

Effects of Post Ingestion and Physical Conditions on PCR Amplification of Host Blood Meal DNA in Mosquitoes

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(Received 3 Dec 2004; revised 3 Sep 2005; accepted 24 Sep 2005)

Abstract

Identification of host blood meal in haematophagous arthropods is an important element in their role in transmission of vector borne diseases. The effects of post ingestion and physical conditions that killed mosquitoes are stored on the success of detecting blood meal DNA of *Anopheles stephensi* and *Culex quinquefasciatus* was investigated by polymerase chain reaction (PCR) amplification at the human mitochondrial DNA cytochromeB (CytB) gene. Host DNA extracted from the blood meal up to 33 h post ingestion in both species acts as an efficient template for PCR amplification. However more DNA concentration needs for meals digested longer time. Successful PCR amplification among meals digested for 36 h dropping to a faint band. There were no differences between PCR success rate for samples stored at +4° C or -20° C, but less successful products were observed in samples kept at 4° C for periods longer than 30 h digestion. The results of this study is important in malaria epidemiological studies to provide valuable information about the degree of contact between human hosts and mosquito vectors, impact of vectors controls such bed nets and repellents, and the transmission dynamics of human malaria and other vector-borne diseases.

Key words: *Blood meal, Anopheles stephensi, Culex quinquefasciatus, CytochromeB, Malaria, Vector-borne diseases, Iran*

Introduction

The feeding preference of mosquitoes on humans is an important element in their vectorial capacity and role in transmission of vector borne diseases (1). For example, the basic reproductive rate (BRR) of malaria parasites could be increased if non-infected mosquitoes prefer to feed on persons carrying infective parasite stages, or if infected mosquitoes prefer to feed on humans (2). The correct identification of the bloodmeal taken by a mosquito species provides information on host preferences

(Anthropophilic Index: AI) under natural conditions. It can also help to understand the efficacy and effectiveness of various control or surveillance tools (e.g. repellents, bed nets and traps), developing effective disease control strategies (e.g. distribution of drugs and insecticides according to the risk of infection), and to estimate the degree of coverage needed for various malaria vaccines when developed (3). Contemporary procedures for arthropod blood meal sources are generally based upon the detection of host antigens by the complement

fixation test (4-5) or by enzyme-linked immunoabsorbant assays (ELISA), using polyclonal antibodies raised against blood components from potential host vertebrates (6). This method, however, requires the preparation of immune sera against the blood of each potential host species, a difficult and laborious process. Pre-adsorption steps are also needed to eliminate cross reactions when using this technique (7). Furthermore, these methods are not suitable for distinguishing between meals obtained from members of the same, or closely related, host species (8). The main criticism of many published papers using monoclonal (and polyclonal) antibodies is that they conducted insufficient cross-reactivity experiments (9).

Recent developments in molecular biology such PCR assay have allowed a significant increase in the efficacy and reliability of bloodmeal identification and have proved to be highly effective and versatile in recent laboratory trials and are likely to rapidly displace all other approaches(8,10). DNA based molecular markers have been used to identify bloodmeals in a few number of arthropods including *Anopheles gambiae* (e.g. 11-12), *Aedes aegypti* and *An. sinensis* (13), *Pthirus pubis* (14-15), *Glossina* spp. (16), *Culex quinquefasciatus* (17), *Simulium damnosum* (18), and *Ixodes ricinus* (19).

Most of mosquito females digest the bloodmeal, oviposit, and begin searching for the next bloodmeal within 48-72 h after the previous bloodmeal. Therefore bloodmeals soon after ingestion begins to be digested and as a consequence the DNA in such blood is degraded and less chance will be remained to be detected for long post ingestion time. The question that how long the host DNA would survive against the action of enzymes in the guts of hematophagous arthropods is very critical in DNA based blood meal identification since having a good quality and quantity of DNA is crucial in any PCR assays. Here in this study we tested the impact of post ingestion and physical condition on PCR amplification of a short fragment

of CytB gene of host bloodmeal DNA in mosquitoes of *An. stephensi* and *C. quinquefasciatus*.

Materials and Methods

Mosquitoes Experiments were carried out using laboratory strains of *An.stephensi* (Iran-shahr-Iran) and *C.quinquefasciatus* (Tehran-Iran) mosquitoes and reared in insectary of Dept. of Medical Entomology and Vector Control, School of public Health & Institute of Health Researches, Tehran University of Medical Sciences, Iran. The mosquitoes were reared under controlled conditions, $27 \pm 1^\circ \text{C}$, $80 \pm 5\%$ relative humidity and 12L: 12D photoperiod. Adult mosquitoes were maintained on a 6% glucose solution. Test mosquitoes were 4-5 d old and starved overnight before feed by blood meal and supplied only with water on cotton wicks. The mosquitoes had not taken any blood meal prior to the experiments.

Test mosquitoes were fed on human blood by introducing the forearm of one of two volunteer test persons inside of cage. Mosquitoes were removed from the arm after voluntary withdrawal of the mouthparts from the skin and feeding had ceased. Blood-fed mosquitoes were held under the rearing conditions ($27 \pm 1^\circ \text{C}$, $80 \pm 5\% \text{RH}$) and freeze-killed in sets ($n=20$) of 1, 6, 12, 18, 24, 27, 30, 33, and 36 h after feeding and then were stored at $+4^\circ \text{C}$ or -20°C . Blood meals of all experimental mosquitoes were processed following storage for one to two days. Control mosquitoes were unfed females ($n=5$).

Ethical clearance Informed consent was obtained from the two adult human participants. The project was approved by the Tehran University of Medical Science Ethical Committee.

DNA extraction and amplification DNA was isolated from human-fed and un-fed female mosquitoes. Isolation was done using the method as described earlier (20). DNA extracts were PCR-amplified at the human mitochondrial DNA Cytochrome B (CytB) gene (18). PCR amplifications were undertaken in 25 μl of

a solution containing 10 mM Tris-HCl (pH 8.3 at 25° C) 50mM KCl, 1.5 mM MgCl₂, 0.001% gelatin 200 mM dATP, dCTP, dGTP and TTP, 0.5 mM each primer, 1 unit of *Taq* DNA polymerase (Cinagene, Tehran, I.R.Iran) and 2.5 ml of 1:2-1:40 dilution of the DNA template solution, prepared as described above. We used the primers introduced earlier (18, 21) for amplification of a portion (358 bp) of CytB gene of vertebrate mtDNA. The sequence of the primers was as follows: 5'-ccatccaacatctcagcatgatgaaa-3' (forward) and 5'-cccctcagaatgatattgtcctca-3' (reverse). They are corresponding to sequences of 14380 to 14405 and 14711 to 14737 of human mtDNA (Accession Number: DQ112962). Amplifications were done on an Eppendorf Master Cycler Personal machine. Reactions began by incubation at 95° C for 3.5 min, followed by 36 cycles comprising 30 s at 95° C, 50 s at 60° C and 40 s at 72° C. The reaction was completed by incubation at 72° C for 5 min. An aliquot (5-10 micro liters) of PCR products was mixed with 6 ml loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) and was loaded onto a 2.5 % agarose gel. Gels were stained in 2 mg/ml ethidium bromide and the products were visualized under UV light.

To test the rate of success in amplification of blood meal DNA within the mosquito gut after digestion and under different physical condition, a total of 240 blood-fed *An.stephensi* and *C. quinquefasciatus* mosquitoes were subjected to PCR amplification at the mtDNA CytB gene. The blood meals were obtained from one of two human volunteers. Analysis of success of PCR amplification for blood meal DNA in mosquitoes killed in sets of 1, 6, 12, 18, 24, 27, 30, 33, and 36 h after feeding.

The success of PCR amplification of bloodmeal DNA kept in different physical conditions and in two mosquito species of *Anopheles* and *Culex* were compared using Chi-square analysis.

Results

Our results showed that host DNA extracted from the blood meal up to 33 h post ingestion acted as an efficient template for PCR amplification and produced a positive PCR products which could be used for further analysis such restriction fragment length polymorphism (RFLP), heteroduplex analysis (HDA), or direct sequencing (Fig. 1-2). A 1/40 dilution DNA concentration of the extracted DNA was enough for amplification. However, for longer digestion time (i.e 30 h and more) more concentrated DNA (>1/20 dilution) was needed. One hundred percent of all samples (40/40) killed 1-6 h after ingestion could be successfully amplified. After 6 h, the number of blood meals that successfully amplified decreased very slowly to 19/20 (95%), 18/20 (90%), and 17/20 (85%) for meals digested for 12, 18-24, and 27-30 h, respectively. Half of the meals digested for 33 h produced a faint PCR product, but a few (20%) meals produced very faint product which was not enough sharp for further analysis (Fig. 1). This trend showed a negative relationship between time since ingestion and the success of PCR amplification among meals digested for between 30 and 36 h. There was no significant ($P<0.01$) difference in amplification success between meals of *An. stephensi* or *C. quinquefasciatus* mosquitoes. Also there was no significant ($P<0.01$) success of PCR amplification for the dead flies stored at +4° C or -20° C following up to 30 h meal digestion.

Negative controls including ddH₂O and un-fed females, yielded no PCR product implying that only human, but not mosquito, DNA patterns were detected in the amplifying specimens.

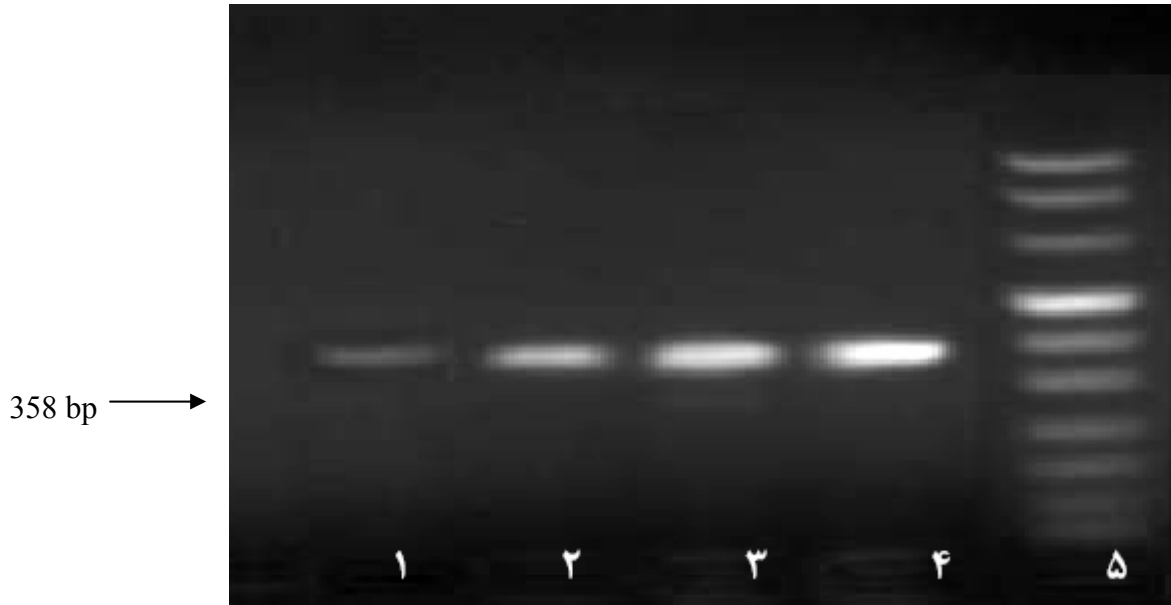


Fig. 1: Effects of post ingestion on PCR amplification of bloodmeals in mosquitoes of *Anopheles stephensi* (Culicidae: Diptera) fed upon a human volunteer and held at 28 C temperature for varying periods of time. DNA was extracted from the flies, and CytB sequences (358 bp) amplified from the bloodmeal as described in Materials and Methods. Lane 1= fly held for 36 h, lane 2= fly held for 24 h, lane 3= fly held for 12 h, Lane 4= fly held for 1 h, lane 5= molecular weight marker (50 bp ladder, Cinna gene, Iran).

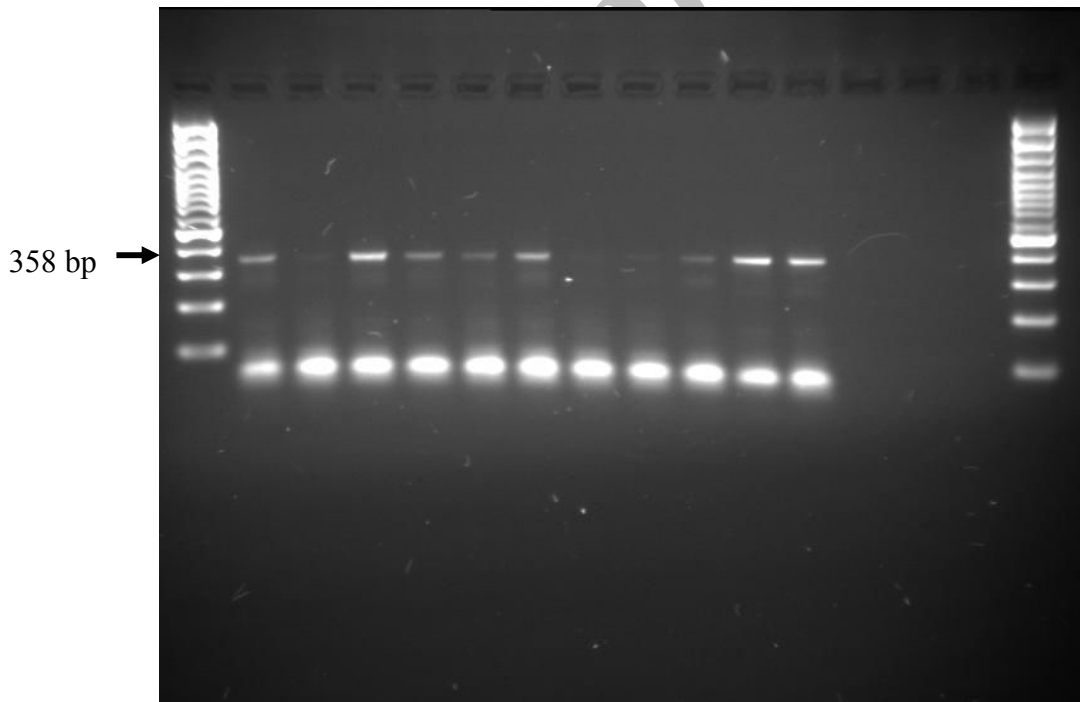


Fig. 2: Effects of post ingestion and physical condition on PCR amplification of bloodmeals in mosquitoes of *Anopheles stephensi* (Culicidae: Diptera) fed upon a human volunteer and held at 28 C temperature for varying periods of time. DNA was extracted from the flies, and CytB sequences (358 bp) amplified from the bloodmeal as described in Materials and Methods. Following numbers indicating time of digestion, temperature dead flies held, and DNA concentration respectively. Lane 1&16 = molecular weight marker (50 bp ladder, Cinna gene, Iran); Lane 2 = 27 h, 4 C, 1/20 DNA dilution; Lane 3 = 27 h, 4 C, 1/40; Lane 4 = 27 h, -20 C, 1/20 DNA dilution; Lane 5 = 27 h, -20 C, 1/20 DNA dilution; Lane 6 = 27 h, -20 C, 1/40 DNA dilution; Lane 7 = 30 h, 4 C, 1/40 DNA dilution; Lane 8 = 30 h, -20 C, 1/20 DNA dilution; Lane 9 = 30 h, -20 C, 1/20 DNA dilution; Lane 10 = 30 h, -20 C, 1/40 DNA dilution; Lane 11= 33 h, -20 C, 1/20 DNA dilution; Lane 12 = 33 h, -20 C, 1/20 DNA dilution; Lane 13-14 = DNA extracted from an unfed mosquito, Lane 15 = PCR negative control.

Discussion

This study demonstrated that the extent of digestion but not the physical condition (temperature between -20 and +4° C) which killed mosquitoes were stored affects the success of amplifying human DNA from blood meals of *An.stephensi* and *C.qinquefaciatus*. Since meals of both species were amplified similarly, it can be inferred from these results that the success rate of DNA amplification was not associated with mosquito and blood meal size. The lack of a positive relationship between mosquito size and success of generating PCR products from the mosquito blood meals was not surprising because PCR amplification can be primed from single target molecules (22). Since 0.01 µl of human blood contains approximately 50 nucleated cells (23) and one nucleated human blood cell contains approximately 6 pg DNA (24) the smallest and largest blood meals which successfully amplified were estimated to contain 2 and 82 ng DNA respectively. In addition, anopheline mosquitoes are known to concentrate erythrocytes during blood feeding by a factor of 1.2–2 times the normal human hematocrit, therefore increasing the concentration of host DNA in the midgut and the probability of obtaining good-quality template for PCR identification (25-26). This study demonstrated that identification of the blood meals in mosquitoes was possible maximum upto 33 h, postfeeding. These data fall within the range of expectations based the results of previous studies. Mukabana and others (12) examined how digestion affected the amplification success of human DNA from the blood meals of *An.gambiae*. They found that digestion between 0 and 8 h had no significant effect on the ability to obtain PCR products; however, between 8 and 32 h post-feeding, the proportion of blood meals that could be successfully profiled decreased steadily, dropping below 50% at 15 h. In literature, various length of time for detection of host blood meal in haematophagous arthropods has been reported by other workers ranging from 24 h to several

days (18, 27- 29). Variation in the DNA yields from different extraction procedures, different digestive processes in different arthropods or the higher DNA content of nucleated bird blood versus mammalian blood may account for these various detection periods.

The decline in amplification success over time particularly after 33 h digestion greatly resembles the time profile observed in the disappearance of blood meal protein from midguts of *An.stephensi* (30) and *Aedes aegypti* L. (Diptera: Culicidae) (31) which were held at 27°C as done in this study. Although it was possible to profile blood meals digested for up to 33 h after feeding, for maximizing the proportion of meals that can be successfully profiled it is essential to time mosquito collection exercises such that fed mosquitoes are collected as early as possible within 24 h of feeding. This emphasizes the need for selecting suitable field-sampling methods that would allow quick and effective collection of blood-fed mosquitoes (32). Besides, if sampling cannot be done at night it is important to collect (and to store at cool temperature, at least less than 4° C) wild-caught mosquitoes at daybreak so that blood meals of mosquitoes that begin to host-seek and subsequently blood-feed in the early evening hours have a higher chance of being profiled successfully.

Even if blood-fed mosquitoes are collected early enough and stored appropriately the probability of obtaining positive amplification reactions can still be limited by the potential effect of ambient temperature on the kinetics of blood meal digestion. Under natural conditions mosquitoes are often exposed to wide variations in environmental temperature, which can affect their metabolism. Further studies describing the efficacy of typing blood meals of mosquitoes with different length of storage and chemical conditions of preservations are necessary in order to know their effects on successful analysis. It is well known that the length and copy number of target amplicon is very important in

success of PCR amplification. Larger sequences simply broke down more rapidly during digestion than shorter sequences and single-copy DNA would be less easy to detect than multiple-copy DNA, simply because the latter provided a larger target (33). In this study we concentrated upon a short fragment of CytB gene of mitochondrial genome with hundreds or thousands of copies per cell (34). However, a large number of loci suitable for identifying mosquito blood meal sources need to be tested so that closely related individuals within houses or from kin networks within villages can be distinguished with increased precision. These methods could be further refined so that the extraction of DNA for mosquito species identification, parasite detection and host identification can be combined. By correctly selecting mosquito sampling time it should be possible to increase the reproducibility of DNA based tests for mosquito blood meal identification as a tool for field entomology and epidemiology.

Acknowledgements

This study was financially supported by the Institute of Public Health Research, Academic Pivot for Education and Research, Tehran University of Medical Sciences: project no.: 241/83/79 -T to M.A.O. The authors would like to thank Mr Abaei and Mrs Raffei Head and staff of Insectary of Dept.of Medical Entomology, the School of Public Health for providing colonies of mosquitoes.

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