Contribution of cattle farms towards river contamination with Giardia cysts and Cryptosporidium oocysts in Sungai Langat Basin

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Abstract. A study to determine the contribution of Giardia cysts and Cryptosporidium oocysts from cattle farms was carried out at the Langat Basin. This study investigated the contribution of cattle farms, located near Sungai Langat and Sungai Semenyih, towards river contamination with these cysts and oocysts. The findings showed that out of 24 samples of water taken from Sungai Semenyih, 4.2% was positive for Giardia cysts with a concentration of 1.3 cysts/L and 20.8% were positive with Cryptosporidium oocysts with a range of 0.7 – 2.7 oocysts/L. At Sungai Langat, from the 43 samples taken, 23.3% were positive for Giardia cysts with a range of 1.5 – 9 cysts/L whereas 11.6% were positive with Cryptosporidium oocysts with a range of 2.5 – 240 oocysts/L. Isolation of cysts and oocysts in bovine faecal materials revealed that 14.6% of faecal samples were positive for Giardia cysts which had a range of 75 – 1.3x10^4 cysts/g and 25% were positive for Cryptosporidium oocysts with a range of 50 - 3.9x10^5 oocysts/g. From the cattle wastewater, 98% were positive with oocysts and 6.7% with cysts. The concentrations were between 20 – 3.1x10^3 oocysts/mL for Cryptosporidium and 4 – 75 cysts/mL for Giardia. Given that the prevalence of Cryptosporidium and Giardia are high amongst the cattle and the positive findings of the (oo)cysts in the river samples, it could be deduced that there is a very high possibility of the cattle farms contaminating the river with Giardia cysts and Cryptosporidium oocysts. Viability study of Cryptosporidium oocysts in the surrounding soil and pond within the cattle farm showed that the viability of Cryptosporidium oocysts decreased with time. It was estimated that it will take 52 days for all the oocysts from both environment to be non-viable. With a viability rate of approximately 2 months in a cattle farm setup, river water contaminated with Cryptosporidium oocysts has a high chance of acting as an agent of transmission. As cattle farms are also inhabited by the owners and their families, this problem may pose a threat to humans (e.g. children) especially if they are dependent on the river water as their source of water for their daily activities.

INTRODUCTION

Cryptosporidium and Giardia have emerged to be pathogens of medical and veterinary importance. Although Cryptosporidium and Giardia infections have been reported for 79 species of mammals, the most frequently reported and most widely recognized host species other than humans are the ruminants (O’Donoghue, 1995). In cattle, these parasites can cause diarrhea and poor performance, predominantly in younger animals.

Cryptosporidium is most commonly found in young calves a few weeks of age. This parasite can cause diarrhea in calves between 1 and 3 weeks of age. Clinically normal calves can also shed Cryptosporidium oocysts in their faeces and serve as a source of infection for other calves and the environment (Becher et al., 2004). However, Giardia is found in cattle of all ages. It is rarely diagnosed as a cause
of clinical diarrhea in calves. A few studies suggest that infection may be associated with reduced performance (Olson et al., 2004).

Economic loss in the cattle farming industry because of cryptosporidiosis has been linked to neonatal diarrhea which causes dehydration, inhibits normal development and even death (de Graaf et al., 1999). In Belgium, mortality caused by neonatal diarrhea is between 5 to 10% and Cryptosporidium parvum is the enteropathogen commonly found in the calves’ first few weeks of live (de la Fuente et al., 1998).

Control of cryptosporidiosis and giardiasis in cattle has become important, not only to reduce the risk of disease in cattle, but also to reduce the risk of infection in humans. If the manure from cattle contaminates water supplies and the water is not properly cleaned or if fresh manure is used on produce, humans may become infected and develop diarrhea. Calves that are infected have been reported to be able to shed 10^6-10^7 oocysts per gram of faeces (Garber et al., 1994; McCluskey et al., 1995). High rate of oocysts and cysts excretion has great potential in contaminating the environment either directly or through its role as manure in the agricultural sector.

The aim of this study was to investigate the contribution of Giardia cysts and Cryptosporidium oocysts from cattle farms, located near Sungai Langat and Sungai Semenyih towards river contamination. In addition, viability study of Cryptosporidium oocysts was also carried out in the surrounding soil and pond within a cattle farm to ascertain how long Cryptosporidium oocysts can survive in the farm environment.

MATERIALS AND METHODS

Isolation of Cryptosporidium and Giardia (oo)cysts from river water

River water from Sungai Semenyih and Sungai Langat which were adjacent to cattle farms were sampled by filtering 10 - 25 L of water through a polypropylene fiber-wound depth cartridge filter (Ametek, Wisconsin, USA, nominal pore size = 1 µm) with a flow rate of 1.5 litres per minute (using a flow restrictor).

River water samples were processed according to the procedures recommended by the U.K. Standing Committee of Analysts (SCA), Department of the Environment (Anonymous, 1990) with some minor modifications. The cartridge filter whose matrix might contained entrapped (oo)cysts was cut longitudinally and teased apart. The filter fibres were eluated with 0.1% Tween 80 (Sigma Chemical, Missouri, USA) for 10 min using a stomacher (Seward Model 3500, Ohio, USA).

The supernatant of the filter eluate was filtered through a 1.2 µm pore size cellulose ester (combination of nitrate and acetate) membrane filter (Catalog no. RAWP 142 SO, 142 mm diameter, Millipore, Bedford, Massachusettes). Membrane filters were eluted using 0.1% Tween 80 and the wash water was concentrated by centrifugation (1500 x g for 10 min) before being brought down to 20 mL. The 20 mL suspension was divided into two equal portions. Portion A was used for the detection of Giardia cysts and portion B for the detection of Cryptosporidium oocysts.

Before the clarification step by discontinous density gradient sucrose flotation, portion A was added with an equal volume (10 mL) of 2% Tween 80 and portion B with 2% Tween 80 in 2% sodium dodecyl sulphate (SDS) (Sigma Chemical, Missouri, USA). The samples were centrifuged (1500 x g for 10 min) and the supernatants were aspirated down to 10 mL.

Samples were slowly underlayered with 10 mL of cold sucrose solution and centrifuged (1000 x g, 5 min). The entire supernatant including the interface was recovered without disturbing the pellet and decanted gently into a clean 50 mL conical centrifuged tube. The residual sucrose was removed by washing three times in distilled water (for portion A) or pH 7.2
phosphate buffered saline (PBS) (Oxoid, Hampshire, UK) (for portion B). The final concentrated sample was reduced to a final volume of 1 to 5 mL, depending on how turbid the samples were.

**Isolation of Cryptosporidium and Giardia (oo)cysts from faecal samples**

Seven cattle farms within the Langat Basin were identified for cattle faecal and wastewater samples. Water-ether technique was employed for the concentration and purification of (oo)cysts from the faecal samples. Each faecal suspension containing 1 gram of faeces was made up to 50 mL with distilled water and vortexed for 20 sec. The resulting suspension was filtered through a sieve into a 250 ml conical flask. The filtrate was concentrated by centrifugation at 1050 x g for 5 min and the supernatant was aspirated down to 10 mL.

Two mL of diethyl-ether was added to the washed faecal suspension and the sample was vortexed for 20 sec and then centrifuged (1050 x g, 5 min). Following the centrifugation of each faecal sample, the fat layer was discarded, pellet was resuspended and sufficient distilled water was added to bring the volume up to 50 mL. Sample was vortexed for 20 sec and centrifuged (1050 x g, 5 min). The faecal washing procedure (as described earlier) was repeated two times and then the pellet was resuspended in distilled water to a final concentrated volume (between 3 to 5 mL depending on the amount of particulate matter).

**Isolation of Cryptosporidium and Giardia (oo)cysts from wastewater samples**

Two hundred ml of wastewater was transferred to a clean 500 mL Schott bottle. Then 2 mL of Tween 80 (10%) was added to the sample. The bottle was closed very tightly and shaken for a few minutes. Twenty mL of sample was then transferred to a centrifuge tube and topped up to 50 mL with distilled water and filtered through a muslin cloth and the filtrate was collected in a conical flask. Distilled water was used to rewash the big particles and filtered through till the volume reaches 500 mL. The filtrate was then transferred to 50 mL centrifuge tubes and concentrated at 1050 g for 15 minutes. The concentration step was repeated till the whole sample was contained in the final 10 mL.

Sample was slowly underlayered with 10 mL of cold sucrose solution and centrifuged (1050 x g, 15 min). The entire supernatant including the interface was recovered without disturbing the pellet and decanted gently into a clean 50 mL conical centrifuge tube. The residual sucrose was removed by washing three times in distilled water. The final concentrated sample was reduced to a final volume of 1 to 5 mL, depending on how turbid the sample was. The sample was further concentrated using the Immuno-magnetisable Separation (IMS) technique. The IMS technique employed followed the protocol suggested by the Dynal manual which used Dynabeads® anti-Cryptosporidium (Dynal® A.S., prod. No. 730.01, Oslo,Norway).

**Detection and enumeration of Cryptosporidium and Giardia (oo)cysts from river water, faecal and wastewater samples**

Twenty five µL aliquots of each sample concentrate was placed onto each of the four wells of a teflon®-coated microscope slide and air dried (room temperature = 24 ± 1ºC). Each well containing concentrates of sample was overlaid with 25 µL of commercially available fluorescein isothiocyanate (FITC)-labelled anti-Cryptosporidium oocysts mAb or 25 µL of commercially available fluorescein isothiocyanate (FITC)-labelled anti-Giardia cysts mAb (Waterborne TM Inc, USA). Slides were incubated in a humidity chamber for 30 ± 5 min at 37°C.

Excess antibody was removed by rinsing the slides twice with 50 µL of PBS dropped onto each well. DAPI (4', 6-diamidino-2-phenylindole) stain (Sigma Chemical, Missouri, USA) was then applied to stain the four nucleus sky-blue. Twenty µL of mounting medium (PBS:glycerol, 1:1
(v/v) was mounted onto each well and coverslips were applied to the slides which were then examined under a x400 blue filter epifluorescence microscope (Carl Zeiss Axioscope, Jena, West Germany, excitation BP 450-490 nm, beamsplitter FT 510 and emission LP 520 nm) to detect FITC stain and Nomarski-DIC optics to observe the internal structures.

(Oo)cysts which were stained with FITC-mAb exhibited bright apple green fluorescence typically concentrated on the periphery of the oocysts when observed under the epifluorescent microscope. Putative *Giardia* cysts are ovoid in shape, and are 8 to 14 µm long and 7 to 10 µm wide, with 2-4 DAPI stained sky-blue nuclei contained within the labelled cyst under the UV filter block. Whilst *Cryptosporidium* oocysts are spherical in shape with a diameter ranging from 4 to 6 µm with 4 sky-blue nuclei contained within the labelled oocyst.

**Assessment of oocysts viability using vital dye assay in soil and pond environments**

The design of the semipermeable container which contained the parasites was developed to allow oocysts to be in contact with defined environments and to allow regular sampling with minimal risk of bacterial contamination (Robertson *et al.*, 1993).

Prior to injecting oocysts into these containers, the containers were thoroughly sterilized in a 1% hypochlorite solution which was then rinsed by repeated immersion of the containers into deionised water. Oocyst suspensions were treated overnight with an antibiotic solution (4 µL of gentamicin (40µg/µL) and 50 µL of penicillin G (1µg/µL) per mL of oocyst suspension) before being injected into the containers.

Approximately 3 x 10⁵ oocysts were spiked into each container and the containers were then placed in defined environments (e.g. soil and pond in one of the cattle farms). For the control, oocysts were injected into the container which stored 4 litres of deionised water at 4ºC in the dark.

Oocyst viability was based primarily on the viability assay described by Campbell *et al.* (1992), which is dependent on the morphology and the inclusion or exclusion of two fluorogenic vital dyes, 4′6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI), by the oocysts.

**RESULTS**

**Detection of *Cryptosporidium* and *Giardia* (oo)cysts from river water**

The results of this study showed that out of 24 samples of water taken from Sungai Semenyih, 1 sample (4.2%) was positive for *Giardia* cysts with a concentration of 1.3 cysts/L and 5 samples (20.8%) were positive with *Cryptosporidium* oocysts with a range of 0.7 – 2.7 oocysts/L (Table 1 and 2). At Sungai Langat, 10 samples (23.3%) from 43 samples taken were positive for *Giardia* cysts (range = 1.5 – 9.0 cysts/L) whereas 5 samples (11.6%) were positive with *Cryptosporidium* oocysts (range = 2.5 – 240 oocysts/L) (Table 1 and 2).

<table>
<thead>
<tr>
<th>Location</th>
<th>No. of samples</th>
<th>No. of positive sample(s)</th>
<th>Percentage of positive sample(s)</th>
<th>Range of concentration of cysts/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sungai Semenyih</td>
<td>24</td>
<td>1</td>
<td>4.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Sungai Langat</td>
<td>43</td>
<td>10</td>
<td>23.3</td>
<td>1.5 – 9</td>
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Table 1. Occurrence of *Giardia* cysts in river water samples at Langat Basin
Isolation of Cryptosporidium and Giardia (oo)cysts from cattle faeces

Isolation of cysts and oocysts in faecal materials revealed that 14.6% of faecal samples were positive for Giardia cysts (range = 75 – 1.3x10^4 cysts/g) and 25% were positive for Cryptosporidium oocysts (range = 50 - 3.9x10^5 oocysts/g) (Table 3). There were 5 samples which had double infections of cryptosporidiosis and giardiasis (Table 4). Occurrence of double infections has also been reported in Sweden among the cattle (Bjorkman et al., 2003).

Detection of Cryptosporidium and Giardia (oo)cysts from cattle wastewater

From the cattle wastewater, 98% were positive with oocysts and 6.7% with cysts. The concentrations were between 20 - 3.1x10^3 oocysts/mL for Cryptosporidium and 4 - 75 cysts/mL for Giardia (Table 5). Given that the prevalence of Cryptosporidium and Giardia are high amongst the cattle and the positive findings of the (oo)cysts in the river water and wastewater samples, it could be deduced that there is a very high possibility of the cattle farms contaminating the river with Giardia cysts and Cryptosporidium oocysts.

Assessment of oocysts viability using vital dye assay in soil and pond environments

Viability study of Cryptosporidium oocysts in the surrounding soil and pond environments.
within the cattle farm showed that the viability of Cryptosporidium oocysts decreased with time. It was estimated that it will take 52 days for all the oocysts from both environment to be non-viable (Figure 1, 2 & 3).

**DISCUSSION**

The results from this study indicated that the rivers were highly contaminated with Cryptosporidium oocysts and Giardia cysts (range = 0.7 – 240 (oo)cysts/L). Outbreaks which occurred in Ayrshire, Swindon, Bradford in UK and Milwaukee in USA had oocysts detected in treated water at densities from less than 0.4 per litre (Smith et al., 1989; Richardson et al., 1991; MacKenzie et al., 1994; Atherton et al., 1995). A concentration of 240 oocysts/L at Sungai Langat is 600 times higher than the concentration of 0.4 oocysts/L which was known to have caused outbreaks.

These data revealed that there is a risk of a possible outbreak if people were to accidentally drink the river water while bathing, playing or swimming. Moreover, the low infectious doses of both cryptosporidiosis and giardiasis would definitely facilitate the transmission of these parasites. Faecal coliform test carried out on these river water samples showed that there was contamination of

<table>
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<tr>
<th>Parasite</th>
<th>No. of samples</th>
<th>No. of positive sample(s)</th>
<th>Percentage of positive sample(s)</th>
<th>Range of concentration of cysts/L</th>
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<td><em>Giardia</em></td>
<td>45</td>
<td>3</td>
<td>6.7</td>
<td>4 – 75</td>
</tr>
<tr>
<td><em>Cryptosporidium</em></td>
<td>45</td>
<td>44</td>
<td>98</td>
<td>20 – 3.1 x 10³</td>
</tr>
</tbody>
</table>

*Table 5. Occurrence of Giardia cysts and Cryptosporidium oocysts in cattle wastewater*

![Figure 1. Linear regression graph showing correlation (r=0.86) between percentage of viable oocysts and duration of days oocysts exposed to soil.](image-url)
faeces into the river water. However, the origin of the faeces whether they were human or animal could not be determined.

The excretion of Cryptosporidium and Giardia (oo)cysts in the cattle faeces was discovered to be between 50 to 3.9 x 10^5 (oo)cysts/g. A calf is said to be able to excrete 40 kg of faeces in a day. Therefore if the concentrations of (oo)cysts detected were to be converted to per 40 kg, it would mean that there would be 2 million to 16 billion of (oo)cysts excreted in a day by a cattle. This finding re-emphasis the high degree of contamination that can occur if
these (oo)cysts contaminates the environment. It becomes hazardous to human health when contamination happens in rivers that are used for abstraction for drinking water supplies.

There is a high probability of this event happening in this study sites because results from the wastewater of the cattle farms suggested that the wastewater do contain (oo)cysts (range of concentration = 4–3110 (oo)cysts/L). Wastewater was seen being discharged into the river signifying contamination of (oo)cysts from the cattle farms into the river system. The viability assay indicated that the oocysts can survive up to 52 days in soil and pond environment. With a long survival period, the presence of C. parvum oocysts in the river water could be a probable source of transmission to humans. This is especially important because Cryptosporidium is known to have a low infectious dose. In another survivability study conducted in Malaysia, it was shown that seeded C. parvum oocysts in the river environment in Malaysia could survive up to 3 months (Lim et al., 1999). Therefore this further confirms that oocysts can survive for about 2 to 3 months in Malaysian climate.

It is interesting to note that besides cattle in the cattle farm other animals can also be sources of Cryptosporidium and Giardia. Szostakowska et al. (2004) reported that filth flies associated with a cattle barn and a municipal landfill were tested positive by combined immunofluorescent antibody and fluorescent in situ hybridization (FISH) for C. parvum and Giardia lamblia on their exoskeletons and in their guts. It was discovered that more pathogens were carried by flies from the cattle barn than from the landfill. Besides that it was also found that 81% of C. parvum and 84% of G. lamblia pathogens were presumptively viable (Szostakowska et al. 2004).

Although this present study did not identify the species of the parasites, it is known that cattle are frequently parasitized with Giardia duodenalis (=lamblia), C. parvum and Cryptosporidium andersoni. In a study by Merle et al. (2004), they discovered that most G. duodenalis from cattle (Assemblage E) are different from those in humans (Assemblages A and B), and C. andersoni does not infect humans. However, molecular tools have shown that humans can be infected with zoonotic C. parvum, as well as anthroponotic Cryptosporidium hominis (Merle et al. 2004).

As cattle farms are also inhabited by the owners and their families, contamination of river water with Cryptosporidium oocysts and Giardia cysts by cattle farms may pose a threat to humans (e.g. children) especially if they are dependent on the river water as their source of water for their daily activities. Therefore, in order to reduce the risk of human infection from cattle faeces, it is recommended that owners of cattle farms practice good manure management practices. Practices should prevent leaching, erosion, and run-off from manure treated fields, pens, and catch basins into lakes, rivers, streams, and ditches. Direct access of cattle to watercourses should be controlled (e.g. rotational grazing, off-site watering, access ramps, fencing). Workers should practice good personnel hygiene. Drinking water for humans should be filtered and chlorinated. Only composted manure should be used as fertilizer for fresh vegetable and fruit crops. These steps should be taken seriously so as to curb contamination of parasites from cattle farms into the river.

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