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RESEARCH ARTICLE

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BLOOD MEAL IDENTIFICATION OF FIELD COLLECTED ON-HOST TICKS SURROUNDING TWO HUMAN SETTLEMENTS IN MALAYSIA

ABSTRACT

The objective of this study is to apply a rapid method for molecular blood meal identification of on- host field-collected ticks. Polymerase Chain Reaction (PCR) using universal primers complementary to the conserved region of the mitochondrial DNA (mtDNA) cytochrome b (cytb) gene fragment was performed on DNA samples of on-host ticks surrounding two human settlements i.e in Janda Baik, Pahang and Labu, Negeri Sembilan. DNA was amplified using PCR and the products were visualized on gel electrophoresis prior to DNA sequencing. The obtained sequences were compared with those in the GenBank database using BLAST program to identify the host species. A total of thirty individuals comprising 4 species of animals were examined. The species were Rattus tiomanicus, Maxomys rajah, Sundamys muelleri and Tupaia glis. From these animals, thirty five on-host ticks from 4 genera namely Ixodes, Dermacentor, Haemaphysalis and Amblyomma were extracted. After PCR amplification and DNA sequencing, only 19 ticks (54.2%) showed amplicons of the expected size with the similarity range of 88 – 99% with those in the GenBank. This study indicates that the PCR direct sequencing system using universal primers for vertebrate cytb gene was a potential convenient alternative rapid screening tool for tick's blood meal identification.

KEYWORDS: Blood meal, On-host tick, Polymerase Chain Reaction, Human settlement

1. INTRODUCTION

After mosquitoes, ticks are the second most important arthropod vector of diseases to both human and other vertebrates [1, 2]. Ticks on animals especially rodents that live in close proximity with humans plays a significant role in the transmission of several diseases [3]. It is essential to identify the natural hosts of ticks which can be potential reservoirs of pathogens. Identification of the hosts via blood meal analysis (BMA) is very important in understanding the feeding behavior of ticks. The analysis has also been used in epidemiological study of diseases transmitted by ticks, forensic medicine and crime scene investigations [4]. Knowledge on vector host preferences of ticks is critical in understanding transmission cycles of tick-borne diseases and provides important information for development of more effective control strategies.

Immunological analyses have been commonly used in the identification of blood meals of arthropods including ticks [5, 6]. The availability of DNA sequence data of various vertebrates has led to development of molecular-based approaches for blood meal analysis, such as Polymerase Chain Reaction (PCR). BMA using PCR is a useful tool for researchers studying disease vectors such as mosquitoes and ticks [7, 8]. An understanding of the source of blood meals will help to identify potential hosts involved in the transmission cycles of tick-borne diseases or those that posed risk to human. In the current study, primers were used to amplify multiple copies of DNA from vertebrate blood meal. One of the commonly targeted DNA is mitochondrial DNA (mtDNA) cytochrome b (cytb) gene which is then sequenced and compared against known sequences in DNA databases to identify the source of blood. MtDNA is a valuable molecule when working with small amounts of DNA due to its potential presence in a single cell and occupying up to 17% of the total cell volume [9]. Moreover, vertebrate mitochondrial DNA evolves roughly 10 times faster than nuclear DNA and the rapid fixation of mutations within species makes it ideal for species identification [10].

In this study, a rapid molecular method was applied to identify blood meals of on-host ticks collected from surroundings of two human settlements.

2. MATERIALS AND METHODS

2.1 Study area.

Human settlements in two different states in Peninsular Malaysia were visited to collect on-host ticks. The study areas were Kampung

Cheringin in Janda Baik, Pahang (3°31'N, 101°55'E) and Kampung Lambar in Labu, Negeri Sembilan (2°46'N, 101°49'E). The settlements were chosen based on the previous data of high numbers of tick infestation on animals. The natural ecology in the settlement in Pahang is mainly pristine tropical lowland rainforest while that in Negeri Sembilan is characterized by secondary growths, scrubs and riverine vegetation. The main occupations of the populations in Janda Baik and Labu are farmers and rubber-tappers, respectively.

2.2 Tick collection and identification.

One hundred wire traps were used to capture wild rodents and tree-shrews in each study site. Traps were placed on the ground and tree branches along existing trails at approximately five meters' interval. Traps were baited with bananas, oil palm fruits, tapioca or potatoes and checked once daily for 5 consecutive days of trapping. Caught animals were placed in cloth bags and brought back to the Institute for Medical Research (IMR) for further processing. The animals were anesthetized with chloroform and identified following Medway, Francis and Payne et al [11-13]. The on-host ticks were collected either using sterile soft forceps or sharpened wooden applicator sticks. Body engorgement index of the ticks was also determined. The ticks were then kept individually in vials containing 70% ethanol prior to identification using specific morphological taxonomic keys.

2.3 DNA extraction of blood meals in ticks.

Prior to DNA extraction, each engorged tick was individually washed 3 times with sterile distilled water. Extraction of DNA using QIAamp Mini Kit (Qiagen, Germany) was performed according to the manufacturer's protocol. The DNA was then used for subsequent PCR.

2.4 PCR amplification of the mtDNA cytb gene.

PCR was conducted with a set of vertebrate-specific primers and reaction conditions as described by Kent & Norris [14] that preferentially amplified a 623-bp region of the cytb gene within the mitochondrial DNA of vertebrates. The PCR reactions were conducted in 50 µl reaction tubes with the following reagents: 25 µl Taq PCR master mix, 0.5 µM of each primer, 10 µl of nuclease free water and 10 µl of DNA template. The amplification program consists of a total of 35 cycles, denaturing at 94^0C for 3 mins, annealing at 52^0C for 1 min, and extension at 72^0C for 1 min, with an initial denaturation at 94^0C for 1 min. For each PCR reaction, a negative control containing distilled water and a positive control containing vertebrate DNA were included. The amplicons were visualized in 1.5% agarose gels stained with ethidium bromide under ultraviolet light.

2.5 DNA sequencing and analysis.

PCR products in the gels were excised with a sterile gel cutter and purified using 5 Prime PCR Agarose Gel Extract Mini Kit (Hamburg, Germany) according to the manufacturer's protocol. DNA sequencing in both directions were done with ABI PRISM ready reaction big dye terminator cycle sequencing kit (Applied Biosystems, Forster City, California, USA), following the manufacturer's manual. To identify the host species, the obtained sequences were compared with sequences in the GenBank database using the Basic Local Alignment Search Tool searches [15].

3. RESULTS

A total of 30 animals were caught surrounding both human settlements; 3 from Janda Baik, Pahang and 27 from Labu, Negeri Sembilan. Four species of hosts were identified namely Rattus tiomanicus, Tupaia glis, Sundamys muelleri and Maxomys rajah. Thirty five immature ticks consisting of larvae and nymphs stages were extracted from the animals and morphologically identified into four genera; Ixodes, Dermacentor, Haemaphysalis, and Amblyomma (Table 1). No adult tick was obtained for the 4 genera and thus the species of the immature stages could not be determined. Nineteen of the 35 ticks examined (54.2%) apparently contained blood meals. The average body engorgement index was in the range of 1.00 to 16.85 and the ticks were estimated to feed on host for approximately more than 3 days based on prediction by Yeh et al [16]. Upon amplification, all 19 ticks showed amplicons of the expected size and no product was

obtained with the negative controls (Table 2 and Figure 1).

The best match of sequences of the PCR products (between 88-99% similarities) with related vertebrate gene sequences from GenBank are shown in Table 3. The single Amblyomma tick had fed on R. tiomanicus while all blood meals of Haemaphysalis ticks corresponded to a single species of a wild rodent, S. muelleri. Rattus tiomanicus and T. glis were fed on by the Dermacentor ticks. In Labu, Ixodes ticks fed only on R. tiomanicus but in Janda Baik, the only host was M. rajah.

4. DISCUSSION

Molecular identification of vertebrate host was achieved in this study by targeting cytb of the vertebrate mtDNA using the whole body of on-host ticks. This particular gene was proven useful for reliable identification of arthropod blood meals due to high copy numbers and sufficient genetic variation at the primary sequence level among vertebrate taxa [17]. In this study, the species of vertebrates identified from the blood meals corresponded with the host from which the ticks were extracted off.

Of particular interest was the high percent (45.8%) of blood meals from the ticks that could not be identified. The possible causes for this were either degradation of residual host DNA or external DNA contamination of the ticks due to shorter duration of attachment on hosts. The former cause was unlikely to occur because appropriate measures have been taken to ensure good preservation and storage condition of the DNA. However, small volumes of hosts' blood inside the ticks as a result of interrupted feeding were reported to contaminate external DNA [18]. The occurrence of co-amplification of ticks' genes with cytb primer sets has also been reported to prevent positive host identification [19]. Another factor for the high failure rate of blood meal identification is possibly the presence of nucleated erythrocytes which probably mask the vertebrate DNA in mixed blood meals and the region of DNA targeted for analysis [20]. Thus a higher number of ticks need to be examined to obtain a higher success rate of blood meal identification.

In this study Haemaphysalis and Amblyomma ticks fed only on one host animal in that particular area whereas 2 host species were fed on by Ixodes and Dermacentor ticks. That appears to indicate that there is limited species of small animals surrounding human settlements for the ticks to feed on. Rattus tiomanicus was the only species of host caught in Labu, Negeri Sembilan and was fed on by all the 3 genera of ticks in that area. The rodent was reported as the dominant species of small animals in oil palm plantations due to its agility and arboreal habits [21]. A stream adjoining the settlement may possibly provide source of water for the oil palm rodents. There were more host species in Janda Baik, Pahang which was probably due to nearly perfect conditions of rainforest area which supported a great number of animal species. Furthermore, the variety and abundance of food from nearby orchards also contributed to the abundance of animal species.

The study also demonstrated that although a single tree shrew was caught in Pahang, it was infested with ticks. The catch of tree shrews was more in Negeri Sembilan but they were all uninfested. The reason for this is not clear but it might due to a very low infestation rate or the environment is probably not suitable for survival of ticks. Tree shrews carrying ticks surrounding human settlement were reported to move as far as 4.5 km from fruit orchards, secondary forest and plantation area, to houses and vice versa [22]. The finding of R. tiomanicus as one of the hosts for Amblyomma ticks was rare because wild reptiles and amphibians were generally reported as the natural hosts for these genera of ticks [23]. However, rodents such as Leopoldamys sabanus, Rattus diardii and Niviventer rapit had also been reported as hosts for Amblyomma ticks [24, 25].

PCR-based methods have been used for diagnosis of many infectious diseases in field collected ticks. DNA from the whole body of ticks not only can be used for blood meal identification but also is useful for detection of parasites [26, 27]. Further study should be undertaken to document more cytb gene sequences of other vertebrate hosts in Malaysia. The availability of a large database is badly needed as it will allow precise identification of a variety of mammalian species and enhance the development of PCR for rapid identification of ticks'

blood meal.

5. CONCLUSION

In conclusion, blood meal identification in field-collected on-host ticks can confirm a strong association between ticks and their natural hosts and may help to improve understanding of the role of rodents and other small wild animals surrounding human settlement in the transmission of infectious diseases caused by ticks. The use of cytb gene is therefore seems to be an effective molecular marker for study of tick-borne zoonoses in identifying the potential host and reservoir of a pathogen. The availability of DNA sequences of hosts for all genera of ticks in Malaysia would be of advantage in the design of more effective future tick-borne diseases control program.

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Location	**Number of host collected**				**Number of ticks collected**			
	R. tiomanicus	**T. glis**	**S. muelleri**	**M. rajah**	**Ixodes**	**Dermacentor**	**Haemaphysalis**	**Amblyomma**
Kg. Cheringin, Janda Baik	0	1	1	1	4	1	9	0
Kg. Lambar, Labu	24	3	0	0	8	10	2	1
TOTAL	**24**	**4**	**1**	**1**	**12**	**11**	**11**	**1**

Table 1. Species of rodents caught and the genera of ticks collected surrounding human settlements in Kampung Cheringin, Janda Baik, Pahang ($3^{o}31'$N, $101^{o}55'$E) and Kampung Lambar, Labu, Negeri Sembilan ($2^{o}46'$N, $101^{o}49'$E)

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RESEARCH ARTICLE

Study area	Code	Host	Tick species	Species of vertebrate identified from the blood meals			
				R. tiomanicus	M. rajah	S. muelleri	T. glis
Kg Lambar, Labu	SBN 01	R. tiomanicus	Ixodes granulatus	R. tiomanicus	-	-	-
	SBN12-1	R. tiomanicus	Dermacentor spp	R. tiomanicus	-	-	-
	SBN23-1	R. tiomanicus	Ixodes granulatus	R. tiomanicus	-	-	-
	SBN16-2	R. tiomanicus	Ixodes granulatus	R. tiomanicus	-	-	-
	SBN26-1	R. tiomanicus	Dermacentor spp	R. tiomanicus	-	-	-
	SBN23-2	R. tiomanicus	Dermacentor spp	R. tiomanicus	-	-	-
	SBN18-2	R. tiomanicus	Dermacentor spp	R. tiomanicus	-	-	-
	SBN19	R. tiomanicus	Amblyomma spp	R. tiomanicus	-	-	-
Kg Cheringin, Janda Baik	JNB10	S. muelleri	Haemaphysalis spp	-	-	S. muelleri	-
	JNB05	S. muelleri	Haemaphysalis spp	-	-	S. muelleri	-
	JBB01	T. glis	Dermacentor	-	-	-	T .glis
	JBB03-2	S. muelleri	Haemaphysalis spp	-	-	S. muelleri	-
	JBB03-1	S. muelleri	Haemaphysalis spp	-	-	S. muelleri	-
	JBB03-3	S. muelleri	Haemaphysalis spp	-	-	S. muelleri	-
	JBB03-4	S. muelleri	Haemaphysalis spp	-	-	S. muelleri	-
	JBB03-5	S. muelleri	Haemaphysalis spp	-	-	S. muelleri	-
	JBB03-6	S. muelleri	Haemaphysalis spp	-	-	S. muelleri	-
	JBB02-1	M. rajah	Ixodes granulatus	-	M. rajah	-	-
	JBB02-2	M. rajah	Ixodes granulatus	-	M. rajah	-	-

Table 2. Vertebrate- derived blood meals identified from 19 samples of ticks collected in two human settlements

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Tick Genus	No. of ticks collected	No. of blood meal identified (%)	No. of host identified (range of % similarity with sequence in GenBank)			
			R. tiomanicus	M. rajah	S. muelleri	T. glis
Ixodes spp	12	5 (41.7)	3 (98-99)	2 (99)		
Dermacentor spp	11	5 (45.4)	4 (98-99)			1 (99)
Haemaphysalis spp	11	8 (72.7)			8 (88-91)	
Amblyomma spp	1	1 (100)	1 (93)			
TOTAL	**35**	**19 (54.2)**	**8**	**2**	**8**	**1**

Table 3. Prevalence of ticks collected and detection of blood meals by PCR-based sequencing analysis

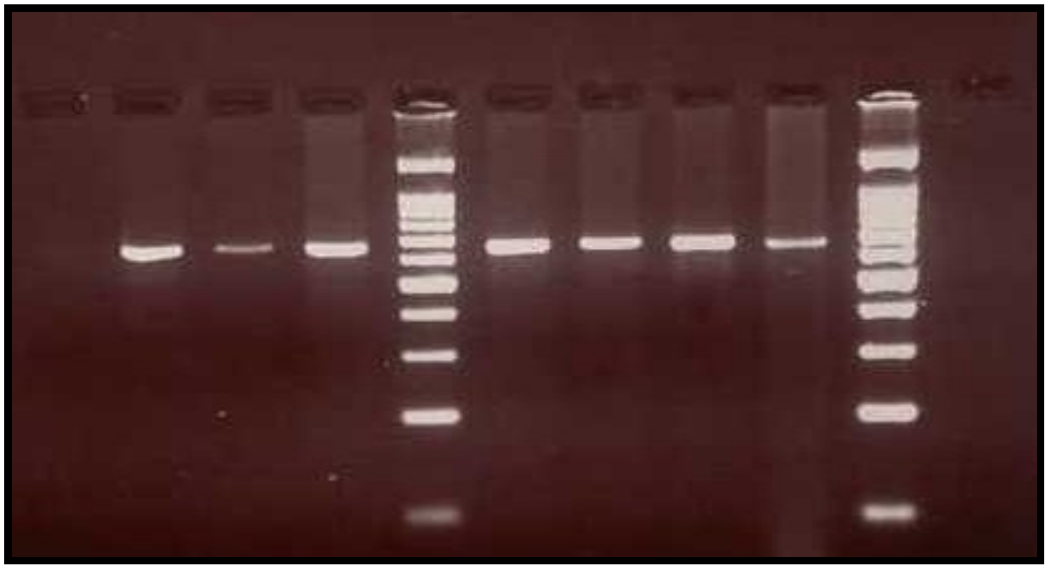

Figure 1. Gel photograph showing partial fragments (623 bp) of the cytb-specific amplification products. Lane 1: unfed ticks (negative control); Lane 2: positive control DNA; Lanes 3-8: DNA of field-collected ticks (Ixodes sp, Dermacentor sp, Ixodes sp, Haemaphysalis sp, Haemaphysalis sp, Amblyomma sp) and Lanes M1, M2: 100 bp marker (Bioron, Germany).

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