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Establishment of a molecular tool for blood meal identification in Malaysia

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ABSTRACT

Objective: To establish a polymerase chain reaction (PCR) technique based on cytochrome b (*cytb*) gene of mitochondria DNA (mtDNA) for blood meal identification. **Methods:** The PCR technique was established based on published information and validated using blood sample of laboratory animals of which their whole gene sequences are available in GenBank. PCR was next performed to compile gene sequences of different species of wild rodents. The primers used were complementary to the conserved region of the *cytb* gene of vertebrate's mtDNA. A total of 100 blood samples, both from laboratory animals and wild rodents were collected and analyzed. The obtained unknown sequences were compared with those in the GenBank database using BLAST program to identify the vertebrate animal species. **Results:** Gene sequences of 11 species of wild animals caught in 9 localities of Peninsular Malaysia were compiled using the established PCR. The animals involved were *Rattus (rattus) tanezumi*, *Rattus tiomanicus*, *Leopoldamys sabanus*, *Tupaia glis*, *Tupaia minor*, *Niviventor cremoriventor*, *Rhinosciurus laticaudatus*, *Callosciurus caniseps*, *Sundamys muelleri*, *Rattus rajah* and *Maxomys whiteheadi*. The BLAST results confirmed the host with exact or nearly exact matches (>89% identity). Ten new gene sequences have been deposited in GenBank database since September 2010. **Conclusions:** This study indicates that the PCR direct sequencing system using universal primer sets for vertebrate *cytb* gene is a promising technique for blood meal identification.

1. Introduction

Vector borne diseases such as scrub typhus, yellow fever, malaria, dengue, chikungunya, and Lyme disease have tremendous health impact on the human population worldwide[1]. Efforts to identify reservoir hosts for vector borne zoonotic pathogens have been a labor-intensive exercise due to several requirements such as capture of potential wildlife hosts and experimental infections with pathogens of interest. It is a challenge to conduct subsequent examination of the host's efficiency to transmit infectious agent to the vector organisms under controlled condition[2]. Moreover, such laboratory-based estimates may fail to capture the true distribution of host competencies because of unknown consequences of host selection behavior by vector organisms or the unmeasured contributions of cryptic reservoir hosts[3,4].

Serological techniques like precipitin test, latex agglutination and ELISA have been developed long time ago to identify the source of vertebrate blood. These techniques however, did not solve some phylogenetic identification problems for closely related species[5]. This may result in a high percentage of samples being identified only to the family level but not to the exact species[6,7]. Moreover, the contemporary techniques are also time consuming and lack sensitivity[8,9].

Recent developments in molecular biology have allowed a significance improvement in the efficacy and reliability of blood meal identification[10]. Polymerase chain reaction (PCR) based techniques have been proven to be highly effective and versatile in laboratory trials and are likely to replace all other approaches. Molecular based assays have been developed to detect and identify blood meal sources of some insect vectors but most studies were focused on human blood[11]. Only few studies concerning

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host preference patterns were conducted for identification of vertebrate species[3,12]. These studies were based on the analysis of mitochondrial cytochrome b (*cytb*) gene which has been widely used[13-17].

Majority of the vector-borne diseases survived in nature by utilizing animals as their vertebrate hosts[18]. To make it worse, environmental changes and deforestations may contribute to the increase of contacts between man and wild animals[19]. It is therefore critical to identify natural hosts of the causative pathogens as fast and accurate possible for an effective outbreak management and control. Thus the aim of this study is to establish a PCR technique for blood meal identification based on the *cytb* gene of mitochondrial DNA (mtDNA) sequences of some vertebrate animals in Malaysia.

2. Materials and methods

2.1. Animal origin and blood collection

Blood from the following laboratory animals (rabbits, Sprague-Dawley rat and BALB/c mice) which reared in the Laboratory Animal Resource Unit, Institute for Medical Research (IMR) were collected for establishment and validation of PCR technique. Fieldworks in 9 locations of Peninsular Malaysia were organized for trapping of wild rodents, shrews and other small animals. The locations studied were Slim River, Perak; Raub, Pahang; Bukit Panchor, Penang; Gunung Inas, Ulu Sedim, Kedah; Janda Baik, Pahang; Seremban, Negeri Sembilan; Hulu Langat, Selangor; Sungai Sedim, Kedah and Setiu, Terengganu. Host was first anaesthetized with zoletil 50 (Virbac Laboratories, France) and 3 mL blood was collected using appropriate humane procedure in EDTA tubes and stored at -20 ℃ until further use. The project was approved by the Animal Use Committee of the Ministry of Health Malaysia (Ref No: ACUC/KKM/02(6)2009).

2.2. Blood dilution

Freeze-thawed blood was diluted 1:10 in sterile double distilled water and used as a template in PCR.

2.3. PCR amplification and gel electrophoresis

DNA of hosts was amplified using PCR with universal primers complementary to the conserved region of mitochondrial DNA (mtDNA) *cytb* gene. The primers, L14841 (F5′-CCATCCAACATCTCAGCATGATGAAA-3′) and H15149 (R5′-CCCCTCAGAATGATATTTGTCCTCA-3′) amplified 359 bp of the *cytb* gene[6,20]. The PCR was performed using KAPA Blood PCR Kit (Kapa Biosystems Inc. USA) according to the manufacturer's manual. A mixture of 25 μL solution containing 2× Kappa Blood PCR mix, 0.5 μM of each primer and 2.5 μL of blood DNA template was prepared and amplified using an Eppendorf Master Cycler Personal machine with conditions of pre-heating at 95 ℃ for 10 min, 35 cycles of consecutive incubations at 94 ℃ for 30 sec, 52 ℃ for 30 sec and 72 ℃ for 5 min. A negative control was included for each batch of assay. Amplified DNA products were confirmed with 1.2% agarose gel in 0.5× electrophoresis buffer and visualized under ultraviolet (UV) light after staining with 2 mg/mL ethidium bromide. A 100 bp DNA ladder (Bioron, Germany) was used as the standard marker for comparison.

2.4. DNA sequencing

PCR products from gel 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 procedure. DNA sequencing in both directions was done in the presence of the ABI PRISM ready reaction big dye terminator cycle sequencing kit (Applied Biosystems, Forster City, California, USA), following the manufacturer's manual.

2.5. BLAST search

To identify vertebrate host species, the obtained unknown sequences were compared with those already deposited in the GenBank database using the BLAST program in Basic Local Alignment Search Tool searches[21]. Sequences of a given pair-wise alignment with the lowest E-value were selected as the most likely species of host.

2.6. Sensitivity of PCR

Ten serial dilutions (1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 μL) of blood DNA were used to detect threshold for determination of sensitivity for the PCR amplification of the *cytb* gene. The PCR followed the procedure as described for the hosts DNA amplification and confirmed by analyzing 6 μL of PCR products on 1.2% agarose gel.

3. Results

A total of one hundred blood samples collected from laboratory and small wild animals were amplified using PCR. Of those, 88 blood samples were from eleven species of wild animals including *Rattus tiomanicus* (*R. tiomanicus*) (19), *Rattus tanezumi* (*R. tanezumi*) (25), *Rattus rajah* (*R. rajah*) (4), *Leopoldamys sabanus* (*L. sabanus*) (7), *Niviventor cremoriventor* (*N. cremoriventor*) (2), *Sundamys muelleri* (*S. muelleri*) (8), *Tupaia glis* (*T. glis*) (16), *Rhinosciurus laticaudatus* (*R. laticaudatus*) (2), *Callosciurus* spp. (1), *Maxomys whiteheadi* (*M. whiteheadi*) (3), *Trpaia minor* (*T.*