Assessing the seroprevalence of Toxoplasma gondii and oocyst shedding in free ranging domestic cats and wild civets in the Lower Kinabatangan floodplain in Sabah, Borneo.

MSc One Health (Infectious Diseases) Project Report

R3451

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A Literature review of *Toxoplasma gondii* in humans, animals and the environment

and approaches to its control

Introduction and life cycle

Anthropogenic changes, causing increased interaction between humans and animals, can lead to a greater threat of parasites emerging and re-emerging and can alter the dynamics of zoonotic disease transmission (Webster et al. 2016). A prime example of this is *Toxoplasma gondii* (*T. gondii*), a protozoan parasite of animals and humans with a wide spread global distribution (Jones and Dubey 2010). The worldwide prevalence of this parasite was recently estimated at 35.8% (Wang et al. 2017) and since its first discovery in 1908 and the elucidation of its lifecycle (Hutchison 1969), it has emerged multiple times in different environments, causing disease in different species (Dubey 2008). The parasite’s complex lifecycle, wide host range and potential to cause congenital disease in its intermediate/secondary hosts makes it of high medical and veterinary concern (Tenter et al. 2000).

The global success of this parasite can be attributed to its complex and variable life cycle, involving a multitude of host species (Fig.1). The lifecycle of *T. gondii* consists of a felid definitive host and all warm blooded animals as intermediate or secondary hosts (Box 1).

Box 1 – definitions of different hosts in relation to *T. gondii* (Webster 2001; Webster et al. 2017).

**Definitive host** – host in which the parasite undergoes sexual reproduction and completes its lifecycle. For *T. gondii* this is any member of the Felidae family.

**Intermediate host** – host in which the parasite undergoes asexual reproduction and is consumed by the definitive feline host. Classic intermediate host for *T. gondii* are rodents or birds.

**Secondary host** – human or animal host in which the parasite undergoes asexual reproduction but is unlikely to be consumed by the definitive feline host. Transmission can still occur congenitally or through consumption of tissue cyst or oocysts.

**Paratenic/transport host** – host in which no parasite reproduction occurs, but can act as transporters of viable oocysts.

(Webster 2001; Webster et al. 2017)
Only the definitive felid hosts are known to actively shed the parasites into the environment through their faeces in the form of oocysts (Hutchison 1969; Jones and Dubey 2010). *T. gondii* undergoes asexual development and reproduction within the intermediate host and sexual reproduction within the definitive cat host (Tenter et al. 2000) (Fig. 1).

**Figure 1** - Lifecycle of *T. gondii* and transmission routes. Intermediate cat or bird host become infected through ingesting environmentally resistant oocysts (1). Within the intermediate host parasite stages known as tachyzoites develop into tissue cysts which contain slowly dividing bradyzoites. These cysts can develop in the central nervous system, as well as the skeletal or cardiac muscles (2). The parasite reaches its definitive host when a cat ingests an intermediate prey host containing tissue cysts (3). Once within the definitive host, the parasite
undergoes another asexual phase followed by a sexual phase occurring in the small intestine of the cat, forming unsporulated oocysts. These oocysts are then released with the faeces into the environment, where sporogony occurs, resulting in oocysts containing infective sporozoites (4). *T. gondii* can also infect humans through ingestion of contaminated water, fruit or vegetables and raw meat containing tissue cysts (6 and 7). *T. gondii* can be transmitted congenitally from pregnant mother to foetus (8) (Tenter et al. 2000). (Diagram adapted from www.CDC.gov 2015).

**Human infection and risk factors**

Secondary animal or human hosts can acquire a *T. gondii* infection through ingesting tissue cysts of infected animals or ingesting food and water contaminated with oocysts. Transmission can also occur transplacentally from mother to foetus if the mother first acquires an infection during pregnancy (Jones and Dubey 2010). There is also evidence of sexual transmission in dogs and sheep (Arantes et al. 2009; Lopes et al. 2013). It is not yet known which transmission route has the biggest epidemiological role in the global prevalence of toxoplasmosis, although consumption of raw meat is thought to have a large contribution to congenital toxoplasmosis in Europe (Cook et al. 2000). However, a protein (TgERP), specific to *T. gondii* sporozoites has been identified and shown to be an effective tool for discriminating between infections from environmental oocysts and those from tissue cysts (Hill et al. 2011). The detection of salivary antibodies against the TgERP protein demonstrated that oocyst infection plays an important role in a *T. gondii* prevalence in humans in Brazil (Mangiavacchi et al. 2016).

Conventionally, most adult-acquired infections in immunocompetent humans have been considered asymptomatic and will not manifest into clinical toxoplasmosis, however it has been shown that the parasite can cause behavioural changes in humans and animals (Webster et al. 2013; Flegr 2015). The biggest threat of toxoplasmosis is to infants that acquire a congenital infection, who can suffer from loss of vision to severe neurological disorders or
death (Hill and Dubey 2002). Ocular toxoplasmosis, which can lead to blindness, was originally thought to only result from congenital infections. However, it is now accepted that it can also occur in patients that acquired an infection postnatally (Holland 2003).

The risk factors associated with human infection with *T. gondii* have been well studied, particularly in relation to pregnant women (Table 1). Despite slight variation between countries, the common risk factors for *T. gondii* infection in pregnant women appear to be eating raw meat or vegetables, contact with cats and drinking of unclean water (Table 1). The risk of toxoplasmosis is also high in immunocompromised individuals. *Toxoplasma*-associated encephalitis became a common opportunistic infection in humans with HIV/AIDS and manifests in one third of HIV patients (Snydman et al. 2005). A recent meta-analysis calculated that globally, over 13 million HIV infected people are co-infected with *T. gondii*, the majority of these being in sub Saharan Africa (Wang et al. 2017).

**T. gondii oocysts in the environment**

To date, only members of the Felidae family are known to shed *T. gondii* oocysts in their faeces (Table 2). Within a shedding period of 1 to 3 weeks, a cat can shed millions of oocysts into the environment. Domestic cats are thought to only shed oocysts after their primary infection and retain immunity for up to 6 years (Dubey 1995; Dabritz and Conrad 2010), whereas wild cats are thought to be intermittent shedders (Kaushick et al. 2014).

*T. gondii* oocysts are extremely environmentally resistant. They have been shown to remain infective after 46 days in direct sunlight and up to 410 days in shaded areas (Yilmaz and Hopkins 1972) and have been detected on vegetables, fruits and within soil (Lass et al. 2009; Lass et al. 2012; Lalonde and Gajadhar 2016). They can also survive in salt water for up to 24 months (Lindsey and Dubey 2009) and for longer in fresh water (Fritz et al. 2012). Environmental contamination with *T. gondii* oocysts can be a source of infection in humans. The largest recorded outbreak of human toxoplasmosis occurred from a municipal water
system in Vancouver, Canada in 1995, in which several thousands of people were estimated to have been infected (Bowie et al. 1997; Aramini et al. 1999). It was later confirmed that cougars were shedding oocysts near the reservoir, contaminating the water supply (Aramini et al. 1999).

*T. gondii* oocysts have also been shown to enter marine environments through water runoff (VanWormer et al. 2016). This environmental transmission from terrestrial to marine ecosystems has increased concern for conservationists as *T. gondii* infections have caused mortalities in marine mammals such as porpoises, dolphins, walruses and sea lions (White et al. 2013; VanWormer et al. 2013; Herder et al. 2015; Roe et al. 2017). Fatal meningoencephalomyelitis in the endangered California sea otter has been attributed to consumption of marine invertebrates containing *T. gondii* oocysts (Miller et al. 2001; Miller et al. 2008). More studies are needed to determine the mechanism for oocyst transmission from terrestrial to marine environments. However, marine snails experimentally exposed to water containing *T. gondii* oocysts, concentrated and excreted the oocysts in their faeces, suggesting that marine snails can act as transport/paratenic hosts for *T. gondii* from land to sea (Krusor et al. 2015).

To date, molecular techniques have been used to isolate *T. gondii* oocyst from environmental samples (Lass et al. 2009; Yang et al. 2009); however, researchers are developing new ways to achieve this. The recent discovery that the outer walls of aged *T. gondii* oocysts bind to a lectin called wheat germ agglutinin (Harito et al. 2016), has enabled the isolation of oocysts from water samples through a technique called lectin magnetic separation, prior to detection with molecular techniques. This technique was shown to be more effective than centrifugation when samples had low concentrations of oocysts, but not when concentrations were high (Harito et al. 2017).

**Behaviour manipulation**
There is a substantial amount of evidence that suggests *T. gondii* can alter the behaviour of its intermediate/secondary host (Webster 1994; Webster et al. 1994; Berdoy et al. 2000; Kreuder et al. 2003; Jones-Brando et al. 2002; Webster et al. 2006; Flegr 2015; Sutterland et al. 2015). Rats are the main intermediate host for *T. gondii*, therefore a large proportion of this research has assessed how the parasite affects their behaviour. These studies have demonstrated that infected rats show decreased aversion to new stimuli (Webster et al. 1994) as well as higher activity levels (Webster 1994) than uninfected rats. Furthermore, rats infected with *T. gondii* were significantly less averse to cat odour than uninfected rats (Berdoy et al. 2000). This is thought to give the parasite an evolutionary advantage, increasing the likelihood of the rats being eaten by a cat, therefore aiding the parasites transmission into its definitive host where it can reproduce (Wester et al. 1994; Webster 1994). Similarly, *T. gondii* has been shown alter the behaviour of sea otters, leading to increased risk of fatal shark attacks (Kreuder et al. 2003).

*T. gondii* has also been shown to subtly alter the behaviour of humans with latent infections, in similar ways to rats. Infected humans are more likely to be involved in traffic accidents and have slower reaction times than uninfected individuals (Havlicek et al. 2001; Flegr et al. 2002; Flegr et al. 2015). *T. gondii* has recently been linked to more severe behaviour changes such as schizophrenia in adults (Webster et al. 2013; Sutterland et al. 2015). Studies show that anti-psychotic drugs can act as anti-*T. gondii* drugs, preventing behaviour change in the intermediate rat host (Webster et al. 2006). Anti-psychotic drugs and mood stabilizers used to treat schizophrenia and bipolar disorder were shown to inhibit *T. gondii* in human fibroblasts (Jones-Brando et al. 2002). A recent meta-analysis showed a significant correlation with antibodies to *T. gondii* and schizophrenia, bipolar disorder, obsessive compulsive disorder and addiction (Sutterland et al. 2015).

**Diagnostics and Control of Toxoplasmosis**
Active *T. gondii* infections in cats can be diagnosed by looking for oocysts in the cat’s faeces, for which there are a number of methods. One method is to use faecal flotations and microscopy (Barutzki and Schaper, 2011); however, the oocysts of *T. gondii* are difficult to distinguish from other coccidian parasites. Therefore, additional methods such as PCR or bioassays in mice are required to confirm a *T. gondii* infection (Dubey et al. 2006; Lilly and Worthum 2013). The most widely used method to detect exposure to *T. gondii* is to test for antibodies against the parasite in the blood using serological tests. A number of studies have assessed the seroprevalence of *T. gondii* in domestic and wild cats around the world (Table 3).

Control of toxoplasmosis is challenging due to the parasite’s wide host range and the multiple routes of transmission. The only commercially available vaccine is a live ovine vaccine of the incomplete S48 strain, used to prevent abortions in sheep (Buxton et al. 1991, 1993; Buxton and Innes 1995). Another live vaccine of the mutant strain, T-263, has been developed, which interrupts the development of oocysts in cats (Frenkel et al. 1991). Field trials of this vaccine, during which cats were vaccinated on a US pig farm, showed that seroprevalence of *T. gondii* in pigs significantly decreased post vaccination. This was attributed to a reduced number of oocysts in the environment (Mateus-Pinilla et al. 1999; Elmore et al. 2010). However, as these vaccines are live strains of the parasite and target oocyst development, they would not be suitable for human use (Verma and Khanna, 2013). There has been recent research into the development of recombinant and DNA vaccines; (Tao et al. 2013; Xu et al. 2014; Ahmadpour et al. 2017; Zulpo et al. 2017) however, many of these are still in early stages of development and have only been tested on mice. One recombinant vaccine, rROP2 was trialled on cats through a nasal spray, which resulted in immunised cats shedding 86% less oocysts than control cats. However, due to small sample size, no significant association could be made (Zulpo et al. 2017). There is still a need to design an appropriate adjuvant for these DNA and recombinant vaccines (Ahmadpour et al. 2017; Zulpo et al. 2017).
With no current cat or human vaccine commercially available, the complex ecology and biology of this parasite calls for a new way of approaching the control of toxoplasmosis. The One Health approach, which advocates for an integrated and transdisciplinary approach to disease control at the human-animal-environment interface, has been suggested to tackle this disease (VanWormer et al. 2013; Webster et al. 2016). Webster et al. (2016) suggest a One Health framework for controlling Neglected Zoonotic Diseases (NZDs) including toxoplasmosis. They highlight the importance of understanding the transmission ecology and ongoing evolution across the parasite’s entire host range and the need for novel molecular tools and mathematical models to aid this understanding. A One Health approach involving collaboration and communication across disciplines could help to fill such knowledge gaps for \textit{T. gondii} which could then be incorporated into toxoplasmosis disease control programs.

**Word count**: 2000 (including in text citations)
Table 1 – Risk factors for toxoplasmosis in pregnant women in different countries

<table>
<thead>
<tr>
<th>Country</th>
<th>Study type</th>
<th>Sample size</th>
<th>Risk factors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>Cross sectional</td>
<td>389</td>
<td>Increased age, drinking of municipal or uncontrolled water supplies</td>
<td>Ertug et al. 2005</td>
</tr>
<tr>
<td>Norway</td>
<td>Case control</td>
<td>191</td>
<td>Eating undercooked or raw meat, cleaning cat litter boxes, eating raw or uncontrolled fruit and vegetables, infrequent washing of knives</td>
<td>Kapperud et al. 1996</td>
</tr>
<tr>
<td>France</td>
<td>Case control</td>
<td>160</td>
<td>Poor hand hygiene, consumption of undercooked beef and lamb, consumption of raw vegetable, having a pet cat</td>
<td>Baril et al. 1999</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>Cross sectional</td>
<td>487</td>
<td>History of intake of immunosuppressant drugs</td>
<td>Almushait et al. 2014</td>
</tr>
<tr>
<td>Thailand</td>
<td>Case control</td>
<td>640</td>
<td>Increased age, living with cats, parity, drinking unclean water</td>
<td>Nissapatorn et al. 2011</td>
</tr>
<tr>
<td>England</td>
<td>Cross sectional</td>
<td>1897</td>
<td>Rural childhood home, feeding a dog raw meat, increased age</td>
<td>Nash et al. 2005</td>
</tr>
<tr>
<td>Naples</td>
<td>Case control</td>
<td>3518</td>
<td>Eating cured pork, eating raw meat, gardening</td>
<td>Buffolano et al. 1996</td>
</tr>
</tbody>
</table>
Table 2 - Different species of the Felidae family that have been shown to shed *T. gondii* oocysts in their faeces.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Latin name</th>
<th>Country</th>
<th>Sample size (n)</th>
<th>Percent of shedding</th>
<th>Identification method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stray and domestic cats</td>
<td><em>Felis catus</em></td>
<td>USA</td>
<td>49</td>
<td>6%</td>
<td>PCR</td>
<td>Lilly and Worthum 2013</td>
</tr>
<tr>
<td>Wild cat</td>
<td><em>Felis silvestrus</em></td>
<td>Czech Republic</td>
<td>175</td>
<td>8%</td>
<td>Faecal flotation</td>
<td>Leukesora and Literak 1998</td>
</tr>
<tr>
<td>Amur leopard cat</td>
<td><em>Felis euptilurus</em></td>
<td>Czech Republic</td>
<td>133</td>
<td>4%</td>
<td>Faecal flotation</td>
<td>Leukesora and Literak 1998</td>
</tr>
<tr>
<td>Geoffroy’s cat</td>
<td><em>Oncifelis geoffroyi</em></td>
<td>Czech Republic</td>
<td>1</td>
<td>100%</td>
<td>Faecal flotation</td>
<td>Leukesora and Literak 1998</td>
</tr>
<tr>
<td>Cheetah</td>
<td><em>Acinonyx jubatus</em></td>
<td>USA</td>
<td>2</td>
<td>100%</td>
<td>Mouse bioassay</td>
<td>Marchiondo et al. 1976</td>
</tr>
<tr>
<td>Bobcats</td>
<td><em>Lynx rufus</em></td>
<td>USA</td>
<td>9</td>
<td>56%</td>
<td>Mouse bioassay</td>
<td>Marchiondo et al. 1976</td>
</tr>
<tr>
<td>Leopards</td>
<td><em>Panthera pardus</em></td>
<td>Thailand</td>
<td>54</td>
<td>1.9%</td>
<td>Faecal flotation</td>
<td>Patton and Rabinowitz 1994</td>
</tr>
<tr>
<td>Jaguar</td>
<td><em>Panthera onca</em></td>
<td>Belize</td>
<td>25</td>
<td>4%</td>
<td>Faecal flotation</td>
<td>Patton et al. 1986</td>
</tr>
<tr>
<td>Ocelot</td>
<td><em>Felix pardalis</em></td>
<td>Belize</td>
<td>8</td>
<td>25%</td>
<td>Faecal flotation</td>
<td>Patton et al. 1986</td>
</tr>
<tr>
<td>Cougar</td>
<td><em>Felis concolor vancouverensis</em></td>
<td>Canada</td>
<td>13</td>
<td>15%</td>
<td>Mouse bioassay</td>
<td>Aramini et al. 1998</td>
</tr>
<tr>
<td>Country</td>
<td>Cat type</td>
<td>Latin name</td>
<td>Sample no.</td>
<td>Test used</td>
<td>Seroprevalence</td>
<td>Reference</td>
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<tr>
<td>--------------------------</td>
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</tr>
<tr>
<td>Thailand</td>
<td>Stray cats</td>
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<td>592</td>
<td>Latex agglutination test</td>
<td>11%</td>
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<tr>
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<td>Domestic cats</td>
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<td>69</td>
<td>methylene blue dye test</td>
<td>41%</td>
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<td>14.5%</td>
<td>Chandrawathani et al. 2008</td>
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<td>Latex agglutination test</td>
<td>18.3%</td>
<td>Lewida and Cabanacan-Salibay 2010</td>
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<td>Philippines</td>
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<td>28.3%</td>
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<td>China</td>
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<td>145</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>Wang et al. 2012</td>
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<td>5.93%</td>
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<td>Qatar</td>
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<td>Sample Size</td>
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<td>-------------</td>
<td>----------------------------------</td>
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</tr>
<tr>
<td>USA</td>
<td>Bobcats</td>
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<td><em>Lynx pardinus</em></td>
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<td>81.5%</td>
<td>Sobrino et al. 2007</td>
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<td>Spain</td>
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<td>92%</td>
<td>Penzhom et al. 2002</td>
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<td><em>Panthera pardus</em></td>
<td>7</td>
<td>Indirect fluorescence antibody test</td>
<td>86%</td>
<td>Penzhom et al. 2002</td>
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Assessing the seroprevalence of *Toxoplasma gondii* and oocyst shedding in free ranging domestic cats and wild civets in the Lower Kinabatangan floodplain in Sabah, Borneo.

Abstract

*Toxoplasma gondii* (*T. gondii*), the causative agent of toxoplasmosis, is a pathogen of major clinical and epidemiological importance to both humans and animals globally. It is therefore imperative to study this parasite at the human-animal-environment interface. This study aimed to determine the presence of *T. gondii* in the Lower Kinabatangan floodplain in Sabah, Malaysian Borneo, where forest fragmentation has led to increased interaction between humans, domestic and wild animals. It aimed to assess seroprevalence in free ranging domestic cats (*Felis catus*) and wild Malay civets (*Viverra tangalunga*) and test the novel hypothesis that civets can, like cats, shed oocysts in their faeces. Seroprevalence was assessed using ELISAs and ILATs and oocyst shedding was determined using faecal flotations/microscopy. A total of 26 cats and 12 civets were sampled. ILATs showed that eight of the 15 village cats were seropositive with titres ≥ 65 (True prevalence = 53.9%) and seven of the 11 plantation cats were seropositive (True prevalence = 65.6%). Of the 12 Malay civets sampled, none were seropositive with titres ≥ 65; however, seven had titres between 16 and 32 suggesting potential previous exposure to the parasite. ILAT and ELISA tests had a high level of agreement (Kappa = 0.8). *T. gondii*-like oocysts were found in two cat scat samples and two civet scat samples; however, molecular analysis is needed to confirm this. This was, to our knowledge, the first study to demonstrate that *T. gondii* is present in Malaysian Borneo, with seroprevalence in domestic cats higher than in Indonesian Borneo. This could pose a threat to both humans and wildlife inhabiting the lower Kinabatangan floodplain.
Introduction

Increased interaction between humans and animals can alter the dynamics of zoonotic disease transmission (Webster et al. 2016). A prime example of this is *Toxoplasma gondii* (*T. gondii*), a protozoan parasite of animals and humans with a widespread global distribution that is shed in the faeces of its definitive felid host (Hutchison 1969; Jones and Dubey 2010). The parasite’s complex lifecycle (Fig. 1), wide host range and potential to cause disease, especially congenital toxoplasmosis, in its intermediate/secondary hosts makes it of high medical and veterinary concern (Tenter et al. 2000). It is therefore imperative to study this parasite at the human-animal-environment interface.

Such an interface is evident in the Lower Kinabatangan floodplain in Sabah, Borneo, which, consists of fragmented secondary rainforest interspersed with oil palm plantations (Davison 2006). This has created an environment which may facilitate the increased interaction between humans, domestic animals and wildlife. Whilst a number of studies have assessed the seroprevalence of *T. gondii* in cats in South East Asia (Table 4), very few studies have assessed this in Borneo. *T. gondii* antibodies have been found in domestic cats, humans and goats in Kalimantan (Indonesian Borneo) with seroprevalence of 41%, 9.7 – 51% and 61% respectively (Durfee and Cross 1976). However, there have been no studies on *T. gondii* exposure in humans or animals in Malaysian Borneo. Furthermore, no studies have looked at *T. gondii* in wildlife in Borneo; although *T.gondii* antibodies were detected in captive Malayan civets and Asian palm civets (*Paradoxorus hermaphroditus*) in the Philippines (Oronan et al. 2014).

A number of civet species inhabit the Lower Kinabatangan floodplains and are known to spend time in both forests and oil palm plantations (Yue et al. 2015). Although civets have been classified into the family Viverridae, they originate from the same sub-order as cats, the Feliformia. To date, only members of the Felidae family are known to shed *T. gondii* oocysts in their faeces (Table 2); however, no study has assessed whether civets are also capable of this. The phylogenetic relationships of the Viverridae have been contested on multiple
occasions and the Asian linsang (*Prionodon linsang*), originally part of the Viverridae, was found to be the closest relative of the Felidae (Gaubert and Vernon 2003). The relative position of Viverridae has still not been confidently established (Eizirik and Murphy 2009). This gives rise to a novel hypothesis that civets are capable of shedding *T. gondii* oocysts in their faeces.

This study aimed to determine the presence of *T. gondii* in the Lower Kinabatangan floodplain and assess the seroprevalence in free ranging domestic cats and wild civets. Furthermore, it aimed to determine the level of oocyst shedding in cats and test the novel hypothesis that civets are capable of shedding oocysts in their faeces. This research is important as toxoplasmosis poses a threat to human, livestock and wild animal health and the findings of this study may help elucidate the potential threat of this disease to humans and animals in the Lower Kinabatangan floodplains.
<table>
<thead>
<tr>
<th>Country</th>
<th>Cat type</th>
<th>Latin name</th>
<th>Sample no.</th>
<th>Test used</th>
<th>Seroprevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand</td>
<td>Stray cats</td>
<td><em>Felis catus</em></td>
<td>315</td>
<td>Sabin-Feldman dye test</td>
<td>7.3%</td>
<td>Sukthana et al. 2003</td>
</tr>
<tr>
<td>Thailand</td>
<td>Stray cats</td>
<td><em>Felis catus</em></td>
<td>592</td>
<td>Latex agglutination test</td>
<td>11%</td>
<td>Jittapalapon g et al. 2007</td>
</tr>
<tr>
<td>Indonesian Borneo</td>
<td>Domestic cats</td>
<td><em>Felis catus</em></td>
<td>69</td>
<td>methylene blue dye test</td>
<td>41%</td>
<td>Durfee and Cross, 1976</td>
</tr>
<tr>
<td>Peninsular Malaysia</td>
<td>Domestic cats</td>
<td><em>Felis catus</em></td>
<td>55</td>
<td>Indirect Fluorescence antibody test</td>
<td>14.5%</td>
<td>Chandrawat hani et al. 2008</td>
</tr>
<tr>
<td>Philippines</td>
<td>Stray and domestic cats</td>
<td><em>Felis catus</em></td>
<td>60</td>
<td>Latex agglutination test</td>
<td>46.67%</td>
<td>Lewida and Cabanacan-Salibay 2010</td>
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<tr>
<td>Thailand</td>
<td>Domestic cats</td>
<td><em>Felis catus</em></td>
<td>348</td>
<td>Modified agglutination test</td>
<td>10.1%</td>
<td>Sukhumavasi et al. 2012</td>
</tr>
<tr>
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<td>Domestic cats</td>
<td><em>Felis catus</em></td>
<td>86</td>
<td>Indirect latex agglutination test</td>
<td>3.48%</td>
<td>Buddhirong awatr et al. 2016</td>
</tr>
</tbody>
</table>
Methods

Study site:

The study was conducted in the Lower Kinabatangan floodplain in Eastern Sabah, Borneo, between the months of May and July 2017. The Lower Kinabatangan floodplain is a rainforest wetland, comprising fragmented secondary forests within a matrix of oil palm plantations. The plantations and villages in the Lower Kinabatangan floodplain are not only home to humans, but also host populations of domestic cats and dogs (Chua et al. 2013) and are used by wild animals such as civets and leopard cats (*Prionailurus bengalensis*) for hunting (Ross et al. 2015; Yue et al. 2015).

Sample collection:

A total of 26 free ranging domestic cats (*Felis catus*) were convenience-sampled in the Lower Kinabatangan floodplain with permission from the cat owners. Of these, 15 cats were from a village called Batu Puteh and 11 were from HillCo plantation (Fig. 2). Cats were restrained in a towel and a maximum of 1ml of blood was drawn by a veterinarian from the femoral vein using a 1cc syringe and a 25cc needle and aliquoted into an EDTA vacutainer. A faecal sample was collected using a rectal swab and stored in a cryovial containing 95% ethanol for future molecular analysis. The cats were then released at point of capture and/or directly to their owners, as appropriate.

Malay civets were trapped and sampled as described by Evans et al. (2016a). Once caught, civets were anesthetised by a licenced veterinarian. For each animal, 2ml of blood was taken from the cephalic or femoral veins and faeces was collected using a rectal swab. After full recovery, civets were likewise released at point of capture. Blood samples from cats and civets were taken back to the laboratory and were centrifuged (model: EBA 21, Hettich Zentrifugen) at 1000rpm for 15 minutes the same day to separate plasma/serum from the blood. 200µl of serum/plasma was transferred into an epindorph and stored at -20°C.
Cat and civet scat was collected between the months of June and July 2017 using walking transects (Fig. 2). Two transects were walked every morning between 6:00am and 9:00am. Cat or civet scat was identified according to Halfpenny (1986) and Chakravarthy and Ratnam (2015) (Fig. 3), collected in a sterile container and the location was marked using a GPS (Garmin).

**Figure 2**- Map of the Lower Kinabatangan Floodplains in Eastern Sabah, Borneo, showing ten walking transects in which cat and civet scat was collected. Transects were 500m separated by approximately 900m intervals between the plantation and village in which the free ranging domestic cats were sampled. Map created using the geographical information system QGIS 2.18.
Figure 3 – Photographs of civet (a) and cat (b) scat found on walking transects in the Lower Kinabatangan floodplains. Civet scat (a) is cylindrical with rounded ends, containing seeds and little hair with no strong odour (Chakravarthy and Ratnam 2015). Cat scat (b) lies in broken cords with pointed ends and contains hair and bones with a strong odour (Halfpenny 1986).

Laboratory analyses:

Enzyme linked immunosorbent assays (ELISAs) were conducted using the Immunocomb® ELISA Feline *Toxoplasma* and *Chlamydophila* IgG antibody test kit (Biogal, Kibbutz Galed, Israel) (specificity 100%, sensitivity 92.3%). This test was conducted according to the manufacturer’s protocol. Briefly, for each sample, 5µl of plasma/serum was pipetted into the first well of the kit. A test strip containing the positive control and the *T. gondii* antigen was then placed into this well for ten minutes. The strip was then moved across the various wells according to the manufacturer’s protocol. A positive result was determined by a colour change on the test strip that was equal to or darker than that of the positive control.
Indirect Latex Agglutination Tests (ILATs) were conducted using Toxoreagent IgG/IgM kit RST7001 (Mast Group Ltd. Liverpool, UK) (specificity 94.1%, sensitivity 93.8%) according to the manufacturer’s protocol. Briefly, 50µl of serum/plasma was diluted with 350µl buffer mix to make a 1:8 dilution. For each sample, 25µl of buffer was pipetted into 8 wells of a 96 well microplate. 25µl of the 1:8 diluted serum was pipetted into the first well and then serially diluted from 1:16 in well 1 to 1:2048 in well 8. Then 25µl of latex was added to each well and the plate was incubated at room temperature for at least 12 hours. The same process was carried out for the positive control. Agglutination that evenly filled the well was considered a positive result, whilst a small dot in the middle of the well was considered a negative result. The cut off point for seropositivity was determined as titres ≥65, specified in the manufacture’s protocol for felines as indicative of clinical infection with *T. gondii*.

Faecal flotations of civet and cat scat were carried out on the same day as collection, as described by Hendrix and Robinson (2006). Briefly, for each sample, two grams of faeces was mixed with 5ml of distilled water. This solution was poured through gauze into a 15ml centrifuge tube and centrifuged at 1500 rpm for three minutes. The supernatant containing fats was discarded. The pellet was re-suspended with 14ml of saturated salt solution (Sg= 1.2) and centrifuged at 1500 rpm for five minutes. The surface film was picked up using a wire loop and transferred to a glass microscope slide. A cover slip was placed on top of the slide and the whole slide was examined for *T. gondii* oocysts under a compound microscope at a magnification of 400x. Any oocysts detected were photographed and measured using image processing software Scopelimage 9.0. *T. gondii* oocysts measure approximately 10 by 12µm in size (Hendrix and Robinson 2006). The remaining scat samples were stored in 95% ethanol for future PCR.

*Data analysis*

Apparent seroprevalence was calculated as the proportion of positive individuals in the sample (Pfeiffer 2010). True prevalence, which accounts for the specificity and sensitivity of the serological test, was calculated with the following equation:
True prevalence = apparent prev. + (spec-1)/sens + (spec-1).

Confidence intervals for true prevalence were calculated using EpiTools according to Reiczigel et al. (2010).

Agreement of the ELISA and ILAT was calculated using the kappa statistic. This calculates the level of agreement that is not expected by chance and ranges from 0 to 1 with 0.4 - 0.6 showing moderate agreement (Pfeiffer 2010). The following calculation was used:

Kappa = observed agreement – expected agreement/1-expected agreement.

Ethical approval

All samples were collected by a licensed veterinarian with approval from the Sabah Biodiversity Centre (access licence Ref. no: JKM/MBS.1000-2/2 JLD.5 (102)) and with ethical approval from the Clinical Research and Ethical Review Board at the Royal Veterinary College (approval no: URN 2017 1699-3).

Results:

A total of 26 free ranging domestic cats were sampled in this study. Of these, 15 were sampled from the village Batu Puteh and 11 were sampled from the HillCo oil palm plantation. A total of 12 Malay civets were sampled in this study.

ILATs showed that of the 15 cats sampled from Batu Puteh, eight were seropositive for T. gondii antibodies with titres ≥ 65. From this, the apparent prevalence for Batu Puteh was calculated as 53.3% and the true prevalence was 53.9% (95% CI [27.5, 78.8]). Of the 11 cats sampled at Hillco plantation, seven were seropositive for T. gondii antibodies with titres ≥ 65. From this the apparent prevalence for Hillco plantation was calculated as 63.6% and the true prevalence was 65.6% (95% CI [33.5, 89.8]) (Fig. 4). The ILAT suggested that no civets had antibody titres ≥ 65. However, seven civets had antibody titres between 16 and 32 (Table 5).

ELISA tests showed that of the 15 cats sampled from Batu Puteh, 11 were positive for T. gondii antibodies. From this the apparent prevalence for Batu Puteh was calculated as 73.3%
and the true prevalence was 79.45% (95% CI [52.1, 96.5]). Of the 11 cats sampled at HillCo plantation, seven were positive for *T. gondii* antibodies. From this the apparent prevalence was calculated as 63.6% and the true prevalence was 68.9% (95% CI [38.3, 91.9]) (Fig. 4). According to the ELISA, no civets were seropositive for *T. gondii*, however two samples were inconclusive. The Cohen’s kappa statistic for the agreement of the two serological tests used in this study was $\kappa = 0.8$, suggesting that the ELISA and ILAT had good agreement (Pfeiffer, 2009); however, the ELISA diagnosed more positives than the ILAT (Table 6).

**Table 5** – Results of ELISA and ILAT for free ranging domestic cats and wild civets in the Lower Kinabatangan floodplain.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample no</th>
<th>Elisa result</th>
<th>ILAT result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>Domestic cat</td>
<td>26</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Malay civet</td>
<td>12</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 6 - Contingency table showing the agreement between the two serological tests (ILAT and ELISA) used in this study for all 38 serum/plasma samples.

<table>
<thead>
<tr>
<th>Test 1 (ELISA)</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>15</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>23</td>
<td>38</td>
</tr>
</tbody>
</table>

Test 2 (ILAT)

Figure 4 – True seroprevalence adjusted for ILAT and ELISA for *T. gondii* in free ranging domestic cats sampled in the plantation and village and wild civets. Showing upper and lower 95% confidence intervals.
A total of 38 scat samples were collected from the walking transects. Of these, 19 were identified as civet scat and 19 were identified as cat scat. A further three more faecal samples were collected from civets that defecated in the traps during sampling. All 41 faecal samples were analysed microscopically for *T. gondii* oocysts. Of these 41 faecal samples, only four were found to contain *T. gondii*-like oocysts, of which two were cat scat and two were civet (Fig. 5).
Discussion

Toxoplasma gondii is an important pathogen of humans and animals and it is therefore vital to study this parasite at the human, animal environment interface. This study demonstrated T. gondii exposure and potential shedding of oocysts in free ranging domestic cats and Malay civets in the Lower Kinabatangan floodplain in Sabah, Borneo.

According to ILATs, true seroprevalence for T. gondii in cats sampled in the village and the plantation was 53.9% and 65.6% respectively. These are both higher than a previous study in Kalimantan which found a seroprevalence of 41% in cats (Durfee and Cross 1975). A recent study in Thailand, also using ILATs, showed a seroprevalence ranging from 0% to 29% in cats in different provinces (titres ≥64) (Buddhirongawatr et al. 2016), again lower than in the current study. The ELISA and the ILAT had a kappa statistic of 0.8 which shows good agreement that is not due to chance. However, the ELISA diagnosed more positives than the ILAT (Table 6) and showed higher seroprevalence than the ILAT for the cats in the village of Batu Puteh (Fig. 4). This could be attributed to the subjectivity of determining the ELISA test results. The results were determined by colour intensity which may be affected by factors such as light intensity in the laboratory. Therefore the ILAT results, which are more objective and quantitative are likely to be more reliable.

Of the 12 Malay civets sampled, none were seropositive at a titre of ≥ 64, the cut off point for this study. However, seven individuals had antibody titres between 16 and 32, suggesting potential previous exposure to the parasite. A previous study in the Philippines that used the same ELISA test kit, showed that five out of 12 Malay civets had antibodies against T. gondii, all with titres between 16 and 32 (Oronan et al. 2014). The current study suggests that the civets in the Lower Kinabatangan floodplains have more exposure to T. gondii than those in
the Philippines. The low antibody titres in the civets compared to the cats could suggest that there is less exposure to the parasite in the forest compared to the plantation and villages. Malay civets are known to enter plantations to hunt (Yue et al. 2015) and also eat rats which are the main intermediate host so they may become exposed to the parasite during this time.

Of the 41 cat and civet scat samples, four contained *T. gondii* like oocysts. Of these, two were from cat scat and two were from civet scat. However, molecular analysis is needed to confirm this as a number of other coccidian parasites such as *Isospora* have morphologically similar oocysts (Dubey 2004). The low number of scat samples containing oocysts is not unexpected as domestic cats will only shed oocysts for a short period of time during their first infection (Dabritz and Conrad 2010). Dabritz et al. (2007) found that only three out of 326 cat faecal samples in California contained *T. gondii* oocysts. Civet and cat scat were distinctive in shape, contents and smell and therefore confident identifications could be made; however, molecular analysis is also needed to confirm this. If future PCR confirms that the civets were shedding oocysts in their faeces, then a number of other wildlife species could be at risk of infection and the environmental oocyst burden could be greater than previously thought.

The seropositive cats in this study are likely to have shed large quantities of oocysts into the environment after their first infection. This environmental contamination could pose a threat to the people living in these areas, especially pregnant women, who can gain an infection from contaminated soil and water. Livestock such as goats and chickens were observed in the village of Batu Puteh. This could be another route of human transmission if the livestock becomes infected and are then consumed. Further research is needed to assess the level of environmental contamination as well as the seroprevalence in livestock. Furthermore, semi-structured interviews are needed to determine the risk factors for people living in the plantations and villages.

Environmental contamination with *T. gondii* oocysts could also be a threat to wildlife inhabiting the Kinabatangan River. Previous studies have shown that *T. gondii* oocysts can enter rivers from land through water runoff (VanWormer et al. 2017), as the Kinabatangan river runs...
through the village of Batu Puteh it is likely that in heavy storms, oocysts could be transmitted from the soil to the river. The Kinabatangan River is home to a number of otter species including the smooth coated otter (*Lutrogale perspicillata*) and the Asian small clawed otter (*Amblonyx cinereus*) (Evans et al. 2016b), both listed as Vulnerable by IUCN. *T. gondii* is known to cause fatal encephalitis in the endangered Californian sea otter (*Enhydra lutris nereis*) (Miller at al. 2001). Therefore otter species in the Kinabatangan may be at risk of fatal infection with *T. gondii*. However, the severity of the infection in Californian sea otter could be due to the strain of the parasite, which could me more virulent than the strain in Borneo. It is therefore important to determine the strain of this parasite through genotyping to assess whether there is a threat to the otters in the Kinabatangan river.

From the findings of this study it can be concluded that the parasite *T. gondii* is present in the Lower Kinabatangan floodplain, Sabah, Malaysian Borneo, with seroprevalence in free ranging domestic cats higher than in Indonesian Borneo. This may pose a threat to both humans and wildlife within the Lower Kinabatangan floodplain. It shows *T. gondii* exposure in wild civets and potential shedding of *T. gondii*-like oocysts in both cat and civet faeces; however, molecular analysis is needed to confirm this. This study assessed just one aspect of the disease dynamics of *T. gondii*; however, in order to understand risks to human and wildlife health in Borneo, more research is needed into the ecology, population genetics and transmission dynamics of *T. gondii* in at the human, animal, environment interface.

**Word count:** 3113 (including in text citations)
Acknowledgements

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References


binturong whitei) and Asian palm civets (Paradoxurus hermaphroditus) at a wildlife facility in Quezon city, phils. " Philippine Journal of Veterinary and Animal Sciences 39(2).


Appendices:

Appendix 1 – Raw data from ILAT and ELISA tests for antibodies against *Toxoplasma gondii* in free ranging domestic cats and wild civets in the Lower Kinabatangan floodplains in Sabah, Borneo.

<table>
<thead>
<tr>
<th>ID</th>
<th>Species</th>
<th>Group</th>
<th>Sample type</th>
<th>Sex</th>
<th>Age</th>
<th>Toxo ELISA titre</th>
<th>Toxo ELISA result</th>
<th>Toxo ELISA titre</th>
<th>Toxo ELISA result</th>
<th>ILAT titre</th>
<th>ILAT result</th>
</tr>
</thead>
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<td>MCF32</td>
<td><em>Viverra tangalunga</em></td>
<td>civet</td>
<td>plasma</td>
<td>female</td>
<td>adult</td>
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<td>negative</td>
<td>32</td>
<td>borderline</td>
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<td>plasma</td>
<td>male</td>
<td>adult</td>
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<td>negative</td>
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<td>negative</td>
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<td>serum</td>
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<td>adult</td>
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<td>inconclusive</td>
<td>32</td>
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<td>serum</td>
<td>male</td>
<td>adult</td>
<td>S0</td>
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<td>BP-b.1</td>
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<td>female</td>
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<td>Age</td>
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As part of our assessment procedure for student projects we are asking you to complete the following short questionnaire. Please tick the most appropriate statements in each section. Please include this page in the body of your Research Project submission.
(Please ensure you tick the correct box)

Type of project
- Original research
- Risk assessment
- Data analysis
- Other: please describe:

Design of project
- Supervisor
- Student
- Designed jointly

Amount of supervision
Discussions between supervisor and student were:
- Regular - at least once a week
- Frequent - not weekly, but at least once/twice a month
- Rare - less often than once a month
- Never

Supervision of project work (Tick all boxes that apply)
- Supervisor advised on the project objectives and analysis plan
- Supervisor advised on literature review
- Supervisor oversaw the risk assessment / data analysis and checked the results
- Supervisor left student entirely alone
- Supervisor delegated supervision

Supervision of written report (Tick all boxes that apply)
- Supervisor advised on the design of the project report
- Supervisor read and advised on one or more drafts of the literature review
- Supervisor read and advised on one or more drafts of the risk assessment / data analysis section
- Supervisor read and corrected the final draft of the complete report prior to submission
- Supervisor has not read the final report prior to submission
- Supervisor has / will* not give advice on the written report (*delete as appropriate)
Declaration

Number of words: 5113 (including in text citations)

a) I have performed all the experiments and/or analyses described in this project.

   YES [✓]
   NO [ ]

If no, please list the procedures/analyses etc described in the project that were performed by research staff in the host laboratory:

b) My supervisor has seen and commented on my project report.

   YES [✓]
   NO [ ]