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Establishment of a molecular tool for blood meal identification in Malaysia

Ernieenor Faraliana Che Lah*, Mariana Ahamad, Mohd Subail Haron, Ho Tze Ming

Acarology Unit, Infectious Diseases Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, 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

*Corresponding author: Ernieenor FCL, Acarology Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia.
Tel: +603 26162445
Fax: +603 26935928
E-mail: erniee@imr.gov.my
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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.*

minor) (1). The amplification of a single fragment encoding a 359 bp sequence of the *cytb* gene yielded the expected amplification products in most blood samples (Figure 1). Negative control (double distilled water) yielded no PCR product implying that only host's DNA patterns were detected in the amplifying specimens.

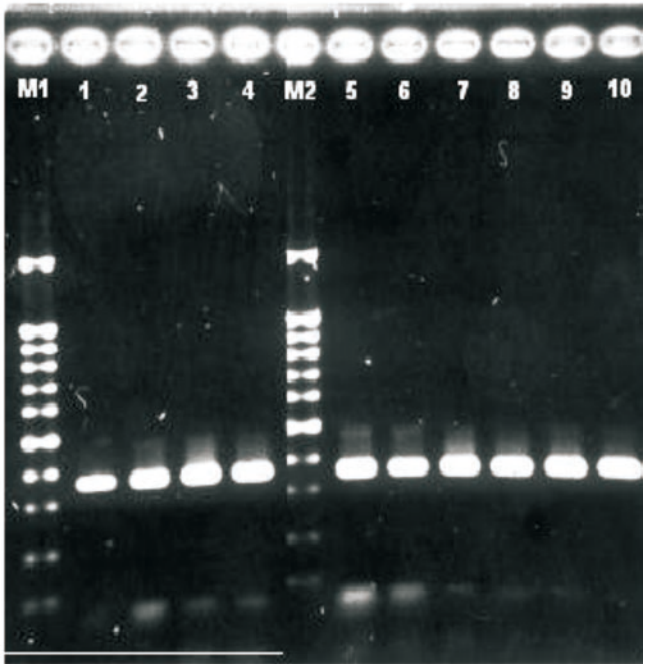

Figure 1. Gel photograph showing 359 bp of *cytb*-specific amplification products.
Lanes 1-3: Laboratory animal blood sample (BALB/c mice, Rabbit, SD rat); Lanes 5-9: Wild rodents blood sample (*T. glis, M. whiteheadi, S. muelleri, R. tanezumi, L. sabanus*); Lanes 4 and 10: Positive control DNA; Lanes M1, M2: 100 bp marker.

Identity of the blood meal recovered in this study ranged from 89%-100% similarities with those of the GenBank sequences. For laboratory animals, the similarities of gene sequences of BALB/c mice, SD rat and rabbit in this study with corresponding sequence in GenBank were 98%-99%, 99%-100% and 94%-95%, respectively. To date, only 38 of the PCR products of wild small animals were sequenced and compared against GenBank. Based on the highest similarity, 19 of the sequences matched corresponding species sequences in GenBank with 96%-100% similarity and 1 with 89% similarity. Ten new gene sequences were deposited with accession number HQ166262 (*L. sabanus*), HQ166263 (*L. sabanus*), HQ180173 (*M. musculus* BALB/c strain), HQ288326 (*S. muelleri*), HQ288327 (*T. glis*), HQ656820 (*M. whiteheadi*), HQ656821 (*N. cremoriventor*), JF417970 (*R. tanezumi*), JF417971 (*R. laticaudatus*) and JF417972 (*R. laticaudatus*) which contribute significantly in GenBank database that constantly growing[10]. A total of 19 sequences were mismatched with other species and the similarity range from 84%-99%; 9 sequences give more than 90% similarity (Table 1).

Table 1
Nine sequences of wild rodents mismatched with other species in GenBank with more than 90% similarity.

Identified as	GenBank species	Similarity (%)
R. tanezumi	*L. sabanus*	98
M. whiteheadi	*S. muelleri*	98
S. muelleri	*T. glis*	98
L. sabanus	*Maxomys* spp.	95
R. rajah	*M. whiteheadi*	92
N. crimoriventor	*M. whiteheadi*	90
Lariscus insignis	*M. whiteheadi*	91
Calloscirus caniseps	*S. muelleri*	97
T. minor	*Tupaia longipes*	91

PCR on blood DNA of both laboratory and small wild animals detected a 359 bp of PCR product using from as low as 0.3 μL blood sample (Figure 2). Faded amplification signals were shown in lanes with lower volume of blood samples. The above-mentioned sensitivity tests were carried out more than two times for each species of host to guarantee the reaction reproducibility.

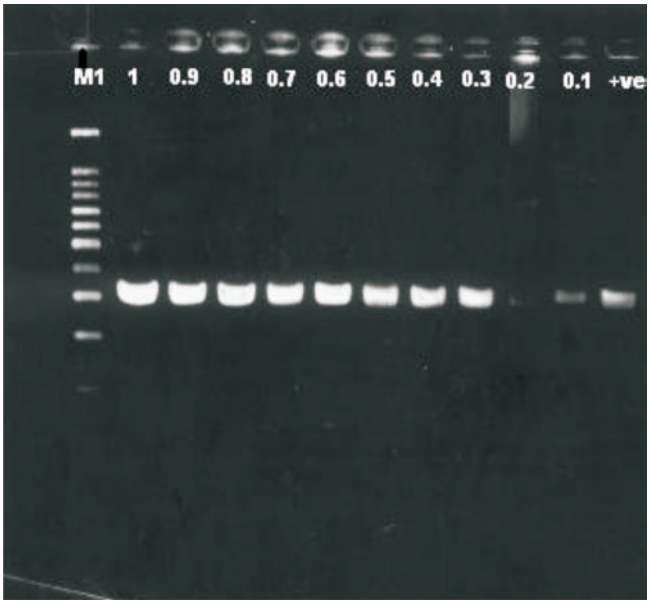

Figure 2. Gel photograph showing 359 bp of *cytb* gene by PCR amplification of 10 serial dilutions tested where each well contains different volume of blood (0.1-1.0 μL).
M1: molecular weight marker (100-bp DNA ladder).

4. Discussion

A PCR technique for blood meal identification of laboratory and wild small animals based on mitochondrial cytochrome b (*cytb*) gene was successfully established in IMR. This is fruitful as PCR has been widely used in the analysis of mitochondrial DNA *cytb* gene for blood meal identification of mammalian and avian host[6,22,23]. In this study, a short fragment of the gene (359 bp) was investigated for blood meal identification due to its widest taxonomic

representation in nucleotide databases. The fragment fulfilled the requirement of relatively short size and enough sequence divergence to achieve objectives of the study. It is therefore likely to find a sequence entry of an unknown sample or at least of a related species[3]. Moreover, *cytb* has been widely used in identifying blood meal due to their high copy numbers and sufficient genetic variation at the primary sequence level among vertebrate taxa[17,23]. Easy support of available sequences from various species deposited in databases is another reason for the application of mtDNA in identifying bloodmeal[24,25]. Currently, there are more than 8 000 *cytb* gene sequences of vertebrate animals available in the GenBank/EMBL/DDBJ and this data set is constantly growing[13]. For studies utilizing direct sequencing of *cytb* to identify bloodmeals, taxonomic coverage of *cytb* in GenBank is also extensive *i.e.* more than 120 000 entries[26].

The most significant aspect of the PCR method is its sensitivity to detect minuscule amounts of DNA. The sensitivity usually depends on both the quantity of target DNA and the progressive degradation of the host DNA. This study has shown that the established PCR technique is sensitive in detection of diminutive volume (0.3 μL) of blood meals. The volume was found lower than the range (0.5-1.0 μL) of sand fly blood meals[27]. One possible reason for the poor and decline of visualization of PCR products signal below 0.3 μL may be due to the increasing insensitivity of *cytb* gene amplification for detecting a small amount of vertebrate DNA in a progressive dilution process.

Throughout the study, only one pair of PCR primers was used to amplify a 359 bp of the *cytb* gene for all investigated animals. This is in agreement with studies that reported the use of universal primers as complements to conserved region of mitochondrial *cytb* gene in vertebrates[20,28,29]. These one set of universal primers, therefore appeared to be effective for almost small vertebrate animals. The primers in this study were already used for various mammalians and avian mtDNA including human[6,7]. The feature of high copy number makes PCR amplification of mtDNA genes a robust option for analyzing vertebrate DNA in tiny arthropod bloodmeals. Furthermore, the mtDNA *cytb* gene has an evolutionary rate 5-10 times faster than nuclear genome[10] and therefore able to not only resolve broader taxonomic groups but also distinct subpopulations within a specific taxon[26,30]. Amplification yielded a single band in most of the species investigated, as the PCR primers annealed in conservative regions of the *cytb* gene as in a study by Kocher *et al*[20]. The nucleotide sequence of the forward primer also gave a clearer signal to noise ratio than the reverse primer and this agreed to the same phenomenon described by Parson *et al*[13] when analyzing other mitochondrial sequences. Nevertheless, the nucleotide sequences of both strands were determined and the consensus sequences were analyzed and served as a basic for the identification of the biological origin of the sample[31].

While the PCR method offers clear advantages over serological identification of blood meals, a small percentage of the blood samples did not exactly match any of the DNA sequences currently available in the GenBank database. That high level of mismatch may be due to many limitations including misidentification of host species in this study, misidentification of host species listed in GenBank, and insufficient gene sequences registered with GenBank. The correct identification of an unknown sample therefore depends on the availability of the sequences in the nucleotide database. To address those limitations, there is a need to optimize training to improve identification of mammalian hosts and to examine larger numbers of local species of mammals and registered their gene sequences in GenBank. Availability of a large database of *cytb* gene sequences of natural hosts in Malaysia is badly needed as it will allow identification of a large variety of mammalian species. Detailed knowledge on the identity of host animals would be invaluable in the design and implementation of vector borne disease control programs.

In conclusion, 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. Direct sequencing of the PCR products allows comparative analysis of the unknown DNA sequences and those already deposited in the databases by using the BLAST program. It should be emphasized that present cost of PCR limits the large scale use of this method but in the case where detailed information on host species of arthropod is required, the PCR analysis has been proven to be a useful technique. However, there are certain limitations that need to be addressed to improve its accuracy. An investigation on the use of this molecular technique application was planned in the future for identification of blood meals in haematophagous arthropods especially on ticks.

Conflict of interest statement

We declare that we have no conflict of interest.

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