UNIVERSITI PUTRA MALAYSIA

# MOLECULAR DETECTION OF POTENTIALLY ZOONOTIC ENTERIC PROTOZOA INFECTING SYNANTHROPIC RODENTS IN SELANGOR AND KUALA LUMPUR, MALAYSIA

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A project paper submitted to the
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In partial fulfilment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
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**CERTIFICATION**

It is hereby certified that we have read this project paper entitled "Molecular Detection of Potentially Zoonotic Enteric Protozoa Infecting Synanthropic Rodents in Selangor and Kuala Lumpur, Malaysia.", by Irdina Binti Zainol and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999- Final Year Project.

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__________________________________

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**DEDICATION**

*To my beloved husband,*
*Shazwan Johari*

*To my lovely parents,*
*Zainol Madrop*
*&*
*Hawayah Syed Abd Rahim*

*To my supportive siblings,*
*Khaliesah Zainol,*
*Muhammad Nabil Zainol &*
*Muhammad Thaqif Zainol*

*For all the love and prayers.*

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Last but not least, a debt gratitude is also owed to my family and friends, without their never ending support and prayers, none of these would indeed be possible.


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# LIST OF ABBREVIATIONS

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| | |
|---|---|
| % | Percent |
| µL | Microliter |
| nm | Nanometer |
| ºC | Degree Celcius |
| µm | Micrometer |
| DNA | Deoxyribonucleic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| gDNA | Genomic Deoxyribonucleic Acid |
| IFA | Immunofluorescence assay |
| PCR | Polymerase Chain Reaction |

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## ABSTRAK

Tikus sinantropik adalah spesis yang hidup berdekatan dengan manusia dan berfungsi sebagai hos untuk pelbagai patogen. Mereka memainkan peranan penting dalam penularan penyakit kepada manusia dan spesis haiwan lain. Salah satu kebimbangan yang mempengaruhi kesihatan awam di seluruh dunia adalah penyakit parasit zoonotik. Sistem penghantaran parasit membenarkan interaksi antara manusia, haiwan, vektor, dan persekitaran. Bagi banyak spesis mikroba patogen, tikus sinantropik juga dikenali sebagai takungan penting dan sumber perantaraan. Kajian ini dilakukan untuk menentukan kelaziman molekul protozoa enterik bawaan tikus terpilih yang mempunyai kepentingan zoonotik, iaitu *Cryptosporidium, Blastocystis dan Giardia* di pelbagai kawasan domestik peri di Selangor dan Kuala Lumpur, Malaysia. Sampel najis dikumpulkan dari 31 tikus di Selangor dan Kuala Lumpur, Malaysia. Sampel diambil dari tikus dari kedua-dua jantina dan dari pelbagai spesis yang mendiami kawasan perumahan, kawasan komersial ( pasar basah, dan Kawasan kedai ) serta taman, dan pinggiran hutan. DNA genom diekstrak dari sampel najis dan reaksi berantai polimerase ( PCR ) dilakukan untuk memperkuat serpihan separa dari gen yang berbeza menggunakan

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primer khusus genus untuk menentukan kehadiran molekul *Giardia, Blastocystis, dan Cryptosporidium.* Prevalensi molekul tertinggi ( 71% ) diperhatikan untuk Cryptosporidium, diikuti oleh Giardia ( 16.1% ) dan Blastocystis ( 9.7% ). Ujian statistik menggunakan Fisher's Exact Test tidak mendedahkan ( $p > 0.05$ ) yang signifikan untuk hubungan risiko jantina, spesies atau habitat. Kajian ini telah menunjukkan prevalensi tinggi protozoa enterik zoonotik di antara tikus yang diperiksa. Ini selanjutnya menyumbang kepada pemahaman tentang kepelbagaian parasit yang terdapat pada tikus sinantropik, dengan implikasi untuk penularan zoonotik dan kesihatan awam.

**Kata kunci**: tikus sinantropik; *Cryptosporidium; Blastocystis; Giardia*; zoonotik

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## ABSTRACT

Synanthropic rodents are species that live in proximity to humans and serve as reservoirs host for various pathogens. They play an important role in disease transmission to humans and other animal species. One of the concerns affecting worldwide public health is zoonotic parasite diseases. The intricate parasite transmission system permits interactions among humans, animals, vectors, and the environment. For many pathogenic microbial species, synanthropic rodents are also known to serve as both a significant reservoir and an intermediate host. This study was conducted to determine the molecular prevalence of selected rodent-borne enteric protozoa of zoonotic importance, namely *Cryptosporidium, Blastocystis* and *Giardia* in various peri domestic areas in Selangor and Kuala Lumpur, Malaysia. Faecal samples were collected from 31 rodents within Selangor and Kuala Lumpur, Malaysia. Samples were taken from rodents of both sexes and from a range of species inhabiting residential areas, commercial areas (wet markets, and shop lots) as well as parks, and forest fringes. Genomic DNA was extracted from faecal samples and polymerase chain reaction (PCR) was done to amplify a partial fragment of the different genes using genus-specific primers to determine the molecular presence of *Giardia, Blastocystis,* and

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*Cryptosporidium*. The highest molecular prevalence (71%) was observed for *Cryptosporidium*, followed by *Giardia* (16.1%) and *Blastocystis* (9.7%). Fisher's Exact Test did not reveal any significant ($p>0.05$) gender, species or habitat risk association. The present study has revealed a high prevalence of potentially zoonotic enteric protozoa among the rodents examined. It further contributes to the understanding of the diversity of parasites found in synanthropic rodents, with implications for zoonotic transmission and public health.

**Keywords:** synanthropic rodents; *Cryptosporidium*; *Blastocystis*; *Giardia*; zoonotic

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## 1.0 INTRODUCTION

Economic development has led to an increase in urbanization, as people migrate from rural areas in search of job and education opportunities (UN-Habitat, 2016). The conditions imposed on urban slum residents, such as lack of adequate sanitation and garbage collection, provide resource-rich environments supporting large populations of synanthropic rodents. (Riley *et al*., 2007; Costa *et al*., 2014a). Synanthropic rodents are species that live in close proximity to humans. These mammals may serve as reservoir host for various pathogens, and play an important role in disease transmission to humans and other animal species (Torres-Castro, 2017). Rodents may transfer zoonotic pathogens humans either directly through environmental contamination where humans become affected as a result of contaminated hands, food, or water, or indirectly, through bites of arthropod vectors (Galán-Puchades *et al*., 2021). These pathogens include a variety of haemotropic bacteria, rickettsias, and protozoa, as well as enteric helminths and protozoa (Paramasvaran and Sani, 2012; Siti Shafiyyah *et al*., 2012; Mohd Zain *et al*., 2012; Gutiérrez *et al*., 2015). The notable zoonotic enteric protozoa include *Cryptosporidium, Blastocystis* and *Giardia. Cryptosporidium* is an enteric apicomplexan protozoan with many species and genotypes that infect the intestinal tract of humans and a wide range of host animals.

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*Blastocystis* is a parasitic organism that can dwell in human intestines and can cause diarrhoea, stomach pain, or other gastrointestinal issues. *Giardia* is a parasite that causes giardiasis, a diarrheal condition. When a person or animal is infected with *Giardia*, the parasite dwells in the intestines and is transferred through the stool. *Giardia* can live for weeks or even months once it has left the body. To date there remains a paucity of information on the enteric zoonotic protozoa of rodents in Malaysia. This study therefore aims at determining the molecular prevalence of selected rodent-borne enteric protozoa of zoonotic importance, namely *Cryptosporidium, Blastocystis* and *Giardia* in various peri domestic areas in Selangor, Malaysia.

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# 2.0 LITERATURE REVIEW

## 2.1 Synanthropic Rodents

Numerous zoonotic pathogens are hosts of rodents. Humans can contract zoonotic diseases either directly that is, when the infectious agent contaminates the environment and human contact with contaminated hands, food, or water or indirectly by way of an arthropod vector that has been infected by the rodent itself. The infections brought on by bacteria or viruses among rodents typically receive more attention than those brought on by parasites [www.cdc.gov/ rodents/diseases/index.html (accessed on 15 October 2020)]. People who live close to rat populations run the risk of contracting an illness. This is why it is essential to take into account the unprecedented rate of global urbanisation in order to establish a full and contemporary understanding of rat-associated zoonoses, particularly in urban areas (Galán-Puchades *et al*., 2001).

## 2.2 Intestinal Protozoan

### 2.2.1 Giardia

*Giardia* is a gastrointestinal parasite that causes the disease giardiasis, which is a very widespread waterborne infection of both humans and animals. It has a global distribution with 250–300 million symptomatic human infections recorded each year. Giardiasis in animals can manifest clinically as anything from asymptomatic to

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diarrhoea and failure to thrive (Einarsson *et al*., 2016). Children are more likely to contract the infection compared to adults, with the exception of epidemics, which affect all age groups equally. Compared to older kids, infants under the age of one year are less likely to get an infection (Chacon-Cruz, 2022). Although the pathogenesis in humans and animals is not fully understood, it is caused by both parasite and host variables, such as parasite virulence, host immunity, and the abundance of cysts in the environment (Feng and Xiao, 2011; Siwila, 2017). One of the most prevalent gastrointestinal parasite disorders affecting people worldwide is giardiasis, which is brought on by the flagellated protozoan *Giardia duodenalis* (also known as *G. lamblia* and *G. intestinalis*) (Geurden *et al*., 2010). *Giardia* is seen as a species complex of organisms with varied zoonotic potential and host preferences that are genetically distinct but physically indistinguishable. It has been hypothesised that rats in the wild could serve as a significant reservoir for the parasites that cause giardiasis in humans.

### 2.2.2 Cryptosporidium

A single-celled eukaryote known as *Cryptosporidium* was first identified as an opportunistic infection in AIDS patients and currently, one of the most common protozoa associated with diarrhoea is *Cryptosporidium* (GBD Diarrhoeal Diseases Collaborators, 2018; Sow *et al*., 2016). Recent human research have identified

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*Cryptosporidium* as the second most common cause of diarrheal disease-related death in children, accounting for 10% of all child deaths worldwide (GBD Diarrhoeal Diseases Collaborators, 2018; Sow *et al*., 2016). *Cryptosporidium* has been found in many different animal species, including domesticated livestock, poultry, companion animals, and wildlife, in addition to human infections, proving its zoonotic nature and hazard to public health (; Ryan *et al*., 2016; Khan *et al*., 2018; Pumipuntu and Piratae, 2018). The huge outbreak in Milwaukee, Wisconsin, in 1993, which resulted in more than 400,000 cases of diarrheal sickness, provided further proof that *Cryptosporidium* can cause disease in people with immune systems that are completely functional (Mac Kenzie *et al*., 1994).

**2.2.3 Blastocystis**

The most widespread protist discovered in humans is the intestinal parasite *Blastocystis*. The main method of transmission is by the faecal-oral pathway using food and water that have been infected with *Blastocystis (*Torgerson *et al*., 2015). *Blastocystis* transmission by zoonotic means may be the root cause of several human diseases (Wang *et al*., 2018; Parkar *et al*., 2010)Studies link *Blastocystis* to a number of gastrointestinal illnesses, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), raising questions about the toxicity of the organism (Jimenez-

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Gonzalez *et al*., 2011; Dogruman-Al *et al*., 2009; Boorom *et al*., 2008). Although thare remains a paucity of information on the life history of *Blastocystis*, current evidence suggest it to be a common commensal in the human gut (Beghini *et al.*, 2017).

## 2.3 Intestinal Protozoal Disease

### 2.3.1 Aetiology and transmission of Intestinal Protozoal Disease

Among the most common illnesses in humans in developing nations are parasitic infections brought on by protozoan parasites and intestinal helminths (Haque, 2007). Compared to helminths, protozoan parasites are more frequently responsible for gastrointestinal illnesses in modern nations. In endemic nations, intestinal parasites significantly increase morbidity and mortality. Faecal-oral transmission is the main method of transmission for all intestinal protozoa. Most, if not all, individual cases and outbreaks are caused by water and/or food contamination. Giardiasis and spore-forming protozoa are influenced by immunologic variables such IgA and T-cell responses. All protozoa are susceptible to malnutrition, which is a major risk factor. (Chacon-Cruz, 2022). When it comes to cryptosporidiosis, it is contracted by ingesting water or food contaminated by human or animal faeces that may contain the parasite. Other than that, the parasite may gain entry by ingestion of oocysts from the soil as well as from close contact with an infected person or animal (Marie & Petri, 2022).

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*Giardia* infection can be contracted through contact with infected person's excrement, drinking water, or eating food that has been contaminated with the parasite (Marie *et al.,* 2022). Food, water, contact with human or animal faeces, and other sources of contamination can all spread *Blastocystis*. People who work with animals and those who reside in or travel to developing countries tend to be more susceptible to *Blastocystis* infection (Mayo Clinic, 2022).

**2.3.2 Treatment**

Asymptomatic *Blastocystis* infection does not require treatment. Mild symptoms and signs may resolve in a few days. Antibiotics like metronidazole (Flagyl) or tinidazole may be used to treat a *Blastocystis* infection and alleviate symptoms. Combination drugs like trimethoprim and sulfamethoxazole (Bactrim, Septra, others) and anti-protozoal drugs like nitazoxanide and paromomycin (Mayo Foundation for Medical Education and Research, 2021)may also be used. For giardiasis, if dehydration is evident, it must be treated. Tinidazole can be given orally with single dose. Other than that, metronidazole can be given orally for three days. Children dose is 30 mg/kg once daily and for adults is 2g once daily (MSF Medical Guidelines, 2022). As for Cryptosporidiosis, no aetiological treatment is necessary in immunocompetent

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patients; the condition resolves on its own in one to two weeks. Dehydration must also be treated if present (MSF Medical Guidelines, 2022).

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# 3.0 MATERIALS & METHOD

## 3.1 Study Area

Sixteen locations in Selangor and Kuala Lumpur were chosen and included five habitat types namely wet market, shop lot, forest and park. The regions' annual temperature has an average of 31.7 °C, with a humidity of 75.16%.

## 3.2 Rodent Sampling

In Selangor, a total of 25 rat traps were set up over the course of three weeks to catch wild rodents. Baits including old coconut, various salted fish varieties, dried fish, bread, and cheese were used. The traps were set in the evening, and collected the next morning. The following day, traps were set up in new locations in the evening. All rodents that were caught were taken to the Post Mortem Laboratory in the Faculty of Veterinary Medicine at Universiti Putra Malaysia for further processing.

## 3.3 Identification of Rodents

All rodents that were successfully captured were subjected to morphometric examination. Euthanisation of rodents was first done using Pentobarbital Sodium 250 mg/kg injected intraperitoneally. It was done according to the protocol stated in the IACUC form. For species identification, length of the body and head, as well as tail

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were measured. The eyes and snout were also observed. Species and sex of the rodents were identified and recorded.

### 3.4 Faecal Sample Collection

Defecated faecal samples were collected from a plastic sheet that was placed beneath each trap. Following euthanasia, the ventral abdomen of the rat was excised to expose the colon and rectum, in order to collect the faecal material. The samples were initially placed inside a small labelled plastic container, then transferred into a 2mL centrifuge tubes and stored at -13ºC at the Parasitology Lab, Faculty of Veterinary Medicine at Universiti Putra Malaysia. Carcasses were then disposed according to the institutional biohazard disposal guidelines.

### 3.5 Molecular Detection

#### 3.5.1 Extraction of genomic DNA

Total gDNA was extracted using a commercial kit, QIAmp® Fast DNA stool Mini Kit (QIAGEN®, Germany) according to the manufacturer's instructions. A 2mL microcentrifuge tube containing 250µL of faeces and buffer solution was placed on ice and the tube was then spun. The stool samples were thoroughly homogenised after being vortexed in 1mL of QIAGEN® samples for 1 minute. The suspension was thereafter heated for five minutes at 70 degrees Celsius. Following a one-minute

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centrifugation to pellet faeces, samples were processed. A fresh 1.5mL microcentrifuge tube containing 15µL of QIAGEN® Proteinase K (QIAGEN®, Germany) was pipetted into the container. To further homogenise the material, 299 QIAGEN® Buffer AL (QIAGEN®, Germany) was added. Then, the sample is incubated for 10 minutes at 70°C. The spin column was then carefully filled with 200µL of ethanol and centrifuged for one minute. Buffer EB (QIAGEN®, Germany) was applied to the membrane in the spin column, allowed to stand for one minute then centrifuged to harvest the eluted DNA.

**3.5.2 Polymerase Chain Reaction (PCR)**

**3.5.2.1 *Cryptosporidium***

Nested PCR was done to amplify a partial fragment of the SSUrRNA of *Cryptosporidium* (Table 1). A total of 4µL of sample DNA, 5µL each of 5X green GoTaq ® Flexi Buffer (Promega, US), 5µL each MgCl2 25mM solution, 0.3µL of each deoxynuceloside triphosphate (dNTP), 1µL each of forward primer P1 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') (Xiao *et al*., 1999) and reverse primer P2 (5'-AAGGAGTAAGGAACAACCTCCA-'3) (Nolan *et al*., 2010), 0.3µL each of GoTaq® DNA polymerase 5u/uL and 8.4 µL OF nuclease- free water (ddH20) were mixed and subjected to PCR.

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Primary amplification included 94ºC (5 minutes) for initial denaturation, followed by 35 cycles of denaturation at 94ºC (45 seconds), annealing at 45ºC (2 minutes), extension at 72 ºC (1.5 minutes) and by one cycle at 72ºC (10 minutes) for final extension. PCR reactions were done in a thermal cycler (BioRad, US). The nested reaction started with initial denaturation of 94ºC (5 minutes), followed by 30 cycles of denaturation at 94ºC (45 seconds), annealing at 45ºC (2 minutes), extension at 72 ºC (1.5 minutes) and by one cycle at 72ºC (10 minutes) for final extension.

Table 1: Primers used for PCR detection of *Cryptosporidium*

| Primers | | Gene | Size (bp) | Reference |
|---|---|---|---|---|
| 1st nest | 2nd nest | | | |
| XF2 (5'-GGAAGGGTTGTATTTATTAGATAAAG 3'), XR2 (5' AAGGAGTAAGGAACAACCTCCA 3') | pSSUf (5'-AAAGCTCGTAGTTGGATTTCTGTT- 3'), pSSUr (5' -ACCTCTGACTGTTAAATACRAATGC- 3') | SSUrRNA | 826-864, 240 | Xiao *et al.*, 1999 Nolan *et al.*, 2010 |

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**3.5.2.2 *Blastocystis***

A partial fragment of the SSUrRNA of *Blastocystis* was amplified in a single step PCR (Table 2). A total of 4µL of sample DNA, 5µL each of 5X green GoTaq ® Flexi Buffer (Promega, US), 5µL each MgCl2 25mM solution, 0.3µL of each deoxynuceloside triphosphate (dNTP), 1µL each of forward primer P1 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') (Xiao *et al*., 1999) and reverse primer P2 (5'-AAGGAGTAAGGAACAACCTCCA-'3) (Nolan *et al*., 2010)., 0.3µL each of GoTaq® DNA polymerase 5u/uL and 8.4µL OF nuclease- free water (ddH20) were mixed and subjected to PCR. The reaction included 94ºC for 5 minutes for initial denaturation, followed by 30 cycles at 94ºC (1 min), 56ºC (1 minute) and 72 ºC for 1 minute and by one cycle at 72ºC (4 minutes) for final extension. PCR reactions were done in a thermal cycler (BioRad, US).

Table 2: Primers used for PCR detection of *Blastocystis*

| Primers | Gene | Size (bp) | Reference |
|---|---|---|---|
| RD5 (5'- ATCTGGTTGATCCTGCCAGT -3'), BhRDr (5'-GAGCTTTTTAACTGCAACAACG -3') | SSUrRNA | 600 | Scicluna *et al*., 2006 |

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### 3.5.2.3 *Giardia*

A partial fragment of the SSUrRNA of *Giardia* was amplified using nested PCR (Table 3). A total of 4µL of sample DNA, 5µL each of 5X green GoTaq ® Flexi Buffer (Promega, US), 5µL each MgCl2 25mM solution, 0.3µL of each deoxynuceloside triphosphate (dNTP), 1µL each of forward primer P1 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') (Xiao *et al*., 1999) and reverse primer P2 (5'-AAGGAGTAAGGAACAACCTCCA-'3) (Nolan *et al*., 2010)., 0.3 µL each of GoTaq® DNA polymerase 5u/uL and 8.4 µL OF nuclease- free water (ddH20) were mixed for the primary PCR amplification. The reaction was run at 96ºC for 4 minutes for initial denaturation, followed by 35 cycles at 96ºC (45 seconds), 55ºC (30 seconds) and 72ºC for 45 seconds and by one cycle at 72ºC (4 minutes) for final extension. PCR reactions were done in a thermal cycler (BioRad, US).

The nested PCR reaction started with initial denaturation of 96ºC (4 minutes), followed by 35 cycles of denaturation at 96ºC (45 seconds), annealing at 59ºC (30 seconds), extension at 72ºC (45 seconds) and by one cycle at 72ºC (4 minutes) for final extension.

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Table 3: Primers used for PCR detection of *Giardia*

| Primers | | Gene | Size (bp) | Reference |
|---|---|---|---|---|
| 1st nest | 2nd nest | | | |
| (GiarF1) Gia2029 (5'-AAGTGTGGTGCAGACGGACTC -3'), (GiarR1)Gia2150c (5'-CTGCTGCCGTCCTTGGATGT -3') | (GiarF2)RH11 (5'-CATCCGGTCGATCCTGCC -3'), (GiarR2)RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG -3') | 16 SSUrRNA | 497, 292 | Appelbee *et al.*, 2003 |

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### 3.5.3 Gel electrophoresis

The amplicons were electrophoresed on a 1.5% agarose gel, stained with Ethidium Bromide for 15 minutes and visualized using a Molecular Imager ® Gel DocTM XR System (Bio-Rad®, US) with UV illumination. A microwaveable flask was used to combine 1.5g of HyagaroseTM (HydraGene) powder with 100 mL of IxTAE to create a 1.5% agarose gel. The HyagaroseTM powder was then fully dissolved by heating the mixture in the microwave for one to three minutes. The dissolved HyagaroseTM

was allowed to cool to 50°C before being poured into the gel tray with the well comb in place, and left to solidify at room temperature. The first well was filled with 5µm of the VivantisTM VC100bp Plus DNA ladder (VivantisTM, Malaysia), and the second well was filled with 5µm of the negative control. The PCR products (5µm) were added to the gel's additional wells and electrophoresed for 50 minutes at 90V. PCR amplicons were also sent for sequencing in order to obtain the exact sequence for the nucleotide/ bases.

**3.6 Statistical Analysis**

Fisher's Exact Test was used for the statistical analysis at a P-value of <0.05. The occurrence and significance of the association of *Cryptosporidium, Blastocystis and Giardia* were evaluated with respect to the gender, species and environmental habitat type.

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# 4.0 RESULTS

A total of 31 rodents were captured from the five sampling habitats. A total of three species were obtained, namely, *Rattus rattus* (18), *R. norvegicus* (11), and *R. tiomanucus* (2). All three enteric protozoa (*Cryptosporidium*, *Giardia* and *Blastocystis*) were successfully detected by PCR. The positive PCR amplicons for each protozoa species examined are presented as visualized on agarose gel electrophoresis (Figures 1 to 6).

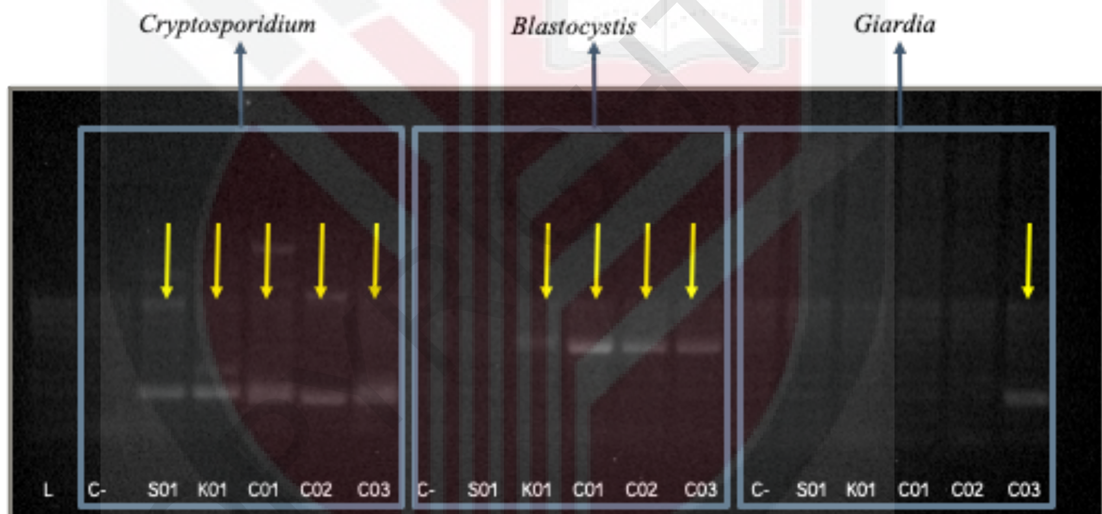


Figure 1. Agarose gel electrophoresis showing the PCR assay results for *Cryptosporidium, Blastocystis* and *Giardia*. Lane L = DNA ladder, C- = Negative control. Yellow arrow = Positive results of protozoa.

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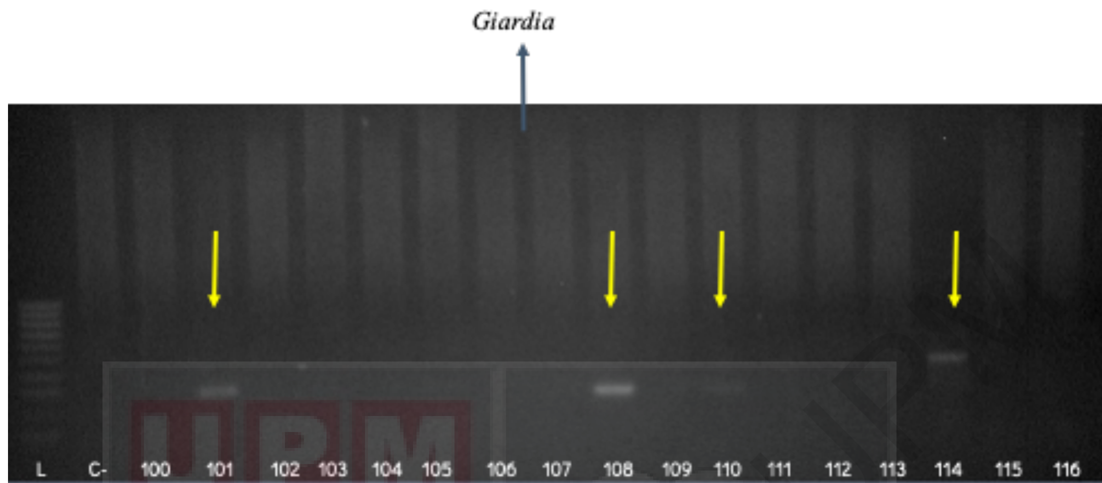


Figure 2. Agarose gel electrophoresis showing the PCR assay results for *Giardia*. Lane L = DNA ladder, C- = Negative control. Yellow arrow = Positive results.

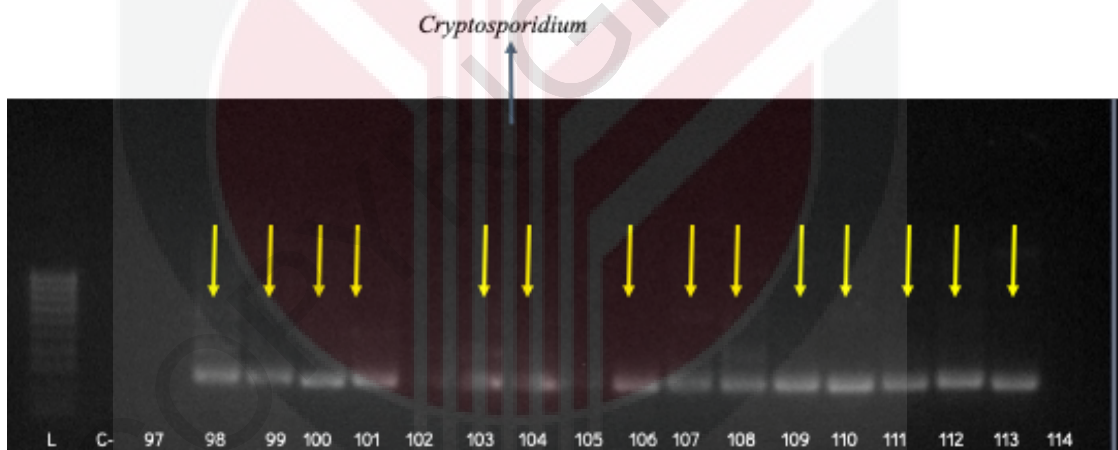


Figure 3. Agarose gel electrophoresis showing the PCR assay results for *Cryptosporidium* and *Giardia*. Lane L = DNA ladder, C- = Negative control. Yellow arrow = Positive results

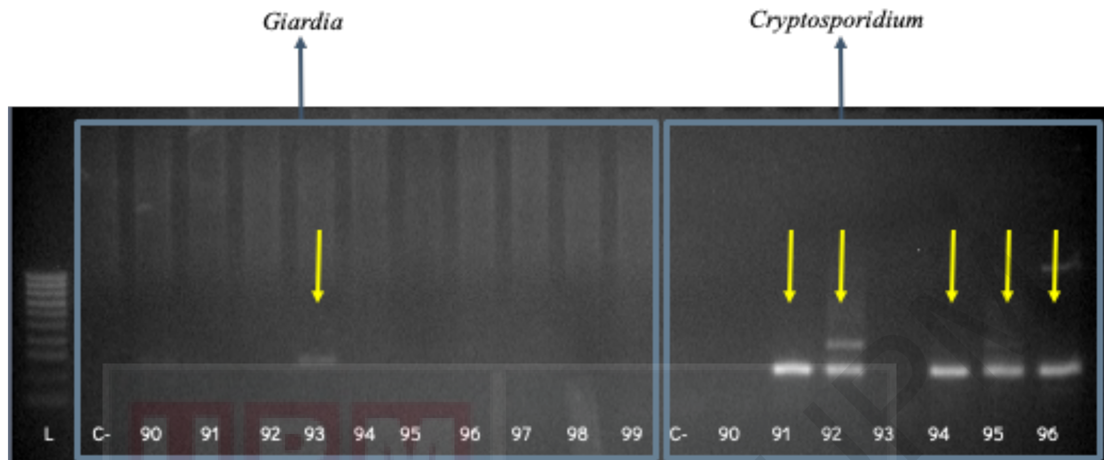


Figure 4. Agarose gel electrophoresis showing the PCR assay results for *Cryptosporidium.* Lane L = DNA ladder, C- = Negative control. Yellow arrow = Positive results

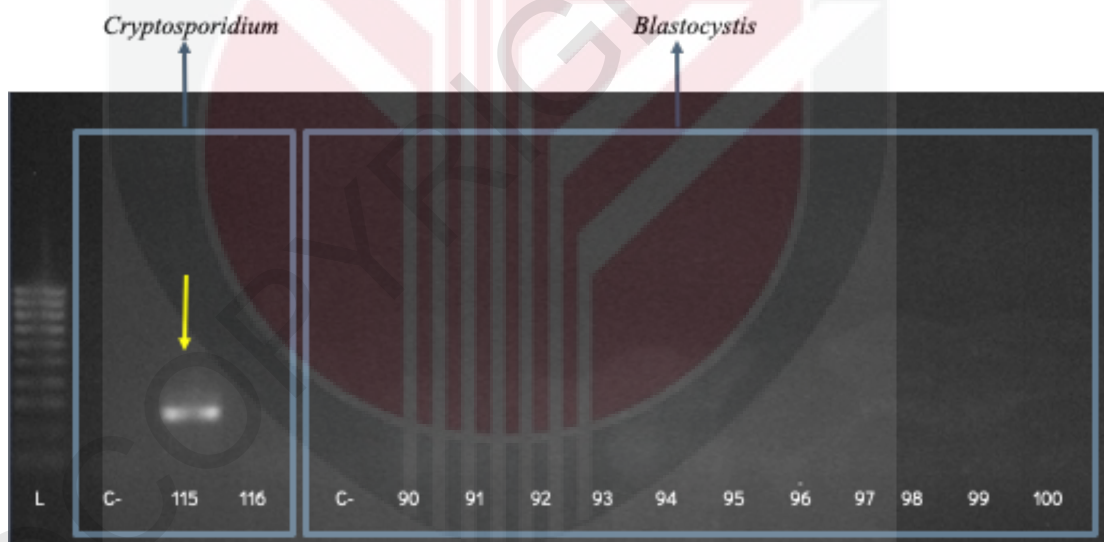


Figure 5. Agarose gel electrophoresis showing the PCR assay results for *Cryptosporidium* and *Blastocystis*. Lane L = DNA ladder, C- = Negative control. Yellow arrow = Positive results

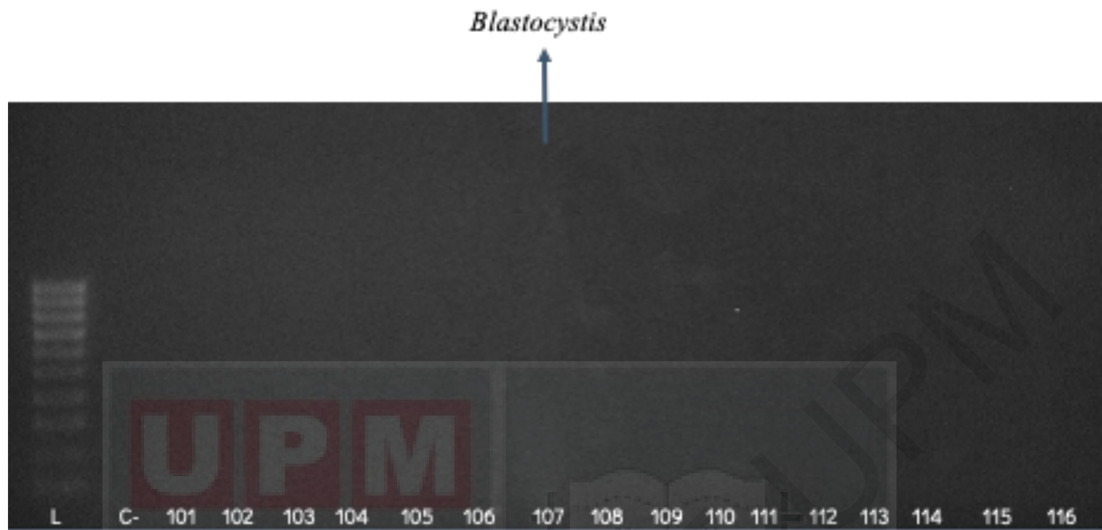


Figure 6. Agarose gel electrophoresis showing the PCR assay results for *Blastocystis.* Lane L = DNA ladder, C- = Negative control. Yellow arrow = Positive results of *Blastocystis*

**Molecular Prevalence**

Prevalence of *Cryptosporidium* was the highest with a percentage of 71% (22 out of 31) (Figure 7). Large difference in prevalence between *Cryptosporidium* with *Giardia* and *Blastocystis. Blastocystis* has the lowest prevalence value with a percentage of 9.7% (3 out of 31) followed by *Giardia* with a prevalence of 16.1 % (5 out of 31).

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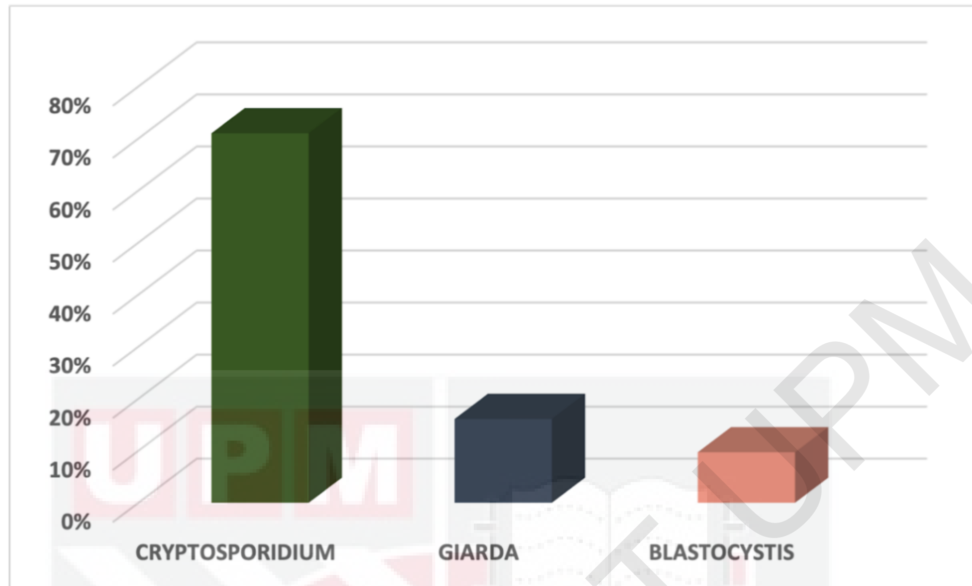


Figure 7. Molecular Prevalence of *Blastocystis, Giardia* and *Cryptosporidium* among peridomestic rodents from Selangor and Kuala Lumpur, Malaysia.

**Rodent Gender**

The overall findings revealed that the males had a higher prevalence of enteric protozoa compared to the females (Tables 4, 5 and 6). *Cryptosporidium* was the most common enteric protozoa detected. Infection rates with *Cryptosporidium* were comparable between the two genders, albeit a slightly higher prevalence in the males (71.2%), compared to the females (64.7%). The prevalence of *Giardia* was much higher (28.6%) among the male rodents compared to the females (5.9%). None of the female rodents examined were positive for *Blastocystis* by PCR detection (Table 5)

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Table 4. Gender of rodents in relation to *Cryptosporidium* infection.

| | +ve | -ve | Prevalence (%) |
|---|---|---|---|
| Male | 10 | 4 | 71.2 |
| Female | 11 | 6 | 64.7 |

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Table 5. Gender of rodents in relation to *Giardia* infection.

| | +ve | -ve | Prevalence (%) |
|---|---|---|---|
| Male | 4 | 10 | 28.6 |
| Female | 1 | 16 | 5.9 |

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Table 6. Gender of rodents in relation to *Blastocystis* infection.

| | +ve | -ve | Prevalence (%) |
|---|---|---|---|
| Male | 3 | 11 | 21.4 |
| Female | 0 | 17 | 0 |

**Protozoa Species**

*Cryptosporidium* was the most prevalent enteric protozoa detected across all three species of rodents examined. *Rattus rattus* showed the highest number of positive *Cryptosporidium* with a prevalence of 77.8%, followed by *R. norvegicus* (54.6%) and

*R. tiomanicus* (50.0%) (Table 7). No *Giardia* and *Blastocystis* were detected in *R. tiomanicus* (Tables 8 and 9). This species also showed the lowest prevalence in relation to *Cryptosporidium* infection (50.0%) compared to *R. rattus* and *R. norvegicus*.

Table 7. Species of rodents in relation to *Cryptosporidium* infection.

| | +ve | -ve | Prevalence (%) |
|---|---|---|---|
| *R. rattus* | 14 | 4 | 77.8 |
| *R. norvegicus* | 6 | 5 | 54.6 |
| *R. tiomanicus* | 1 | 1 | 50.0 |

Table 8. Species of rodents in relation to *Giardia* infection.

| | +ve | -ve | Prevalence (%) |
|---|---|---|---|
| *R. rattus* | 3 | 15 | 16.7 |
| *R. norvegicus* | 2 | 9 | 11.2 |
| *R. tiomanicus* | 0 | 2 | 0 |

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Table 9. Species of rodents in relation to *Blastocystis* infection.

| | +ve | -ve | Prevalence (%) |
|---|---|---|---|
| *R. rattus* | 1 | 17 | 5.6 |
| *R. norvegicus* | 2 | 9 | 18.2 |
| *R. tiomanicus* | 0 | 2 | 0 |

**Habitat**

Enteric protozoa were detected in the rodents inhabiting at least one habitat out of five (Tables 10, 11 and 12). *Cryptosporidium* was the most widely distributed enteric parasite, and was detected in four out of the five habitats examined. Sampling distribution across the habitats was not equal with a low number of rodents captured from the forest (3), Shop lot (1) and park (3). The most number of rodents were captured in the wet market (17), and from these samples, the highest prevalence (70.0%) was for *Cryptosporidium*. *Blastocystis* was only detected among the forest-dwelling rodents.

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Table 10. Location of sampled rodents in relation to *Cryptosporidium* infection

| | +ve | -ve | Prevalence (%) |
|---|---|---|---|
| Forest | 3 | 0 | 100 |
| Shop lot | 0 | 1 | 0 |
| Wet market | 17 | 7 | 70 |
| Park | 1 | 2 | 33.3 |

Table 11. Location of sampled rodents in relation to *Giardia* infection.

| | +ve | -ve | Prevalence (%) |
|---|---|---|---|
| Forest | 1 | 2 | 33.3 |
| Shop lot | 0 | 1 | 0 |
| Wet market | 4 | 20 | 16.7 |
| Park | 0 | 3 | 0 |

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Table 12. Location of sampled rodents in relation to *Blastocystis* infection.

| | +ve | -ve | Prevalence |
|---|---|---|---|
| Forest | 3 | 0 | 100.0 |
| Shop lot | 0 | 1 | 0 |
| Wet market | 0 | 24 | 0 |
| Park | 0 | 3 | 0 |

**Prevalence of Protozoa Co-infection**

Single enteric protozoa species infection was the most common. The highest prevalence among single enteric protozoa infection was *Cryptosporidium* (48%), followed by Blastosystis (6.5%) and Giardia (3.2%) (Table 13). Dual co-infection involving *Cryprosporidium* and *Giardia* infecting the rodents had a prevalence of 9.7%, while that of *Cryptosporidium* and *Blastocystis* was 6.5%, and the lowest prevalence for dual infection was *Giardia* and *Blastocystis* with prevalence of 3.2%. Triple co-infection comprising *Cryptosporidium, Giardia*, *Blastocystis* was only observed in one rodent with a prevalence 3.2%. (Table 13).

Table 13. Prevalence of mixed protozoal infection among the sampled rodents.

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| | *Cryptosporidium* | *Giardia* | *Blastosystis* |
|---|---|---|---|
| Single infection | 15 (48%) | 1 (3.2%) | 2 (6.5%) |
| Dual infection (*Cryptosporidium* + *Giardia*) | 3 (9.7%) | | |
| Dual infection (*Cryptosporidium* + *Blastocystis)* | 2 (6.5%) | | |
| Dual infection *(Giardia* + +*Blastocystis)* | | 1 (3.2%) | |
| Triple infection | 1 (3.2%) | | |

**Fisher's Exact Test**

Association between gender, species and location with the prevalence of *Cryptosporidium, Giardia* and *Blastocystis* were assessed using a contingency table analysis and Fisher's Exact Test (Table 14). The results indicated that overall, there was no significant association between gender, species and location ($p>0.05$) with the prevalence of *Cryptosporidium, Giardia* and *Blastocystis*, with the exception of a significant ($p<0.05$) association between location of rodents and the prevalence of *Blastocystis*.

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Table 14. Significance of association between the protozoa prevalence and epidemiological variables (gender, species, location) tested using Fisher's Exact Test. P value set at <0.05.

| | Gender | Species | Location |
|---|---|---|---|
| *Cryptosporidium* | 0.71 | 0.31 | 0.11 |
| *Giardia* | 0.15 | 1 | 0.96 |
| *Blastocystis* | 0.08 | 0.87 | 0.002 |

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**5.0 DISCUSSION**

This study provides and insight on current prevalence rates of *Cryptosporidium, Giardia* and *Blastosystis* infecting rodents in Selangor and Kuala Lumpur, Malaysia. Results from this study have shown that these rodents harbour high infection rates of enteric protozoa. The overall prevalence rate of *Cryptosporidium* infection was the highest (71.0%), followed by *Giardia* (16.1%) and *Blastocystis* (9.7%). A previous study (Taghipour *et al*., 2020) has revealed a global prevalence of 17% for *Cryptosporidium*. However, prevalence rates reported in this study vary among the different countries. In China 11% of the rodents examined were positive for this enteric protozoa infection, infection among rodents in the USA was high (30%). The highest infection rate (42%) was reported from Poland (Taghipour *et al*., 2020). Interestingly, this study has revealed a very high prevalence (71.0%) of *Cryptosporidium* among the rodents examined. The moderate prevalence (16.1%) for *Giardia*, the prevalence is similar to another morphological identification study that reported the prevalence of *Giardia* in rodents to be 14.6% (David, 2006). However sampling variables such as sample size and detection methods vary among the studies done in the various countries. This may yield different findings which will confound comparative prevalence analyses to be done.

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A previous study (Feng & Xiao, 2011), demonstrated the co-occurrence of two protozoa parasites was observed in ten rats, with six of them shedding *Cryptosporidium and Giardia.* This shows that, one rodent can harbour multiple enteric protozoa at a time. Apart from that, in this study one rodent was found to have triple enteric protozoa infection which were *Cryptosporidium, Giardia* and *Blastocystis.*

Gender association with parasite infection have been investigated in a number of previous (Grossman, 1989; Folstad and Karter, 1992; Mafiana *et al*., 1997; Nicola *et al*., 2004; Zain, 2008). That prevalence of endoparasite infection based on host gender was slightly higher in males (18.6%) than females (16.4%) rats. The present study has shown that the prevalence of *Cryptosporidium, Giardia* and *Blastocystis* was higher in the males compared to the females. The immunosuppressive impact of male sex hormones on male rats may have an influence on this finding of higher infection prevalence. This would explain a greater tendency for parasite infection. However, the results of this study may not be conclusive as yet due to relatively small number of male and female rodents sampled.

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Epidemiological variables are known to influence parasite infection rates in rodents. Variation in infection may be attributed to intrinsic (sex, age, and species) as well as extrinsic (habitat, season, and density) factors (Easterbrook *et al*., 2007). However, there remains a paucity of data reporting statistical associations of both intrinsic and extrinsic factors of rodents in the specific context of enteric protozoa infection among synanthropic rodents.

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## 6.0 CONCLUSION

This study has revealed a high molecular prevalence of *Cryptosporidium* (71.0%) in synanthropic rodents within Selangor and Kuala Lumpur, Malaysia. The infection with other enteric protozoa is moderate with prevalence rates of 16.1% for *Giardia* and 9.7% for *Blastocystis.* The present study has also found that overall, there is no significant association of gender, species and environmental habitat with *Cryptosporidium* and *Giardia* infection among the synanthropic rodents. However, there was only one statistical significant association between environmental habitat (location) and *Blastocystis* ($p < 0.05$). The rodents sample from the wet market showed the highest positive results of *Cryptosporidium*. This is most likely due to damp environment at the wet market which gives longer survival time for the protozoa to multiply and increase the transmission rate.

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## 7.0 RECOMMENDATION

More rigorous sampling would be the direction for future studies in order to get a better representation of the enteric protozoa infection among synanthropic rodents in various habitats and life history categories. This would add robustness to the statistical analyses and provide more accurate data on the effect of both epidemiological intrinsic and extrinsic variables on parasite infection rates. In addition, more habitat types could be included in future studies. This may include residential areas, schools, and areas with high numbers of eateries. This could give more information on the infection risk among a wide spectrum of humans as there are known differences in the clinical manifestation of enteric protozoa among adults and children. Risk factors for infection could also be studied to detect the transmission rate according to different factors but in order to get a high accuracy result, large sample size is needed.

Results from the present study revealed a high prevalence of enteric protozoa among the synanthropic rodents studied. It is highly recommended that the management of areas like shop lots and wet markets initiate regular disinfection to control the transmission of enteric protozoa. This would minimize the transmission among rodents as well as to humans. Efforts on getting rid of rodents should be done such as

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rat trapping and hiring pest control companies. This is because rodents are known to multiply rapidly in the presence of abundant food resources. This will lead to the increase in transmission rate of enteric protozoa of zoonotic and public health concern.

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