UNIVERSITI PUTRA MALAYSIA

PREVALENCE OF ZOONOTIC ENTERIC PROTOZOA AMONG CAPTIVE PRIMATES IN PENINSULAR MALAYSIA

TAN YOU KEN

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A project paper submitted to the

Faculty of Veterinary Medicine, University Putra Malaysia

In partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

University Putra Malaysia

Serdang, Selangor Darul Ehsan

It is hereby certified that we have read this project paper entitled "Prevalence of Zoonotic Enteric Protozoa among Captive Primates in Peninsular Malaysia", by Tan You Ken and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

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Specially dedicated to my family, friends, and the special one that I love.

ACKNOWLEDGEMENTS

I would like to express my profound appreciation and gratitude to my family for their continuous support, motivation and encouragement. I have gained invaluable knowledge from Dr. Reuben Sharma who has guided and supervised me and it is extreme gratitude I would like to express for his encouragement and great supervision.

Furthermore, the guidance and excellent experience from Dr Nur Mahiza Mohd Isa, Madam Maizatul Akmal Moktar, Mr Abd Rashid Abdul Rahman, Ms Amlizawaty Amzah, Ms Ruviniyia Kalaiselvam, Dr Michelle Tan Li Ping, Ms Norhadila Zulkifli, Ms Zarith Suraya, Ms Donea Abdulrazak Abdullah, Mr Kiven Kumar and Ms Vynter Low who were guiding and assisting me with my experimental work and statistical analysis to complete my project are greatly appreciated.

My deepest gratitude, appreciation and thanks to Zoo Negara, Zoo Melaka, Zoo Taiping and Bukit Merah Orang Utan Island for allowing me to collect sample from their facilities. I would like to appreciate the assistance from the Veterinary Officer and the Assistance Veterinary Officer, Dr Jessie Ho, Dr Kavitha, Dr Yeoh Boon Nie, Mr Gan, Dr Ridhwan, and Dr Sabapathy.

Finally I would like to acknowledge the contribution of my friends, Chan Shin Yee, Robinn Teoh, Ginny Lim, Joy Lee, Rabbani, Chong Zheng Zhe, Kunambiga, Lim Mei Yi, Stephanie Magdalene and Muhammad Hafiz Abu Hassan for their continuous support and assistance. I would also like to further express my gratitude to all my course mates and everyone who have contributed directly or indirectly in completing this study.

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Abstrak daripada kertas projek dikemukakan kepada Fakulti Perubatan Veterinar bagi memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir.

KAJIAN BERKAITAN DENGAN KELAZIMAN PROTOZOA ENTERIK ZOONOTIK ANTARA PRIMATE TAWANAN DI SEMENANJUNG MALAYSIA

Oleh

TAN YOU KEN

2017

Penyelia: Dr Reuben Sharma

Transmisi penyakit zoonotik hidupan liar telah meningkat dengan pesat, terutamanya dalam primate bukan-manusia disebabkan pengubahan dalam social, demografik dan persekitaran. Kini, adalah kekurangan dalam data terbitan terhadap kejadian dan kelaziman protozoa enteric zoonotic antara primate bukan-manusia dalam negara ini. Oleh itu, kajian ini adalah diadakan untuk menentukan kelaziman protozoa enteric zoonotic antara primate tawanan ditempatken dalam empat kemudahan zoology di Semenanjung Malaysia. Jumlah 40 sampel kumpulan tahi telah dikumpulkan dari Sumatra orangutans, Borneo orangutans, Cimpanzi, Ungka tangan putih, Siamang, Beruk ekor singa, Beruk kentoi, Beruk bonet, Mandrill, Capuchin coklat, Monyet kecil, dan Lemur coklat. Smear tahi disediakan dan bewarna dengan Giemsa dan Modified Ziehl-Neelsen untuk pemeriksaan mikroskopik. Polymerase chain reaction (PCR) dengan menggunakan primer genus tertentu telah dijalankan untuk menguatkan Subunit kecil ribosom RNA (SSU) gen *Giardia*, *Cryptosporidium*, *Blastocystis*, *Balantidium* dan *Entamoeba*. Pemeriksaan mikroskopik menunjuk kehadiran *Blastocystis* (30%), *Entamoeba* (12.5%), *Cryptosporidium* (10%), dan *Balantidium* (2.5%). *Giardia* tidak dijumpai dalam pemeriksaan mikroskopik smear tahi. Penguatkan PCR telah menunjukkan *Entamoeba* (65%) adalah protozoa enteric yang paling dominan antara primate, diikuti oleh *Blastocystis* (50%), *Balantidium* (20%), *Cryptosporidium* (20%) dan *Giardia* (5%). Tiada hubungan yang ketara antara kelaziman pelbagai protozoa dan primate atau kemudahan zoologi. Kelaziman protozoa enteric zoonotic yang tinggi antara primate tawanan dalam negara ini melayakan kajian lanjut pada epidemiologi, faktor risiko untuk jangkitan, dan potensi mereka dalam transmisi zoonotic kepada kakitangan zoo dan pelawat.

Kata kunci: Primate bukan-manusia, protozoa enterik zoonotik, *Giardia*, *Cryptosporidium*, *Blastocystis*, *Balantidium, Entamoeba*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD4999 – Final Year Project

PREVALENCE OF ZOONOTIC ENTERIC PROTOZOA AMONG CAPTIVE PRIMATES IN PENINSULAR MALAYSIA

By

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2017

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The transmission of zoonotic wildlife diseases has increased exponentially, especially in non-human primates mainly due to social, demographic and environmental alterations. At present, there remains a dearth of published data on the occurrence and prevalence of zoonotic enteric protozoa among non-human primates in the country. This study was therefore conducted to determine the prevalence of zoonotic enteric protozoa among captive primates housed in four zoological facilities in Peninsular Malaysia. A total of 40 pooled fecal samples were collected from Sumatra Orangutans, Borneo Orangutans, Chimpanzees, White-hand Gibbons, Siamangs, Lion-tailed Macaques, Stump-tailed Macaques, Bonnet Macaques, Mandrills, Brown Capuchin monkeys, Common Marmosets and Brown Lemurs. Fecal smears were prepared and stained with Giemsa and

Modified Ziehl-Neelsen for microscopy examination. Polymerase chain reaction (PCR) using genus-specific primers was carried out to amplify the Small Subunit Ribosomal RNA (SSU) gene of *Giardia*, *Cryptosporidium*, *Blastocystis*, *Balantidium* and *Entamoeba*. Microscopy examination revealed the presence of *Blastocystis* (30%), *Entamoeba* (12.5%), *Cryptosporidium* (10%), and *Balantidium* (2.5%). *Giardia* was not detected in the fecal smears. PCR amplification demonstrated that *Entamoeba* (65%) was the most dominant enteric protozoa among the primates, followed by *Blastocystis* (50%), *Balantidium* (20%), *Cryptosporidium* (20%) and *Giardia* (5%). There was no significant association between the prevalence of the various protozoa and primate hosts or captive facility. The high prevalence of zoonotic enteric protozoa among captive primates in the country merits further investigation on the epidemiology, risk factors for infection, and their potential for zoonotic transmission to the staff and visitors.

Key words: Non-human primates, zoonotic enteric protozoa, *Giardia*, *Cryptosporidium*, *Blastocystis*, *Balantidium, Entamoeba*

INTRODUCTION

The incidence and frequency of epidemic transmission of zoonotic diseases have increased exponentially in the past 30 years, mainly due to social, demographic and environmental transformation (Wilcox *et al.,* 2005). Among these zoonotic diseases are those vectored by non-human primates, and include gastrointestinal protozoa like *Entamoeba, Giardia, Cryptosporidium, Balantidium.* and *Blastocystis.* With the ever changing landscape in the country where forests are cleared for industrial purposes, these wild primates are driven out of their natural habitat and are now commonly seen in urban settings. This reduces the disease transmission interface and could contribute significantly to the rise in zoonotic diseases in the country. In addition, zoonotic enteric protozoa have been documented in both wild and captive primates worldwide (Lim *et al.,* 2008; Kilbourne *et al*., 2003; Abe *et al.,* 2002; Munene *et al.*, 1998; Pourrut *et al.,* 2011; Legesse *et al.,* 2004; Gomez *et al.,* 2000; Levecke *et al.*, 2007).

Captive primates pose a serious risk of zoonosis to visitors and staff due to constant direct or indirect contact between humans and primates. Various factors including captive related stress, high stocking density, and close proximity among various groups of animals might contribute to a high prevalence of zoonotic disease in captive primates. In addition, there could also be a risk of cross infection from wild feral primates which could introduce new diseases to the captive primates, some of which may be zoonotic. A number of genotypes of the enteric protozoa are known to be shared among humans and primates and thus these pathogens pose a serious zoonotic risk (Jonckheere *et al.*, 1989).

At present there remains a paucity of published information of the species diversity and prevalence of zoonotic enteric protozoa among captive primates in Peninsular Malaysia. In addition, previous reports have focused on microscopy examination which has a lower detection sensitivity compared to molecular techniques like Polymerase Chain Reaction (PCR) amplification. As such, it is necessary to employ more sensitive molecular detection techniques to determine the prevalence of infections among these primates, especially those that are readily transmitted by environmental contamination and the faecal–oral route like the enteric protozoan parasites.

The objectives of the present study are:

1. To determine the prevalence of zoonotic enteric protozoa among various species of captive primates in Peninsular Malaysia.
2. To ascertain if the prevalence is related to host species the captive facility.

The results from this study provides a baseline for further investigation on the epidemiology, risk factors for infection, and their potential for zoonotic transmission to humans.

LITERATURE REVIEW

Zoonotic parasites of primates

Zoonotic infections have given a huge impact on human health and human well-being, especially those who staying in rural areas of developing countries (Meslin, 1995). This is mainly due to the slow pace of the 'public health revolution' in these areas, which including environmental hygiene improvement, nutrition and housing, leading to the spread of infectious diseases cycling in the population. According to Warren 1989, health of human and animals living in the tropical regions are believed to be challenged by mainly helminthes and protozoa. The route of transmission of these zoonotic diseases between primates and human is primarily through environmental contamination, although direct contact of persons in constantly close proximity with primates such as zoo workers, researchers in primate facilities, and pet owners is also a major route of infection. Studies have shown that primates can be infected with a number of zoonotic protozoan genera, including *Giardia* (Berrilli *et al.*, 2011), *Cryptosporidium* (Salzer *et al.*, 2007), *Blastocystis (*Abe *et al.*, 2003), *Balantidium.* and *Entamoeba.* (Muriuki *et al.*, 1998; Chunge *et al*., 1991; Muller-Graf *et al*., 1996; Munene *et al*., 1998). This could be due to the free interaction of susceptible host and the contaminated environment.

Zoonotic enteric protozoa

Giardia is an anaerobic flagellated protozoan parasite, classified in the Hexamitidae family of the phylum Sarcomastigophora. They are diplomonads, which have two nuclei. They are pear-shaped with a median body, four pairs of flagella, and a ventral disk. The cyst are approximately 5 x 7-10µm in diameter. The trophozoites are 12-15µm long by 5-9µm wide (Adam, 2001). After infection, the incubation period varies from 9-15 days. They reproduce in the small intestines of their host causing a disease known as giardiasis. This includes uneasiness of intestine, nausea, anorexia, low-grade fever, chills, follow by explosive, watery, foul-smelling diarrhea, marked abdominal gurgling and distension, together with passage of foul gas or belching with a foul taste. Clinical signs will last three to four days (Wolfe, 1992).

Cryptosporidium is an Apicomplexan parasite. Their oocyst are round to oval in shape, measuring 4-6µm in diameter, and may be appreciated under light microscopy with acid-fast staining (Yu, 2003). Clinical signs will develop 1 to 12 days after exposure (Vogue *et al.*, 2011). They reproduce in the microvillus border of gastrointestinal epithelium of the host and cause a disease known as cryptosporidiosis (Xiao *et al.,* 2004). This includes watery diarrhea, dehydration, weight loss, abdominal pain, nausea, vomiting and fever. Destruction of the microvilli of the small intestine causes and impairment of digestive enzyme secretion leading to indigestion and malabsorption. Clinical signs will last one to two weeks especially in immunocompetent person (Yu, 2003).

Blastocystis is a single-celled heterokont parasites belonging to a group of organisms known as the Stramenopiles. They are predominantly rounded in shaped but may sometimes be ovoid. These organisms measure a diameter of 4-15µm, and are surrounded by a thick cyst-like wall which contains visible mitochondria (Zierdt, 1991). The disease known as blastocystosis manifests in diarrhea, abdominal pain, weight loss, anal itching, constipation and excess gas production. However, many host shows no symptoms at all. *Blastocystis* can remain in the intestine for weeks, months, or even years (CDC, 2014).

Balantidium is a Ciliophora and the only ciliated protozoon that known to infect humans. It is also the largest protozoon infecting non-human primates and humans. The cyst has a spherical or slightly ovoid shape measuring 40-60µm. The trophozoite however is ovoid, covered with cilia, and larger in size with a length of 30-150µm and width of 25-120µm. The trophozoite possesses two nuclei, with a sausage-shaped macronucleus and rounded micronucleus (Schuster *et al.*, 2008). The trophozoite invades the large intestine and the incubation period is 4-5 days. The disease balantidiasis has mainly three categories of clinical manifestation. Firstly, the host may be an asymptomatic carrier of disease and play a role as a reservoir. Secondly, the host will pass mucoid, bloody stools, and experience weight loss, and tenesmus. Lastly, the host will have a chronic infection with diarrhea, cramping, halitosis, and abdominal pain (Schuster *et al.*, 2008).

Entamoeba is from the family of Entamoebidae under the phylum of Amoebozoa. The cyst are spherical and measure 10-35µm in diameter with a protection wall to survive in the environment from days up to weeks until transmission happens. They also contain one to eight nuclei. The trophozoite is ovoid in shape, measuring 15- 50µm in length, with ectoplasmic pseudopodia. It has a large irregular shaped karyosome with the nucleus arranged eccentrically (Yu, 2003). *Entamoeba* infections usually develop into clinical disease in 2-4 weeks, but may range from a few days to years. Clinical manifestation of *Entamoeba* infection (also known as amebiasis), include non-invasive intestinal tract infection, annular colonic granulation, and liver abscess. The onset of intestinal amebiasis is usually gradually for a few weeks of symptoms. Ameboma is another rare complication of intestinal amebiasis where it forms an annular colonic granulation which results in a large local lesion of the bowel (commonly cecum), and can be easily mistaken for colonic carcinoma (Dhawan, 2016). Development of liver abscess is by dissemination of the trophozoite *via* the hematogenous route lead to the liver or less commonly lung or brain abscessation (Manitoba, 2015; Heymann, 2008). The symptoms of these abscesses are fever, cough, and a constant, dull, aching abdominal pain in the upper quadrant or epigastrium (Petri *et al.*, 2010).

MATERIALS AND METHODS

Study area and animals

The study was conducted on the captive non-human primates from four different zoos in Peninsular Malaysia. The animals and numbers examined in the study were Bonnet macaque (*Macaca radiata*) (19), Borneo Orang Utan (*Pongo pygmaeus*) (17), Chimpanzee (*Pan troglodytes*) (13), Stumped tail macaque (*Macaca arctoides*) (12), Brown capuchin (*Sapajus apella*) (6), Sumatra Orang Utan (*Pongo abelii*) (4), White handed gibbon (*Hylobates lar*) (3), Siamang (*Symphalangus syndactylus*) (3), Mandril (*Mandrillus sphinx*) (3), Brown lemur (*Eulemur fulvus*) (3), Lion tailed macaque (*Macaca silenus*) (1), and Common marmoset (*Callithrix jacchus*) (1).

Sample collection, transportation, storage

Fresh fecal samples were collected early in the morning by 0800hrs, before the cages were cleaned in the morning. Fecal samples were collected from primates housed individually, or pooled if a number of individuals of the same species were housed together. The samples were transported chilled to the Parsitology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Fecal samples meant for microscopy were processed within the same day, while those meant for molecular detection were stored at -30°C prior to DNA extraction.

Microscopic identification

Fecal smears were prepared from fresh samples, fixed in methanol for five minutes, air-dried and stained with Giemsa and Ziehl-Neelsen acid-fast stains. The slides were examined under a light microscope at oil immersion 1000x magnification. A total of 100 microscope fields were examined per fecal smear.

DNA extraction and PCR amplification

Genomic DNA extraction was done on the fecal samples using the QiAamp DNA Stool Mini Kit (Qiagen, Italy) following the manufacturer`s instruction. To identify the enteric protozoa, fragments of nuclear genes were amplified by PCR using primers and protocols listed in Table 1. The PCR products were load on a 1.5% Agarose gel and electrophoresed for 50 minutes at 110 Volts. gels were stained with 0.01% Ethidium Bromide for 30 minutes, de-stain in distilled water for a few seconds and visualized under ultraviolet (UV) light.

Table 1. Primers used for PCR detection of the various enteric protozoa from faecal samples of primates in this study. Their respective thermocyclic profiles and expected amplicon size are indicated. ID = initial denaturing, D = denaturing, A = annealing, E = extension, FE = final extension.

Protozoa	Primers	Fragment	Thermocyclic profile	Size (bp)	Reference
Giardia	**Primary PCR** RH11 (5'-CAT CCG GTC GAT CCT GCC-3') RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3') **Nested PCR** Giar-F (5'-GAC GCT CTC CCC AAG GAC-3') Giar-R (5'-CTG CGT CAC GCT GCTC-3')	SSU-rRNA	ID: 95°C /2mins D: 95°C /20sec A: 59°C /20sec E: 72°C /20sec No of cycles: 35 FE: 72°C /7mins Similar protocol for the 2nd nested PCR.	200	(Lim *et al.*, 2013)
Cryptosporidium	**Primary PCR** XF2 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') XR2 (5'-AAGGAGTAAGGAACAACCTCCA-3') **Nested PCR** pSSUf (5'-AAAGCTCGTAGTTG- GATTTCTGTT-3') pSUr (5'-ACCTCTGACTGTTAAATACRAATGC-3')	pSSU-rRNA	ID: 94°C /5mins D: 94°C /45sec A: 45°C /2mins E: 72°C /90sec No of cycles: 30 FE: 72°C /10mins ID: 94°C /5mins D: 94°C /30sec A: 55°C /30sec E: 72°C /30sec No of cycles: 35 FE: 72°C /10mins	240	(Yap *et al.,* 2016)

Blastocystis	BhRDr (5'- GAGCTTTTTAACTGCAACAACG -3') RD5 (5'-ATCTGGTTGATCCTGCCAGT-3')	SSU-rDNA	ID: 94°C /5mins D: 94°C /1min A: 56°C /1min E: 72°C /1min No of cycles: 30 FE: 72°C /7mins	600	(Scicluna *et al.,* 2006)
Balantidium	B5D (5'-GCT CCT ACC GAT ACC GGGT-3') B5RC (5'-GCG GGT CAT CTT ACT TGA TTTC-3')	18s-rRNA	ID: 94°C /10mins D: 94°C /1min A: 59°C /1min E: 72°C /1min No of cycles: 35 FE: 72°C /5mins	500	(Gordo, 2008)
Entamoeba	Entam1 (5'-GTT GAT CCTGCCAGTATTATATG-3') Entam2 (5'-CAC TATTGG AGC TGG AAT TAC-3')	SSU-rRNA	ID: 94°C /5mins D: 94°C /1min A: 55°C /1min E: 72°C /1min No of cycles: 35 FE: 72°C /7mins	550	(Sukprasert *et al.,* 2008)

RESULTS

The overall PCR prevalence of zoonotic enteric protozoa among captive primates in Malaysian zoos using PCR detection were 65% for *Entamoeba*, 50% for *Blastocystis*, 20% for *Cryptosporidium*, 20% for *Balantidium*, and 5% for *Giardia.* However, the microscopy examination revealed a prevalence of 30% for *Blastocystis*, 12.5% for *Entamoeba*, 10% for *Cryptosporidium*, and 2.5% for *Balantidium*. *Giardia* was not detected by microscopy examination of the fecal smears. Positive amplicons detected by PCR are depicted in Figure 1, while microscopy images of the protozoa are provided in Figure 2.

The prevalence of *Giardia* was the lowest with only two positive samples namely from Siamang and Chimpanzee, accounting for 7.1% and 11.1%, of the samples from Zoo A and C, respectively (Figures 3 and 4). Fecal samples positive for *Cryptosporidium* include 1/3 of the Chimpanzee samples, 1/1 of White-handed Gibbon, and 1/2 of the Siamang samples from Zoo A. This accumulated to a prevalence of 14.3% for *Cryptosporidium* at this zoo. In Zoo B, fecal samples of 1/1 White-handed Gibbon and 1/2 Brown Capuchin were positive for *Cryptosporidium*, amounting to a prevalence rate of 25.0%. In Zoo C, 1/1 of the Borneo Orang Utan pooled sample, 2/2 of the White-handed Gibbon pooled samples and 1/1 of the Mandrill pooled sample were positive for *Cryptosporidium* accounting for a prevalence rate of 44.4% at the facility. *Cryptosporidium* was not detected from the samples collected at Zoo D.

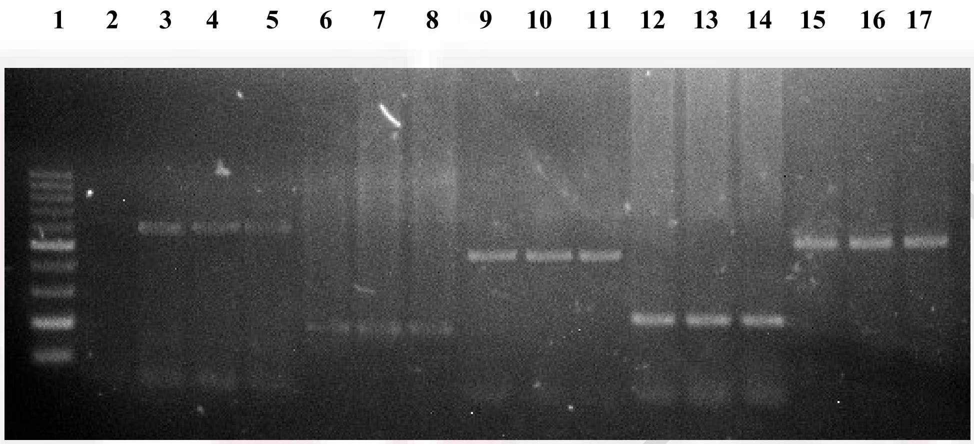

Figure 1. Gel electrophoresis showing PCR amplicons of the various zoonotic enteric protozoa detected in primates from zoos in Peninsular Malaysia. Lane 1 (100bp size ladder), Lane 2 (negative control), Lane 3 (*Blastocystis* positive control), Lanes 4 & 5 (*Blastocystis* positive samples), Lane 6 (*Giardia* positive control), Lane 7 & 8 (*Giardia* positive samples), Lane 9 (*Balantidium* positive control), Lane 10 & 11 (*Balantidium* positive samples), Lane 12 (*Cryptosporidium* positive control), Lane 13 & Lane 14 (*Cryptosporidium* positive samples), Lane 15 (*Entamoeba* positive control), Lane 16 & 17 (*Entamoeba* positive samples)

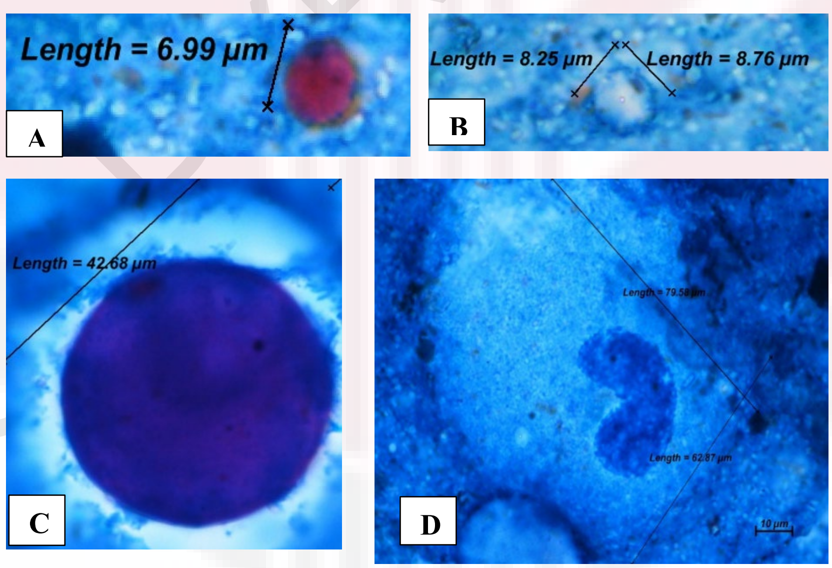

Figure 2. Microscopy detection of various zoonotic enteric protozoa detected in primates from zoos in Peninsular Malaysia. (A) *Cryptosporidium*, (B) *Blastocystis*, (C) *Entamoeba* and (D) *Balantidium*.

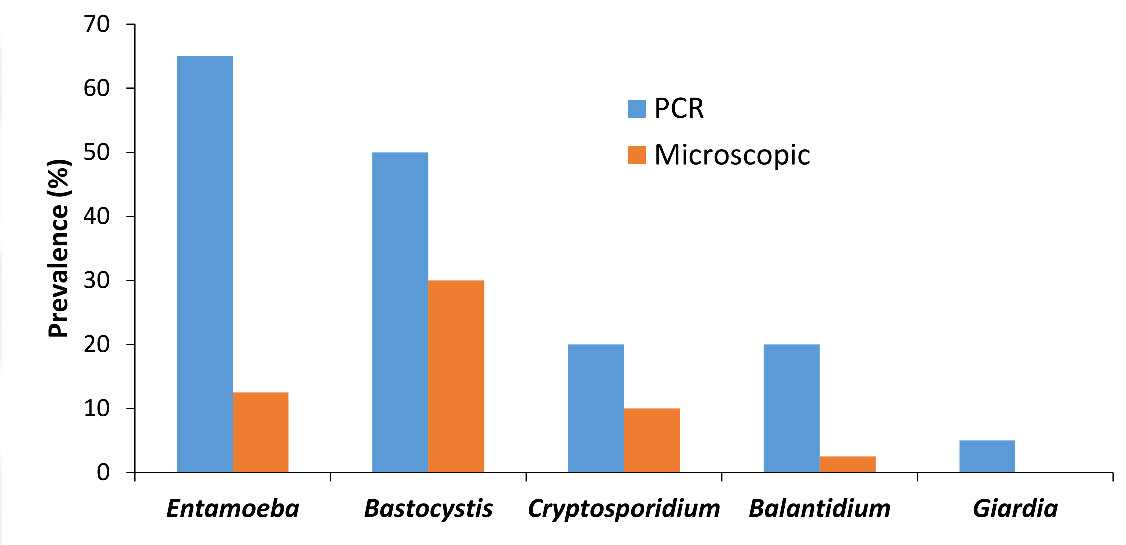

Figure 3: Overall prevalence of zoonotic enteric protozoa among captive primates in Peninsular Malaysian.

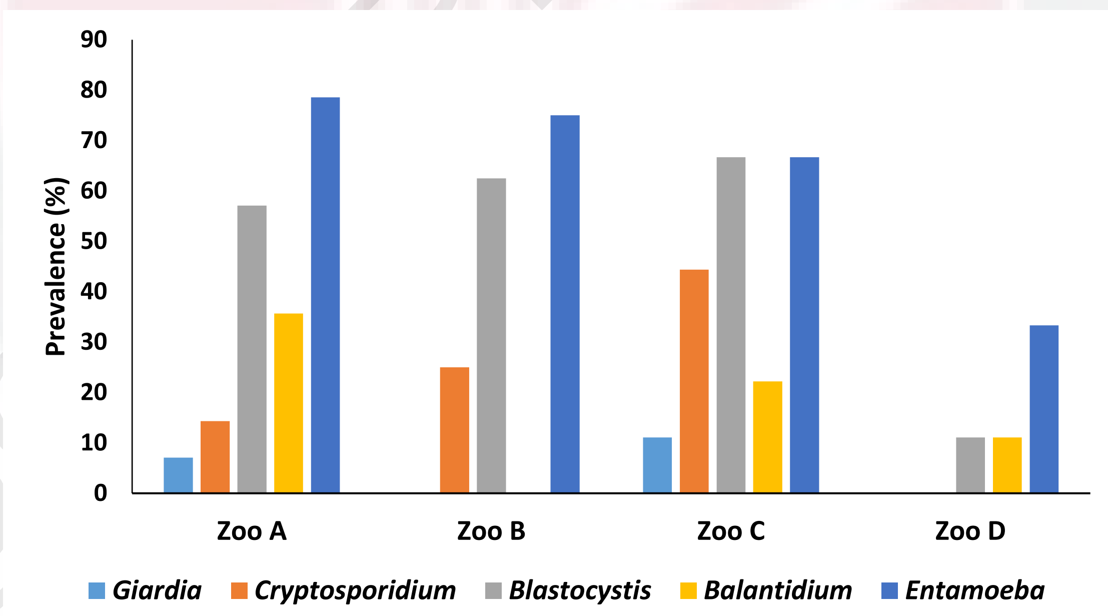

Figure 4. Comparative prevalence of zoonotic enteric protozoa (PCR detection) among zoos in Peninsular Malaysia.

Blastocystis infection was detected in 3/4 Sumatra Orang Utan fecal samples, 1/1 of the White-handed Gibbon, 2/2 of the Siamang, 1/1 of the Stump-tailed Macaque, and 1/1 of the Bonnet Macaque samples collected in Zoo A. This amounted to a prevalence rate of 57.1% for *Blastocystis* at the zoo. In Zoo B *Blastocystis* was apparent in 1/1 of the White-handed Gibbon pooled sample, 1/1 of the Siamang, 1/2 of the Brown Capuchin, 1/1 of the Common Marmoset, and 1/1 of Brown lemur pooled sample, which contributed to a 62.5% prevalence rate at Zoo B. In Zoo C, 1/1 of Borneo Orang Utan pooled fecal sample, 2/2 of White-handed Gibbon, 2/2 of Stump-tailed Macaque, and 1/1 of Mandrill samples were positive for *Blastocystis*, resulting in a prevalence of 66.7%. Only 1/9 (11.1%) pooled fecal samples collected from the Borneo Orang Utans was PCR positive for *Blastocystis*.

Balantidium infection was detected in 2/4 of the Sumatra Orang Utan pooled fecal samples, 1/2 of the Chimpanzee, 1/1 of the Stump-tailed Macaque, and 1/1 the Bonnet macaque kept at Zoo A. This accounted for a *Balantidium* prevalence rate of 35.7% at Zoo A. *Balantidium* was not detected among the primates kept in Zoo B. In Zoo C, the protozoa was present in 2/2 of the Stump-tailed macaque pooled fecal samples, leading to a prevalence rate of 22.2%. There was a single fecal sample (11.9%) positive for Balantidium among the nine Borneo Orang Utan pooled fecal samples collected at Zoo D.

Entamoeba was detected in 4/4 of the Sumatra Orang Utam pooled fecal samples, 2/3 of the Chimpanzee, 1/1 of the White-handed Gibbon, 2/2 of the Siamang, 1/1 of the Stump-tailed Macaque, and 1/1 of the Bonnet Macaque samples from Zoo A. The prevalence of this protozoa at this zoo was 78.6%. In Zoo B Melaka, 1/1 of the Borneo Orang Utan, 1/1 of the White-handed Gibbon, 1/1 of the Siamang, 1/2 of the Brown Capuchin, 1/1 of the Common Marmoset, and 1/1 of the Brown Lemur pooled fecal samples were found to be positive for *Entamoeba*, with a prevalence rate of 75.0%. In Zoo C, 1/1 of the Borneo Orang Utan pooled fecal sample, 2/2 of the White-handed Gibbon, 2/2 of the Stump-tailed Macaque, and 1/1 of the Mandrill samples were positive for *Entamoeba*, contributing to 66.7% prevalence rate. In Zoo D, 3/9 (33.3%) of the Borneo Orang Utan were positive for this protozoa.

A summary of the presence and co-infection with *Giardia*, *Cryptosporidium*, *Blastocystis*, *Balantidium*, and *Entamoeba* among various species of captive primates in the four zoological facilities in Peninsular Malaysia is presented in Tables 2 and 3. By running Pearson Chi-Square, the relationship between the prevalence of these zoonotic enteric protozoa to the captive facilities is not significant ($P<0.05$). However, by comparing the co-infection of *Giardia*, *Cryptosporidium*, *Blastocystis*, *Balantidium*, and *Entamoeba* among various species of captive primates in four zoological facilities in Peninsular Malaysia, the Siamang (*Symphalangus syndactylus*) and Stump-tailed Macaque (*Macaca arctoides*) had a higher diversity of zoonotic enteric protozoa. The overall occurrence of protozoa genera co-infection is presented in Figure 5.

Table 2. Summary of the presence and co-infection with *Giardia* (*Gi*), *Cryptosporidium* (*Cr*), *Blastocystis* (*Bl*), *Balantidium* (*Ba*), and *Entamoeba* (*En*) among various species of captive primates in four zoological facilities in Peninsular Malaysia. Back slash (/) indicates the absence of the particular primate species in the facility, while "x" indicates the absence of infection among the primates examined.

Primate species	Zoological Facility				No. of genera
	A	B	C	D	
Sumatra Orangutan (*Pongo abelii*)	*Bl, Ba, En*	/	/	/	3
Borneo Orangutan (*Pongo pygmaeus*)	X	*En*	*Cr, Bl, En*	*Bl, Ba, En*	4
Chimpanzee (*Pan troglodytes*)	*Cr, En*	/	*Gi*	/	3
White-handed Gibbon (*Hylobates lar*)	*Cr, Bl, En*	*Cr, Bl, En*	*Bl, En*	/	3
Siamang (*Symphalangus syndactylus*)	*Gi, Cr, Bl, Ba, En*	*Bl, En*	/	/	5
Lion-tailed Macaque (*Macaca silenus*)	X	/	/	/	0
Stump-tailed Macaque (*Macaca arctoides*)	*Bl, Ba, En*	/	*Cr, Bl, Ba, En*	/	4
Bonnet Macaque (*Macaca radiata*)	*Bl, Ba, En*	/	/	/	3
Mandrill (*Mandrillus sphinx*)	/	X	*Cr, Bl, En*	/	3
Brown Capuchin (*Sapajus apella*)	/	*Cr, Bl, En*	/	/	3
Common Marmoset (*Callithrix jacchus*)	/	*Bl, En*	/	/	2
Brown Lemur (*Eulemur fulvus*)	/	*Bl, En*	/	/	2

Table 3. Collective number of positive samples for *Giardia* (*Gi*), *Cryptosporidium* (*Cr*), *Blastocystis* (*Bl*), *Balantidium* (*Ba*), and *Entamoeba* (*En*) among the various species of captive primates in four zoological facilities in Peninsular Malaysia.

Primate species	N	*Gi*	*Cr*	*Bl*	*Ba*	*En*
Sumatra Orangutan (*Pongo abelii*)	4	0	0	3	2	4
Borneo Orangutan (*Pongo pygmaeus*)	12	0	0	2	1	5
Chimpanzee (*Pan troglodytes*)	6	1	1	0	0	2
White-handed Gibbon (*Hylobates lar*)	4	0	2	4	0	3
Siamang (*Symphalangus syndactylus*)	3	1	1	3	1	3
Lion-tailed Macaque (*Macaca silenus*)	1	0	0	0	0	0
Stump-tailed Macaque (*Macaca arctoides*)	3	0	0	3	3	3
Bonnet Macaque (*Macaca radiata*)	1	0	0	1	1	1
Mandrill (*Mandrillus sphinx*)	2	0	0	1	0	2
Brown Capuchin (*Sapajus apella*)	2	0	1	1	0	1
Common Marmoset (*Callithrix jacchus*)	1	0	0	1	0	1
Brown Lemur (*Eulemur fulvus*)	1	0	0	1	0	1

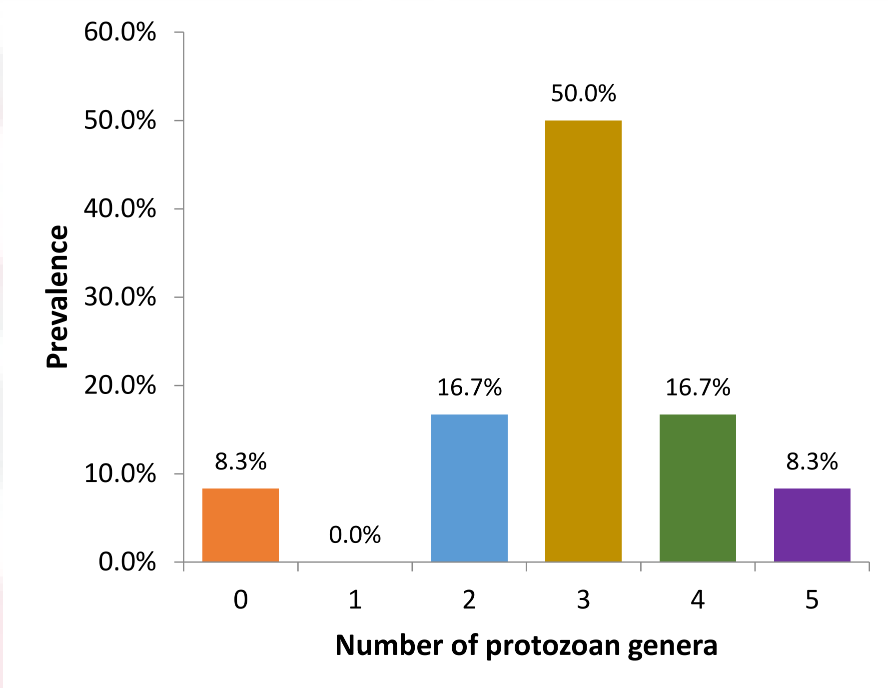

Figure 5. Prevalence (%) of enteric protozoa genus co-infection among captive primates in Peninsular Malaysia. Triple species co-infection recorded the highest prevalence, followed closely by double species and four species co-infection, next is free from any infection and five species co-infection, and lastly none of the primates have infected by only one single species.

DISCUSSION

The present study constitutes the first attempt in the country to detect zoonotic enteric protozoa among captive primates using PCR methods. Results revealed a high prevalence of *Entamoeba* (65%) which was the most dominant enteric protozoa among the primates, followed by *Blastocystis* (50%), *Balantidium* (20%) and *Cryptosporidium* (20%). The least prevalent enteric protozoa was *Giardia* (5%). previous studies have also demonstrated the presence of these pathogens among captive primates, and have stressed to implications of zoonotic transmission (Lim *et al.*, 2008; Kilbourne *et al*., 2003). These zoonotic enteric protozoa are easily transmitted from one animal to another, mainly due to the simplicity of their lifecycle, and the fact that there is no requirement for an intermediate host or vector. These pathogens are readily transmissible once shed by the patient or carrier. Furthermore, the incubation period require by these protozoa are short and they require a low dose to be infective, which makes them hard to control and increases the prevalence (Tanyuksel *et al*., 2003).

Compared to a previous microscopy screen among captive primates at a local zoo (Lim *et al*., 2008), *Balantidium* was present at a prevalence of 19.2%, followed by *Cryptosporidium* (14.1%) and *Blastocystis* (2.1%). Both *Giardia* and *Entamoeba* were not detected in this study. The microscopy identification method employed may be less efficient at detecting these protozoa compared to the present investigation. Microscopy screens among captive and semi-captive orang utans in Sabah, Malaysia revealed a prevalence of 14% for *Balantidium coli* (Kilbourne *et al*., 2003). Cage and environmental hygiene may be an important contributing factor to the high prevalence of zoonotic enteric protozoa encountered in this study. Most of the zoos only clean the cages once a day and fecal material built-up throughout the course of the day is left unattended. In addition, the primates frequently feed on the ground and the food is easily contaminated with these pathogens. The movement of keepers between enclosures is also a contributing factor to the spread of these pathogens. The absence of a functional disinfectant protocols will enable the protozoa to be easily transmitted around the zoo via fecal material attached to boots and clothing of the staff. Furthermore equipment are shared among cages with no proper disinfection. .

Pearson Chi-Square revealed a lack of significant association between the prevalence of zoonotic enteric protozoa and the particular captive facility. This could be explained by the husbandry practices at these zoos which in most instances are similar. These include feeding, deworming and vaccination protocols, biosecurity, interior design of the cages and flooring, and cleaning routine among others. To be able to reduce the prevalence of these zoonotic enteric protozoa, preventive measures are crucial. The animals should be treated for these infections and the cages should be cleaned more frequently to reduce the possibility of fecal-oral transmission. The contamination of drinking water is also an important facet in the transmission of these pathogens (Schuster, 2008). It is crucial to provide a clean and uncontaminated water source for animal consumption and also for cleaning purposes. The major feeding area should also be elevated from the ground to minimize transmission (Schuster, 2008). The practice of proper disinfection for the keeper's boots, and the use of disposable gloves and equipment,.

have also been suggested to avoid spreading of the infection among the cages (Berrilli *et al.*, 2011). To prevent zoonotic transmission to the zoo keepers, it is also advisable for them to wear disposable gloves during cleaning the cages and feeding the animals. A way to prevent zoonotic transmission to the visitors, is increasing the space between the animal cage and visitor, and to prevent any form of contact between the primates and the visitors. Another way to protect the visitors or prevent pathogens from being introduced into the zoo by the visitors is to introduce a foot disinfection dip at the entrance and exit of the captive facility.

The zoos that have infected primates should be notified in order for treatment and control measures to be carried out. They should also be advised about the potential for zoonotic transmission among the staff and visitors. It is also advisable for the zoo keepers to screen themselves for any possible infection. Metronidazole is an effective drug to treat infection with these pathogens especially for Giardiasis, Blastocystis infection and Amebiasis (Escobedo *et al.*, 2007; Bratt *et al.*, 1990; Haque *et al.*, 2003). Another study advocated the use of Secnidazole (nitroimidazole) for effective treatment for *Balantidium coli* (Bilal *et al.*, 2009). At present there are no effective drugs available to treat human or animal Cryptosporidiosis (Fayer *et al.*, 2007; Chen *et al.*, 2002; O'Donoghue *et al.*, 1995). However, O'Donoghue *et al.* (1995) advocates supportive treatment with oral or intravenous fluid for electrolyte replacement as it has been shown to effectively correct the dehydration caused by the acute diarrhea. In order to rest the gut from digesting more food, parenteral nutrition may be necessary, and anti-diarrheal medication could be used to control the fluid loss from diarrhea.

CONCLUSION

The present molecular investigation has revealed a high prevalence of zoonotic enteric protozoa among the captive primates in this country. These findings merit further investigation on the epidemiology of these zoonotic enteric protozoa, risk factors of their infection, and their potential for zoonotic transmission to humans. Basic epidemiological data and genotyping is crucial for these infections are to be managed, and to prevent outbreaks. Captive facilities play an important role in controlling the diseases as they are a source for human infection. The staff who are in close contact with the primates are at a high risk of being the first line of human transmission. Proper biosecurity measures must be instituted at the zoos and this will greatly reduce the chances of zoonotic transmission to humans. Prophylaxis plays an important role to prevent shedding of these pathogens as most of the infected host can be asymptomatic carriers. The prevalence of these enteric pathogens are present in all of the zoos studied, indicating that lack of proper hygiene and biosecurity is a common element that needs urgent attention. Controlling zoonotic diseases should be a concerted effort by all the stakeholders which include the zoo facility and its staff, veterinary and medical officers, and the public at large. Education and awareness on hygiene and biosecurity at personal and facility-wide levels are crucial to ensure the containment of these zoonotic infections.

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