SEASONAL VARIATION AND POTENTIAL SOURCES OF CRYPTOSPORIDIUM CONTAMINATION IN SURFACE WATERS OF CHAO PHRAYA RIVER AND BANG PU NATURE RESERVE PIER, THAILAND

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Abstract. Using molecular techniques, a longitudinal study was conducted with the aims at identifying the seasonal difference of Cryptosporidium contamination in surface water as well as analyzing the potential sources based on species information. One hundred forty-four water samples were collected, 72 samples from the Chao Phraya River, Thailand, collected in the summer, rainy and cool seasons and 72 samples from sea water at Bang Pu Nature Reserve pier, collected before, during and after the presence of migratory seagulls. Total prevalence of Cryptosporidium contamination in river and sea water locations was 11% and 6%, respectively. The highest prevalence was observed at the end of rainy season continuing into the cool season in river water (29%) and in sea water (12%). During the rainy season, prevalence of Cryptosporidium was 4% in river and sea water samples, but none in summer season. All positive samples from the river was C. parvum, while C. meleagris (1), and C. serpentis (1) were obtained from sea water. To the best of our knowledge, this is the first genetic study in Thailand of Cryptosporidium spp contamination in river and sea water locations and the first report of C. serpentis, suggesting that humans, household pets, farm animals, wildlife and migratory birds may be the potential sources of the parasites. The findings are of use for implementing preventive measures to reduce the transmission of cryptosporidiosis to both humans and animals.

Keywords: Cryptosporidium species, river and sea waters, seasonal variation, potential sources, Thailand

INTRODUCTION

Cryptosporidium spp contamination in water source is a major concern. Due to its relative low infective dose (30 oocysts) and the robustness of oocysts in difficult environments, this parasite has become an important waterborne diarrheal causative agent (DuPont et al, 1995; Carey et al, 2004). Cryptosporidiosis has an impact on both public health and economy of 106 countries (Xiao and Fayer, 2008). The Milwaukee outbreak in 1993, for example, infected 403,000 individuals with more than 100 deaths, costing 92.6 million US
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Cryptosporidium contamination in river and sea waters (Mac Kenzie et al., 1994; Corso et al., 2003). Water contamination was incriminated as the major route of transmission. Since the various causes cryptosporidiosis have been reported worldwide, resulting in health authorities, especially in the US and European countries, paying high attention on Cryptosporidium contamination in water.

The concentration of Cryptosporidium in water depends on such factors as seasonal variations affected by runoff water and difference of water temperature, source distribution and species of parasite, and type of water usage (viz. household, agriculture or waste). Thus an understanding of the nature of water source of Cryptosporidium contamination as well as the original source is very useful for appropriate water treatment and efficacious management.

The Chao Phraya River flows from the northern part of Thailand, through the capital city, Bangkok in the central region, before emptying into the Gulf of Thailand at Samut Prakan Province. The river is the source of water supply for approximately 12 million people in Bangkok and the surrounding provinces. At the mouth of Chao Phraya River, Bang Pu in Samut Prakan Province is where >10,000 seagulls migrate every year from China and remain during the cool season. There are three seasons in Thailand: summer season from March to May with an average temperature of 33-36°C, rainy season from June to October with average temperature of 28-32°C, and cool season from November to February with average temperature of 24-28°C.

At present, there are 20 species of Cryptosporidium reported among humans, domestic animals and wild vertebrates (Plutzer and Karanis, 2009; Cabada and White, 2010) but only 7 species have been identified as being zoonotic, namely, C. parvum, C. hominis, C. meleagridis, C. canis, C. felis, C. suis and C. muris (Xiao and Frayer, 2008), of which the former three are of major concern with C. parvum and C. hominis being responsible for >90% of human cryptosporidiosis (Xiao and Ryan, 2004). Although 7 species of Cryptosporidium (C. parvum, C. hominis, C. meleagridis, C. canis, C. felis, C. muris and C. parvum bovine genotype 5) have been reported in HIV patients in Bangkok (Gatei et al., 2002; Tiangtip and Jongwutiwes, 2002; Saksirisampant et al., 2009), epidemiological studies concerning sources of such protozoa, transmission vehicle or zoonotic potential are limited in Thailand. Hitherto, there is no study concerning Cryptosporidium spp contamination in river and sea waters especially during the three seasons. Therefore, we conducted a longitudinal study with the aims at identifying seasonal variations of Cryptosporidium contamination in two settings of surface water, along the Chao Phraya River and sea, as well as analyzing the potential source of the contamination using species or genotype identification.

MATERIALS AND METHODS

Study sites

The study sites were 3 districts located at the north, central and south of Chao Phraya River, namely, Bang Sue with population of 139,750, Samphan Thawong with 28,762 and Rat Burana with 89,610 (wikipedia.org/wiki/List_of_Districts_of_Bangkok). Households occupying by humans and domestic pets as well as farm animal husbandry and wildlife along the river are potential sources of contaminants. In addition, the Bang Pu Nature Reserve pier, located about 12 km...
east of the town center of Samut Prakan Province, where more than 10,000 seagulls migrate annually from China after visiting Myanmar or Cambodia during the cool season was chosen.

**Water samples**

At the study sites, 20 liters of water samples were collected in plastic containers at 3 districts located along the Chao Phraya River during the 3 seasons: from November 2010 to February 2011 (cool season), March 2011 to May 2011 (summer) and from June 2011 to October 2011 (rainy season). Twenty liters of samples were collected at Bang Pu Nature Reserve pier during: 1) presence of migratory seagulls (January to March 2011), 2) absence of the birds (May to July 2011) and 3) prior to presence of migratory seagulls (August to October 2011). Temperature, turbidity and pH of each water sample were immediately determined and recorded, and then all water samples were transported to the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand for further laboratory processing.

**Isolation of Cryptosporidium from water samples**

Twenty liters of each water sample were filtered through cellulose acetate membrane, 1.2 µm pore size (PALL Life Science, Ann Arbor, MI). The plastic container was washed with 1 liter of washing buffer solution pH 7.2 (Gracyzk et al, 1997) and filtered through the same filter membrane. The filter membrane was washed with 10 ml of washing buffer and mixed with sucrose solution to generate a final solution with specific gravity of 1.3 (Jenkins et al, 2010). The mixture was then left to stand for 10 minutes before centrifugation at 1,000g for 10 minutes at 8ºC. Cryptosporidium oocysts, if any, will float to the top of the solution and 15 ml of the upper layer were added to 30 ml of distilled water to make the specific gravity of solution of 1.0, which then was centrifuged at 2,700g for 15 minutes at room temperature. The sedimanted samples were subjected for DNA amplification.

**Amplification of Cryptosporidium DNA by nested PCR**

The sedimented sample was suspended in 100 µl of phosphate-buffered saline buffer. DNA was extracted using QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocols. The suspension was subjected to lysis by 5 cycles of freezing at -83°C for 10 minutes and thawing at 56°C for 5 minutes. The extracted DNA was stored at -20°C until used. Nested PCR employed specific primers targeting 18S rRNA gene was carried out (Xiao et al, 1999, 2004b). In brief, 4 volumes (0.5 µl to 3 µl) of DNA solution were made up to 100 µl reaction volume containing 200 µM each dNTP, 200 nM each primary primer, 6 mM MgCl₂, 400 ng/µl non-acetylated BSA, 2.5 U Taq polymerase, and 1X PCR buffer. Primary PCR conditions consisted of a total of 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 60 seconds. The initial hot start was at 94°C for 3 minutes and final step at 72°C for 7 minutes. For the second PCR, BSA was not added, and double concentrations of each second primers (400 nM) were added, and 2 µl of the primary amplicon template were used. Thermocycling conditions were identical to those of the primary PCR except that the annealing temperature was increased to 58°C. Amplicons were visualized on 1.5% agarose gel with ethidium bromide under UV light. The primary amplicon was about 1,500 bp and second round amplicon was about 823 bp. These prim-
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ers are specific for genus Cryptosporidium allowing detection of all species of Cryptosporidium.

Sequencing and genotyping

The nested PCR amplicons were purified by PureLink Quick Gel Extraction kit (Invitrogen, Carlsbad, CA) and sequenced using the second primer pair (Pacific Science, Bangkok, Thailand). The sequences were compared with published sequences of Cryptosporidium species and genotypes deposited at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The reference accession number for C. parvum were HQ651731.1, AB513881.1, C. meleagridis AJ493205.1 and C. serpentis AF093502.1.

RESULTS

Altogether 144 samples, 72 from the Chao Phraya River and 72 from Bang Pu Nature Reserve pier, were collected. The river and sea water samples were similar in terms of pH and temperature, but the mean turbidity was 3 times higher in sea water samples than in river water samples with the highest mean turbidity of the former of 188.6 (34.1-403.0) NTU during the period following the presence of seagulls and 79.8 (44.2-153.5) NTU in the latter in the summer season (Table 1).

Total prevalence of Cryptosporidium contamination was 11% (8/72) in the river water samples and 6% (4/72) in the sea samples. The highest contamination (29%) of Cryptosporidium spp found in the Chao Phraya River samples was during the cool season and 12.5% in sea water during the presence of seagulls (January to March which was also the cool season) (Table 1). Low Cryptosporidium contamination (4%) was found during the rainy season in river water and in sea water before the presence of seagulls, but none was found in the summer and after the birds had gone, respectively. The highest prevalence of Cryptosporidium spp contamination was when the average temperature was 28.3°C.

The distribution of Cryptosporidium spp along the Chao Phraya River indicated that the highest prevalence (17%) was at the northern part of Bangkok (Bang Sue District) where there is a moderate population density (Table 2). Water samples from both Samphanthawong District, with the highest population density, at the central and Rat Burana District, with the lowest population density, in the southern part of Bangkok were contaminated with Cryptosporidium spp at 8.3%. From PCR sequencing, all 8 positive samples from the Chao Phraya River were identified as C. parvum, whereas of the 4 positive samples from sea water, were 2 C. parvum and 1 each of C. meleagridis and C. serpentis.

DISCUSSION

In the present study, the overall prevalence of Cryptosporidium spp contamination in the Chao Phraya River was twice that in sea water at Bang Pu, Samut Prakan Province which was in agreement with a previous study in the southwest costal of Thailand (12% of water samples were contaminated with Cryptosporidium) (Srisuphanunt et al, 2010). In another study in Thailand using 1,000 liters of underground water, a combination of immunomagnetic separation (IMS) and fluorescence assay revealed a higher Cryptosporidium contamination (35%) (Sutthikornchai et al, 2005). Temperate zone countries showed high contamination levels of up to 93% (Yang et al, 2008; Xiao et al, 2011; Budu-Amoaka et al, 2012a,b). These different results might not only reflect actual difference in Cryptosporidium contamination levels from various loca-
Table 1
Seasonal prevalence of *Cryptosporidium* spp contamination and physical properties of Chao Phraya River and sea water of Thailand during 2010 - 2011.

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Season</th>
<th>Turbidity (NTU) Mean (min-max)</th>
<th>Temperature (°C) Mean (± SD)</th>
<th>pH (mean ± SD)</th>
<th>No. of positive samples/total (%)</th>
<th>Identified species (No. of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Chao Phraya River</td>
<td>Summer</td>
<td>79.8 (44.2 - 153.5)</td>
<td>31.1 ± 0.4</td>
<td>7.04 ± 0.02</td>
<td>0/24</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Rainy</td>
<td>66.8 (26.0 - 192.0)</td>
<td>30.5 ± 0.4</td>
<td>7.23 ± 0.01</td>
<td>1/24 (4%)</td>
<td>(99% identity with HQ651731.1) C. parvum (1)</td>
</tr>
<tr>
<td></td>
<td>Cool</td>
<td>9.4 (3.3 - 76.8)</td>
<td>28.3 ± 1.2</td>
<td>6.61 ± 0.25</td>
<td>7/24 (29%)</td>
<td>(99% identity with AB513881.1) C. parvum (7)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8/72 (11%)</td>
<td></td>
</tr>
<tr>
<td>Sea water</td>
<td>Presence of seagulls</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Before</td>
<td>188.6 (34.1 - 403.0)</td>
<td>31.7 ± 0.6</td>
<td>7.52 ± 0.01</td>
<td>1/24 (4%)</td>
<td>(85% identity with AJ493205.1) C. meleagris (1)</td>
</tr>
<tr>
<td></td>
<td>During</td>
<td>81.0 (11.4 - 270.0)</td>
<td>30.1 ± 2.3</td>
<td>6.48 ± 0.39</td>
<td>3/24 (12%)</td>
<td>(99% identity with JN247404.1) C. parvum (2)</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>247.3 (44.3 - 529.0)</td>
<td>31.2 ± 0.7</td>
<td>6.39 ± 0.04</td>
<td>0/24</td>
<td>-</td>
</tr>
<tr>
<td>Total Cryptosporidium contamination in sea water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4/72 (6%)</td>
<td></td>
</tr>
</tbody>
</table>

aMarch - May, bJune - October, cNovember - February, dAugust - October, eJanuary - March, fMay - July
tions, but also the difference in techniques used. We used a 20 liters water sample, which is the requirement of surface water sample volume recommended by the USEPA method 1623 (Yang et al., 2008). The sucrose flotation technique used was as effective as the IMS method (Koomapong et al., 2009).

The highest water turbidity was observed in the summer season in both river and sea water samples, and was higher than in other studies (Yang et al., 2008; Srisuphanunt et al., 2010). Particles turbidity in water may affect purification processes. Because the normal specific gravity of Cryptosporidium oocyst is 1.009-1.08, adhesion of oocysts to particles may increase the specific gravity of the oocysts resulting in less floatation (Dumetre et al., 2011), which will decrease the recovery rate. However, the sucrose solution used in this study has a specific gravity of 1.3, which is high enough to float oocysts with attached particles. Temperature and pH of water samples may have little effects on the recovery rate as temperature and pH of the water samples in this study differed from other studies (Fayer, 1994; Li et al., 2010).

Studies have indicated that the highest contamination of Cryptosporidium is in the rainy season, since the runoff water carries the parasite into surface water (Kaucner et al., 2005; Cizek et al., 2008; Miller et al., 2008). However, large amounts of runoff water can also dilute oocyst concentration. In Thailand, the rainy season in 2011 caused severe flooding in the north and central regions of Thailand as well as in Bangkok and including the Chao Phraya River. This could account for the 4.2% Cryptosporidium spp concentration in this season. Thus by the end of the rainy season and continuing into the cool season, the highest contamination

<p>| Table 2: Prevalence of C. parvum contamination in Chao Phraya River in different seasons and locations. |
|--------------------------------------------------|--------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Population density (persons/km²)</th>
<th>No. of positive (% of total samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer (Mar-May)</td>
<td>Cool season (Nov-Feb)</td>
</tr>
<tr>
<td>Bang Sue (North)</td>
<td>13,040</td>
</tr>
<tr>
<td>Samphan Thawong (Central)</td>
<td>21,993</td>
</tr>
<tr>
<td>Rat Burana (South)</td>
<td>5,928</td>
</tr>
<tr>
<td>Jitramontree, 2008</td>
<td></td>
</tr>
</tbody>
</table>
level (29%) was found.

The highest contamination level of Cryptosporidium (12.5%) in sea water samples corresponded with the presence of migratory seagulls. The absence of Cryptosporidium in sea water after the departure of the birds might be attributed to high turbidity of the water causing low recovery rate.

The upper stream of the Chao Phraya River in Bangkok had twice the contamination prevalence than in the middle and lower stream. The upper stream receives the water from many tributaries from the northern part of Thailand, which run through animal farms, communities, and forests thus facilitating Cryptosporidium oocyst contamination. In the central region the highest population density of Bangkok (~22,000 persons/km² in Samphanthawong District) should have contributed to Cryptosporidium spp contamination of the Chao Phraya River more than other parts of Bangkok. However, contamination in this area and in the lower part was lower than in the upper part of Bangkok. This might be due to the waste water management being better in this area than in other parts of Bangkok.

All positive water samples in Chao Phraya River were contaminated with C. parvum, the zoonotic and the most common causative agent of cryptosporidiosis (Xiao et al, 2004a). Reports from Thailand have revealed that patients and cattle are infected with C. parvum (Gatei et al, 2002; Jittapalapong et al, 2006; Nuchjangreed et al, 2008; Saksirisampant et al, 2009; Inpankaew et al, 2010). Thus domestic animals, wildlife, farm animals and humans are potential sources of C. parvum in the river.

Mammalian animals and humans can be the sources of C. parvum in sea water. Avian species are potential sources of C. meleagris. C. serpentis is recognized as being snake and lizard parasite (Xiao et al, 2004a). To the best of our knowledge, this is the first report of C. serpentis in Thailand sea water.

In summary, this is the first study of the seasonal variation of Cryptosporidium contamination and species in Chao Phraya River and marine water of Thailand. The cool season (from November to February) favored contamination than other seasons. Three species, C. parvum, C. meleagris and C. serpentis were identified in marine water, but only C. parvum in river water. Further studies are needed to identify the original hosts of Cryptosporidium spp present in the water sources. The results of this study indicate that Bangkok water authorities should be aware of potential cryptosporidiosis outbreak among the 12 million citizens by paying attention to water treatment processes. Appropriate preventive measures utilizing the epidemiological findings of the present study will be beneficial in reducing the transmission of human and animal cryptosporidiosis.

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