuantan River than Balok River[9]. In contrast, a previous study conducted in three different points of river in Pos Betau, Pahang reported that all river water samples were negatively detected[10]. Besides, 5.6% ($n = 8$) of cryptosporidium infection was detected from a study carried out in a Temuan indigenous community[11]. In addition, 14.9% of the river water samples taken from Langat River Basin were contaminated with *Cryptosporidium* oocysts[12]. For occurrence of *Cryptosporidium* studies in Selangor, it was reported to be higher in Congkak River than Batu River[13]. Other studies have reported that water samples from Kemensah and Kuala Pangsun were positive with *Cryptosporidium* oocysts. Meanwhile, there was negative occurrence of *Cryptosporidium* oocysts from other sampling sites at Pos Piah, Paya Lebar and Bentong[14].

To date, the occurrence of *Cryptosporidium* species in river water especially in Kuantan, Pahang is still unclear. For this reason, genotyping and phylogenetic analysis have been carried out to provide the sufficient data on 18S rRNA sequence of *Cryptosporidium* oocysts isolated from three different points of two major rivers in Kuantan, Pahang.

2. Materials and methods

2.1. Collection of river water samples

This study was conducted in Kuantan, Pahang ($3^{\circ}49'00.1''$

N, $103^{\circ}19'59.9''$ E) (Figure 1) and Balok, Pahang ($3^{\circ}55'59.2''$ N, $103^{\circ}22'03.8''$ E) (Figure 2). 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.

2.2. Water processing

United States Environmental Protection Agency 1623.1 method was performed to detect *Cryptosporidium* from river water samples[15]. River water samples were concentrated using continuous flow centrifuge (CFC Express System) (Scientific Methods Inc, Granger, Indiana, USA). Immunomagnetic separation was implemented based on the manual instruction of Dynabeads Dynabeads® GC-Combo (Dyna, Cat. no. 730.02, Oslo, Norway).

2.3. 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 $400\times$ magnification.

2.4. DNA extraction from water samples

DNA extraction from river water sample was carried out by using QIAamp DNA Mini Kit (QIAGEN, Germany) referring to manual

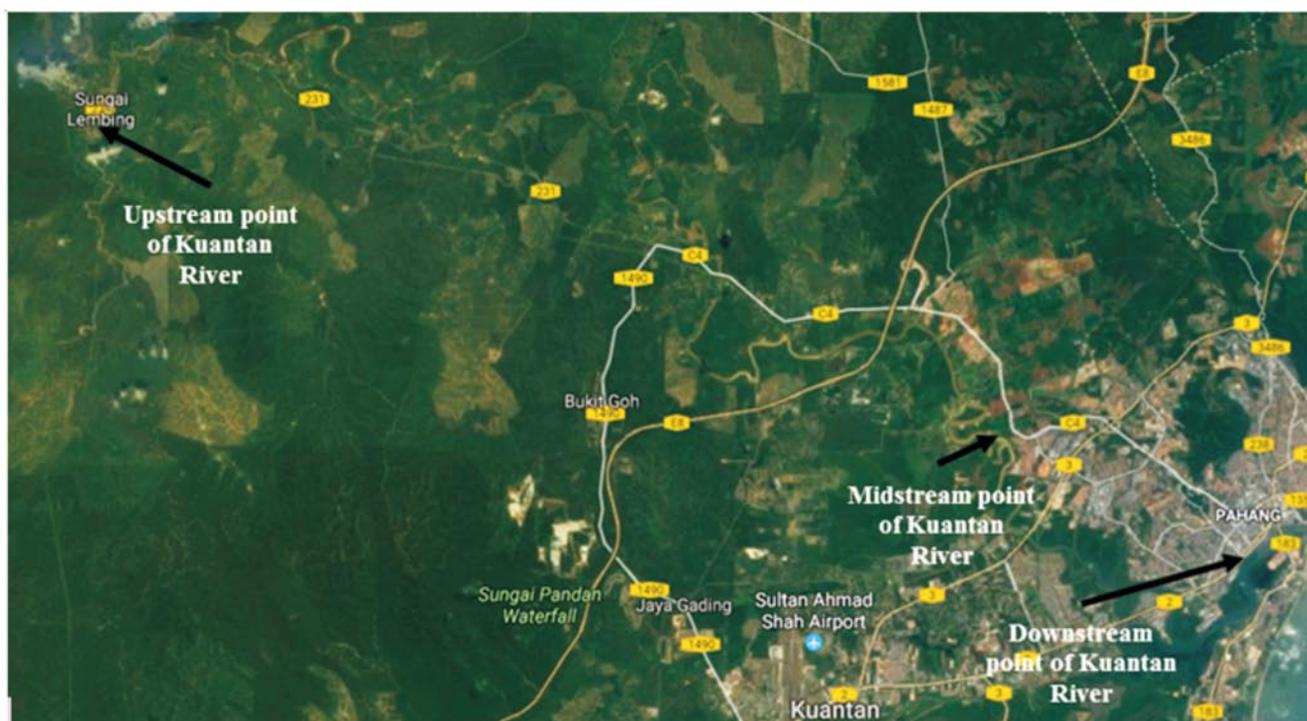


Figure 1. Sampling sites of upstream, midstream and downstream points in Kuantan River.

GPS location: downstream point of Kuantan River (3.791770, 103.306760); midstream point of Kuantan River (3.829910, 103.264931); upstream point of Kuantan River (3.919227, 103.035311).

instructions with minor modifications.



Figure 2. Water sampling sites of upstream, midstream and downstream points in Balok River.

GPS location: downstream point of Balok River (3.938092, 103.376077); midstream point of Balok River (3.961288, 103.387420); upstream point of Balok River (3.987227, 103.388099).

2.5. Identification of 18S rRNA sequence

Nested PCR of 18S rRNA was performed for molecular detection of *Cryptosporidium* [16]. Purified commercially genomic DNA of *C. parvum* was used as a positive control. Distilled water was used as a negative control for nested PCR. Agarose solution (1.2%) was prepared by mixing 0.6 g of agarose with 50 mL of 1× TBE buffer with 1 µL GelRed. The gel was electrophoresed at 100 V for 40 min and then visualised using GelDoc Ez System (Bio-Rad). The desired gel bands then were excised prior to gel purification using QIAquick Gel Extraction Kit (QIAGEN, Germany).

2.6. Analysis of DNA sequencing

DNA sequencing was conducted by First BASE Laboratories, Malaysia. Purified PCR products were run using the same primers used for nested PCR. After DNA sequencing, nucleotide sequences were edited using Bioedit sequence alignment editor for getting a consensus sequence [17]. The nucleotide sequences were placed in the NCBI GenBank database under accession numbers KX817974,

KX817975, KX817976, KX817977 and KX817978.

2.7. Phylogenetic tree construction

MEGA 7.0 software was used to generate phylogenetic tree with default of maximum probability method and the Kimura two-parameter model [18]. Those sequences were then used for generating a phylogenetic tree that also aligned with other multiple nucleotide sequences of *Cryptosporidium* denoted with these accession numbers (KC662502, KX151740, AY864317, KJ162058, AB777188, JX914624, AY282721, AY204241, FJ984565, KP004206, KT151554, KX259139, KM199757, KM199753, KF128753, JX948123, KU679364, HQ651731 and AB513881). The sequence of 18S rRNA gene of *Eimeria tenella* strain Bangalore (nucleotide accession number: JX312808) was selected as the out-group for rooting the phylogenetic tree.

3. Results

3.1. Genotyping

PCR product-based DNA sequencing was implemented to distinguish and determine the species of *Cryptosporidium* oocysts. 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. Then, five other samples which had a single band were finally subjected to proceed for gel purification and then sent for DNA sequencing. The obtained nucleotide sequences were aligned with those available nucleotide sequences retrieved from NCBI GenBank database. From the result of DNA sequencing on positive PCR products of interest, only *C. parvum* was highly matched in identity against several 18S rRNA sequences of *C. parvum* as a result of computing with BLASTn search of NCBI GenBank database. Thus, all PCR products from 5 river water samples sent for DNA sequencing were positively classified as *C. parvum*. More importantly, all of five identified nucleotide sequences from those *C. parvum* positively-river water samples have been deposited to BankIt program in NCBI GenBank database with these accession numbers (KX817974, KX817975, KX817976, KX817977 and KX817978). Those five sequences were identical to several partial sequences of 18S rRNA gene in NCBI GenBank database with high sequence similarity between 99% to 100%.

3.2. Phylogenetic analysis

Based on the phylogenetic result, the water samples were clustered together with the strain denoted as *C. parvum* of isolate 337 (accession number: KX25139), strain KSU-1 (accession number: AH006572) and other *C. parvum* strains which were of origin from environmental samples (Figure 3). This clade formed a group with the sequence in the same sister taxa representing *C. parvum* strain from Iran river (HQ651731) and another isolate IQ. Cp-15.

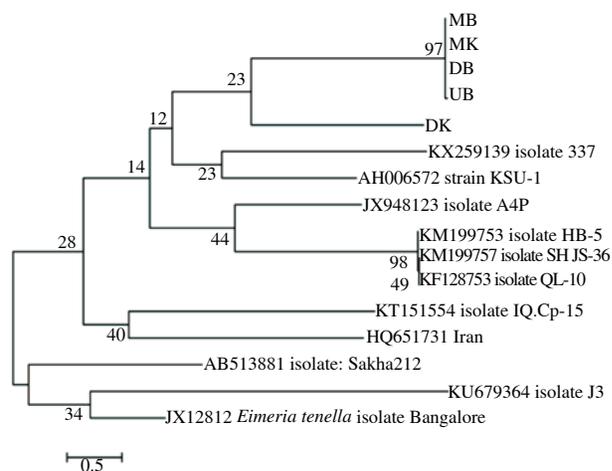


Figure 3. A phylogenetic tree of *Cryptosporidium* constructed based on partial 18S rRNA nucleotide sequences.

The phylogenetic tree was constructed using Neighbour Joining method. Figures on the branches are bootstrap probabilities (> 50%) based on 1000 bootstrapped trees. The outgroup sequence used was *Eimeria tenella* (JX312812). A phylogenetic analysis was constructed using MEGA 7.0. DK: Downstream point of Kuantan River; MK: Midstream point of Kuantan River; DB: Downstream point of Balok River; MB: Midstream point of Balok River; UB: Upstream point of Balok River.

4. Discussion

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. This is the first positive genotyping report on *Cryptosporidium* from Kuantan River and Balok River by using molecular approach of 18S rRNA gene. A previous study had revealed distribution of *Cryptosporidium* species in river water samples of Kuantan River and Balok River, especially at downstream point[9]. The *Cryptosporidium* contamination of both rivers originated most probably from human and animal as *C. parvum* can infect both hosts. Moreover, there were ruminant farms and mainly inhabited areas near to Kuantan River, especially at midstream and upstream point where sewage and housing discharge from farms and residential areas, respectively. On the other hand, the downstream point of Kuantan River and Balok River is surrounded by inhabited and recreational areas. Meanwhile, three different points of Balok River also were detected with the positive identification of *C. parvum*. This is could be due to the fact that the location of downstream and midstream point of Balok River is near to residential areas whereby the housing discharge from residential areas might be dumped to Balok River from the nearby housing drains. In particular, downstream point of Balok River is also near to mangrove forest area that might increase the probability of getting *Cryptosporidium* oocysts on surface river water even this area is also adjacent to estuary of Balok River heading to South China Sea[19]. In fact, the upstream point of Balok River is also located near to forest area where zoonotic transmission of *Cryptosporidium* oocysts can possibly happen and infect nearby river water stream[19]. In contrast, only one water sample from the upstream point of Kuantan River was detected to be negative for *C. parvum*. This could be due to the location of upstream point in Kuantan River (normally shallow), which is due to the sedimentation process resulting in reduction of aquatic life[20]. Furthermore, fast river water flow due to high number rainfall from the higher area at Tapis Mountain in Lembing

River, Kuantan also could be one of the contributing factors[21]. On the other hand, polluted heavy metals from abandoning tin mines in Lembing River town also could be a possible factor for negative result in molecular detection of *Cryptosporidium*[22]. However, extensive studies should be done in future since this study was the first ever reported so far.

In phylogenetic studies of this gene, a constructed phylogenetic tree generally showed the interrelationship of genotypes and species of *Cryptosporidium*[23]. Multiple sequence alignments of similar type of genes evolutionarily has been extensively used to determine the genotypes of *Cryptosporidium* species with minimal bootstrap values[23,24]. However, previous studies had reported that constructed phylogenetic trees showed a poor interpretation of bootstrap values regarding to the partial sequence of 18S rRNA for *Cryptosporidium*[24-28]. The percentage of bootstrap value for branching pattern of phylogenetic tree was influenced by the number of species and genotypes[24]. In fact, 16S rRNA of some bacterial species had demonstrated the weakness to resolve the internal branching supported by bootstrap value[29]. Thus, this may also be true in a case of *Cryptosporidium* especially for the partial sequence of 18S rRNA. In this study, the topology of phylogenetic tree is somehow consistent with the overall topologies of phylogenetic trees from other previous studies in terms of low proportion of branches that were supported with low bootstrap values normally resulting from the unknown sequences like identified 18S rRNA partial sequences from both rivers in this study[25-28]. In fact, phylogenetic tree construction was used to root novel *Cryptosporidium* sequences with known species or genotypes based on the genotypic variations of sequence diversity, thereby resulting in non-homologous copies of this gene due to different factors like host adaptation and geographical distribution[30]. In this study, for all five positive samples with *C. parvum*, the samples might contain sequence polymorphisms at the 18S rRNA gene location as reported in many cases of 18S rRNA partial sequences[31-33]. Moreover, it is troublesome to verify either variation of sequences corresponding to species diversity or reflecting non-homologous copies of ribosomal RNA[28,34].

In conclusion, this is the first study to report the genotyping of *C. parvum* detected in Kuantan River and Balok River by 18S rRNA gene. So far, there is no case that has officially reported on human cryptosporidiosis due to waterborne transmission of *C. parvum* oocysts from Kuantan River and Balok River even though this present study clearly showed the positive genotyping of *C. parvum*. Therefore, preventive measures should be taken regularly by local water authorities to avoid the risk of *Cryptosporidium* contamination to water supplies.

Conflict of interest statement

We declare that we have no conflict of interest.

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