

Genotyping of *Toxoplasma gondii* Isolates from Wild Boars in Peninsular Malaysia

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Abstract

Toxoplasma gondii is a parasitic protozoan that infects nearly one-third of the world population. The present study was done to isolate and genotype *T. gondii* from wild boar from forests of Pahang, Malaysia. A total of 30 wild boars' blood, heads and hearts were obtained for this study and 30 (100.0%) were found to be seropositive when assayed with modified agglutination test (MAT \geq 6). The positive samples were inoculated into mice and *T. gondii* was only isolated from samples that had strong seropositivity (MAT \geq 1:24). The isolates were subjected to PCR-RFLP analysis and all the Peninsular Malaysia isolates of *T. gondii* are of clonal type I.

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Introduction

Toxoplasma gondii is a cosmopolitan protozoan that causes an important zoonosis. It is considered as the most successful parasitic pathogen worldwide [1]. Although all warm blooded animals including marine mammals [2] and birds are susceptible to the infection, only felids are the definitive host of the parasite. Infected felids shed environmentally resistant oocysts which become infectious upon sporulation [3]. Once these oocysts are ingested, the protozoa alters its form to rapidly dividing tachyzoite, and then to slower dividing bradyzoites that encysts in brain, muscle and nervous tissues [4]. This provides the parasite an important pathway to successfully transmit the infection via consumption of uncooked or undercooked meat [5]. One such incidence was reported by Choi et al. [6] who described a case whereby *T. gondii* infection was acquired from eating raw wild boar (*Sus scrofa*) meat that led to blindness and loss of vision.

Wild boar meat is considered a delicacy in Malaysia and often enjoyed by the ethnic Chinese community. The Act 76 of Malaysian Protection of Wild Life Act (1972) lists wild boar under "Protected Wild Animals" category. However, the act also recognizes wild boar as a game animal and thus hunting is allowed but the numbers that can be hunted are seriously limited and the licensed hunters are monitored accordingly.

Genotyping study on local isolates of *T. gondii* in South-East Asian region is minimal with only one report from Vietnam by Dubey et al. [7]. In relation to this, only seroprevalence of *T. gondii* in animals has been studied in Malaysia. In one such study, Rajamanickam et al. [8] reported 15.6% infection in the pigs; while in another study, Chandrawathani et al. [9] reported no infection in pigs. With the aim of increasing our knowledge on the *T. gondii* genotypes in this region, the present study was designed to determine whether the local wild boars in Malaysia are infected with *T. gondii*.

Materials and Methods

Wild boar sampling

All the animals used in this study originated from forests in Pahang, a state in the east coast of Peninsular Malaysia. A total of 30 samples from adult boars were obtained from multiple licensed hunters who hunted the wild boars with rifles. The hunters did not hunt the animals especially for the purpose of this study; rather, they hunt the boars for the meat and the samples collected for this study are usually deemed as waste by them. Blood, brain and heart of the animals were collected and shipped to our laboratory in ice boxes to maintain the temperature at 4°C. The samples were processed within 5 days from the day it was collected from the hunters. Serum samples were kept at -80°C.

Serological examination

Serum samples from the wild boars were tested for IgG antibodies using the modified agglutination test (MAT), as described by Dubey and Desmonts [10]. Whole RH tachyzoites were obtained from intraperitoneum of experimentally infected mice and stored in -20°C until further use. The samples were diluted two fold starting at 1:6 dilutions, and both positive and negative controls were included. Agglutination reaction at a titer of 1:6 and above was regarded as positive.

Animal ethics

Approval (USM/PPSF/50(088)Jld.2) was obtained from USM animal research ethics committee for use of mice for intraperitoneal infection of wild boar samples and to maintain the RH strain *in-vivo*. All animal work carried out in this research were in accordance to the guidelines set by The Animal Research Ethics Committee of USM (JEHUSM).

Isolation and bioassay for *T. gondii* infection

The brain and heart from the same wild boar sample with MAT titer of $\geq 1:6$ were pooled, homogenized with phosphate buffered saline (PBS), digested with acidic pepsin solution by incubation at 37°C for 1 hour, and then neutralized with 1.2% sodium bicarbonate as described by Dubey [11]. The neutralized mixture was then filtered through a 100 μ m glass filter and the filtrate was pelleted in order to remove unwanted particles and concentrate the parasite in order to reduce the number of mice needed for infection as explained by Puvanuesuaran et al. [12]. The pellet was then mixed with PBS containing 100 units of penicillin and 100 μ g of streptomycin per ml before infecting a group of two mice per sample. Mice from Swiss albino strain used in this study were obtained from USM Animal facility. Brains of the mice that died were collected and stored in PBS at 4°C. In addition, intraperitoneal fluids were collected if present.

Table 1. RFLP markers and restriction enzymes used in this study.

Markers	Restriction Enzyme
3'-SAG2	Hha I
5'-SAG2	Sau3A I
BTUB	BsiE I and Taq I
cB21-4	Hae III
GRA1	Hind III and Nla IV
GRA6	Mse I
SAG3	Nci I
SRS1	ApaI I and Hae II

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Table 2. MAT titer of wild boar samples, isolation information from infected mice and MAT titer results of infected mice.

Sample ID	MAT titer of wild boar	Isolation in mice	MAT titer of mice	Time of death (p.i)
WB 1	96	2/2	ND ^a ,ND	72 h/72 h
WB 2	64	2/2	ND,ND	96 h/96 h
WB 3	6	0/2	0,0	E/E
WB 4	6	0/2	0,0	E/E
WB 5	6	0/2	0,6	E/E
WB 6	6	0/2	0,0	E/E
WB 7	6	0/2	0,12	E/E
WB 8	6	0/2	0,0	E/E
WB 9	24	2/2	ND,ND	144 h/144 h
WB 10	24	1/2	ND,6	144 h/144 h
WB 11	6	0/2	0,0	E/E
WB 12	6	0/2	0,0	E/E
WB 13	6	0/2	6,6	E/E
WB 14	6	0/2	0,0	E/E
WB 15	64	2/2	ND,ND	96 h/120 h
WB 16	48	2/2	ND,ND	120 h/120 h
WB 17	6	0/2	12,0	E/E
WB 18	6	0/2	0,0	E/E
WB 19	6	0/2	0,0	E/E
WB 20	6	0/2	0,0	E/E
WB 21	12	0/2	6,12	E/E
WB 22	6	0/2	0,6	E/E
WB 23	12	0/2	12,6	E/E
WB 24	6	0/2	0,0	E/E
WB 25	6	0/2	0,0	E/E
WB 26	6	0/2	0,0	E/E
WB 27	6	0/2	0,0	E/E
WB 28	6	0/2	6,0	E/E
WB 29	6	0/2	0,0	E/E
WB 30	6	0/2	6,0	E/E

ND^a: No data.

E: Euthanized.

h: hours.

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Table 3. Genotypes of *T. gondii* isolates from wild boar in Pahang, Malaysia.

Isolate Designation	3'+5' SAG2	BTUB	cB21-4	GRA1	GRA6	SAG3	SRS1	Genotype
WB1-a*	I	I	I	I	I	I	I	I
WB1-b*	I	I	I	I	I	I	I	I
WB2-a	I	I	I	I	I	I	I	I
WB2-b	I	I	I	I	I	I	I	I
WB9-a	I	I	I	I	I	I	I	I
WB9-b	I	I	I	I	I	I	I	I
WB10-a	I	I	I	I	I	I	I	I
WB15-a	I	I	I	I	I	I	I	I
WB15-b	I	I	I	I	I	I	I	I
WB16-a	I	I	I	I	I	I	I	I
WB16-b	I	I	I	I	I	I	I	I
RH	I	I	I	I	I	I	I	Ref# (I)
ME49	II	II	II	II	II	II	II	Ref (II)
VEG	III	III	III	III	III	III	III	Ref (III)

*a,b: are isolates from two different mice.

#: Indicates reference strains.

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Mice that survived 21 days post-infection (p.i) were bled through their orbital sinus as described by Hoff [13] to check for *T. gondii* antibodies by MAT. Surviving mice were euthanized at 45 days p.i and their brains were collected and stored in PBS at 4°C.

The mice organs were separately homogenized and centrifuged at 500 × g for 2 min. The resulting supernatant was collected and pelleted by centrifugation at 3,000 × g for 15 min. The pellet was used for DNA extraction using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the protocol suggested by the manufacturer. The quality and concentration of the isolated DNA was checked using the Nanophotometer (Implen, Munich, Germany), then stored at -20°C. The DNA templates used in this study were between 50–100 ng/μl and the ratios of A₂₆₀/A₂₈₀ were between 1.8–2.0.

PCR-RFLP analysis

PCR amplification was performed using eight different markers (3' SAG2, 5' SAG2, BTUB, cB21-4, GRA1, GRA6, SAG3 and SRS1) under previously optimized conditions as reported by Puvanesuaran et al. [14]. The PCR amplification process was carried out in the 96-well thermal cycler (MyCycler, Bio-Rad, California, USA). The reaction mixture was denatured at 95°C for 5 minutes; followed by denaturing step for 30 seconds, annealing for 30 seconds and extension at 72°C for 1 minute for 40 cycles. Final extension was at 72°C for 10 minutes. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and digested using appropriate restriction enzymes as listed in Table 1; each digestion was carried out according to the conditions suggested by the manufacturer (New England Biolabs, Massachusetts, USA). The RFLP analysis was carried out and analyzed by referring to the data in *Toxoplasma* Genome Map Database [http://toxomap.wustl.edu/Toxo_Genetic_Map_Table.html]. The positive controls used in this study were from previously isolated genomic DNA from RH (type I), ME49 (type II) and VEG (type III) strains of *T. gondii*. Water was used as negative control in this study.

Results and Discussion

The peninsular of Malaysia has an abundance of rainforest and is estimated to contain 20% of all animal species in the world [15]. The vast forests in the state of Pahang are a known domain for a variety of wild felids such as tigers (*Panthera tigris*), panthers (*Panthera pardus*), clouded leopards (*Neofelis nebulosa*) and many others. Sampling of wild boar samples from this area can help determine if the soil is contaminated with *T. gondii* and of which genotype.

Shaapan et al. [16] reported that MAT was more specific and sensitive in comparison to ELISA, thus MAT was used as the serological test in this study. All the 30 samples were found to have a minimal MAT titer of 1:6 and were bioassayed into 30 groups of mice. Six samples (6/30, 20.0%) had MAT titer of ≥1:24 which indicated strong positive reaction [17]. The results of MAT are summarized in Table 2. Among the mice that were inoculated with samples from animals that had MAT titer of ≥1:24, 11 (11/12, 91.67%) died within 1 week p.i. Estimated time of death in hours p.i for these mice are listed in Table 2, while the symptoms observed on these mice were consistent to the observations made by Hisaeda et al. [18] on outbred mice infected with RH (type I) strain of *T. gondii*. This is also an indicator to the virulence of the isolates which are similar to the archetypal type I strains that are uniformly lethal in outbred mice [19]. These mice showed signs of sickness as described by Hrda et al. [20]; MAT was not performed on these mice as blood was not collected from the carcasses while the organs were harvested. The rest of the infected mice were tested for *T. gondii* serology; the results showed that 49 (49/49, 100.0%) had MAT titer of ≤1:24. These results too have been summarized in Table 2 along with information on the number of successful isolations obtained from the infected mice.

Dubey [17] reported that when MAT titers are 1:24, the specificity for detection of *T. gondii* antibodies are very high. This study showed that 6 of the 30 (20.0%) wild boar samples had MAT titer of ≥1:24. The wild boar samples with lower titers were still processed and inoculated into mice as there have been studies reporting isolation of viable *T. gondii* from samples of low MAT titers (MAT>1:5) [21]. Recent serological studies carried out in

China by Cong et al. [22] to detect seropositivity of *T. gondii* in market sold animals also used similar MAT titer. However, when inoculated into mice only the samples with MAT titer of $\geq 1:24$ had *T. gondii* infection in current study, thus corroborating the above observation by Dubey [17]. Nevertheless, not all samples with MAT titer of $\geq 1:24$ had successful isolation of the parasite from the infected mice as observed in sample WB10 in which only one mouse had the infection resulting in a successful isolate, while the other mouse had a MAT titer of 1:6 and was negative by PCR amplification. Thus for future studies, MAT titer should be set at higher values ($\geq 1:24$) when the parasite must be isolated to reduce false positive results as well as to help reduce the number of mice sacrificed for similar studies.

The 49 mice which survived 45 p.i were euthanized and DNA was extracted from their brains, and tested with two PCR markers (*GRA6* and *cB21-4*), and the results were negative for all samples. DNA extracted from mice that died within a week p.i was also tested using the same markers and all were positive. The positive samples were then subjected to PCR amplification and RFLP analysis using other markers namely *3'SAG2*, *5'SAG2*, *BTUB*, *GRA1*, *SAG3* and *SRS1*. The results are summarized in Table 3.

All the isolates obtained in this study were determined to be of type I strain of *T. gondii*. Type I strains are known to be very virulent to mice and causes parasitemia; they are also closely associated with human congenital toxoplasmosis [23]. The only

other similar study carried out in this region was on dogs in Vietnam; it was observed that among the 42 samples tested, 50% were seropositive and two different atypical strains were successfully isolated from 8 of the 21 positive samples [7].

To the best of our knowledge, this is the first reported study on *T. gondii* serology and genotyping from wild boar samples in this region. Nimir et al. [24] reported that in Malaysia, most people who were found seropositive to *T. gondii* infection were from rural areas and at some point of their life has consumed undercooked or raw meat. Since free-range meat products are quite commonly consumed in Malaysia and regional countries, further genotyping of local isolates of *T. gondii* should be carried out.

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Author Contributions

Conceived and designed the experiments: VRP RN VB. Performed the experiments: VRP. Analyzed the data: VRP VB. Contributed reagents/materials/analysis tools: RN VB. Wrote the paper: VRP RN VB.

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