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DETECTION OF MALARIAL PARASITES IN *ANOPHELES STEPHENSI* BY NESTED POLYMERASE CHAIN REACTION FROM KARACHI- PAKISTAN

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ABSTRACT

Anopheles stephensi mosquitoes were trapped from Karachi and its suburbs -Pakistan and examined for the detection of malarial parasites by nested polymerase chain reaction (PCR). *Plasmodium* genus specific nested PCR amplification was applied on all samples. Only head and thorax of mosquitoes were used for pools preparation. Nested 1 product of *Plasmodium spp*. positive samples were further processed for PCR amplification with species specific primers for *P. falciparum* and *P. vivax*. Total 610 pools of single mosquitoes were processed. Out of which 3.27% pools were found positive for *P. falciparum* and 0.8% for *P. vivax* and mixed infection was found in 2.13%. Nested PCR considered as a significantly high sensitive technique for the detection.

KEYWORDS: **Nested PCR; *Anopheles stephensi; Plasmodium falciparum; Plasmodium vivax;* Karachi- Pakistan.**

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INTRODUCTION

A number of mosquito borne diseases including malaria, dengue fever and yellow fever are prevalent throughout the world especially in tropical and sub tropical countries, about 500 million people are affected every year. Malaria is transmitted by *Anopheles* female mosquitoes. It has its victim in almost all parts of Pakistan. Several *Anopheles* species have been identified but their role as a malarial vector in Pakistan was not confirmed. Presently *Anopheles stephensi* Liston (1901) was identified as a vector of malaria from Karachi-Pakistan. The major breeding sites identified were stagnant water especially at construction places, tyres tracks, water overhead tanks and uncovered tanks, open water collected containers and flower pots. Over many decades Plasmodial infections in *Anopheles* mosquitoes have been assessed using traditional methods such as dissection of salivary glands of mosquitoes to detect the actual carrier of malaria, which is time consuming, cumbersome and lacks accuracy. It is often difficult to distinguish the parasites morphologically taken from the salivary glands. Two accurate methods ELISA and Nested PCR were introduced to overcome to these difficulties. Monoclonal antibodies against the circumsporozoites (CS) protein are most commonly used in ELISA and the PCR is more popular due to its effectiveness and has been applied to overcome, the difficulties of cross reactivities in serological tests. The first demonstration of these DNA based applications was made by [5] Franzen et al (1984) which was followed by [10, 18] Oquendo et al (1986), Zolg et al. (1987) and [9] Mullis and Faloona (1987). By using PCR, it has been established that it is possible to synthesize millions of copies of a specific target DNA sequence. [2, 17, 7]. It appears that the detection of *Plasmodium falciparum* and *P. vivax* specific DNA sequences in mosquitoes by PCR has a higher sensitivity compared to ELISA tests [16]. In recent years, the polymerase chain reaction (PCR) has been used to amplify specific DNA sequences of *P. falciparum* for highly sensitive detection of the parasites in mosquitoes. It employs a set of primers derived from repetitive DNA sequences. [6] The PCR procedure can detect as few as 10 sporozoites in salivary glands therefore, a useful tool for screening small numbers of anopheline for detection of specific infection [14]. As PCR is useful tool for the detection of *Plasmodium* species in carrier mosquitoes and has been successfully used to confirm the vector status. Hence the main objectives of this study were: (a) to find out the anopheline species involved in malaria transmission, from Karachi and its suburb and (b) to assess the sensitivity and specificity of nested PCR for the detection of *P. falciparum and P. vivax* in anopheline.

MATERIALS AND METHODS

Karachi, the capital of the province of Sindh is most populated city of Pakistan. It is located on the coast of the Arabian Sea in Southeastern Pakistan, northwest of the Indus Delta as a result has a relatively mild climate. The level of precipitation is low for most of the year approximately 10 inches per annum; humidity levels usually remain high throughout the year. According to Census report 2006, the estimated population of Karachi is 11,969,284. Prior to the implementation of the devolution plan in 2001, the administrative area of Karachi was known as Karachi Division, which was subdivided into five districts: Karachi Central, Karachi East, Karachi West, Karachi South, and Malir, in 2000, the five districts were merged into Karachi District and officially became a City District in 2001. [See Fig-1] The present study was carried out in five districts of Karachi and its suburbs. The concerned area supervisors and related people were fully informed of the nature and aim prior to the inspection and asked their active participation. An informed approval was also obtained from each household head. The collection was done in the dry and wet seasons and tried to cover whole areas throughout the year with the interval of three months [11]. All houses were plotted and their residents were fully identified especially the room in which they

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slept. The survey was conducted from 6.00 A.M. to 7.00 P.M. Blood fed resting *Anopheles* females were collected especially in the morning. Collection was done by sweeping, netting and aspirating methods but spraying was not applied. The spraying with insecticide to habitats is time consuming and expensive. It is difficult to cover all mosquito habitats and causes environmental pollution [19]. All captured mosquitoes were kept in paper-cups then transferred in Eppendorf tubes and stored in refrigerator until identification.

(a) Identification of Mosquitoes Species

Species identification was made by the help of entomological key at the Department of Zoology, University of Karachi-Pakistan.

(b) Preparation of Mosquitoes Pools

Each pool consists of single head and thorax of mosquitoes. Collin's (1986) technique was applied for DNA extraction. 50µl of lyses buffer mixed with mosquitoes homogenate, crushed with pestle. The sample was incubated at 65ºC for 30 minutes. (16.68µl of 3mM) Sodium acetate was added to give 1M concentration. The sample was incubated at ice for 30 minutes, centrifuged for 10 minutes at 1000rpm. Supernatant was discarded and washed with pallets twice with 100 µl cold 70% ethanol, centrifuged for 5 minutes at 1000 rpm. Pallet was air dried and re-suspended in TE or ddH2O. [4]

(c) Nested Polymerase Chain Reaction

This method was applied for the detection of sporozoites on the extraction of dead, dried and frozen *A. stephensi* mosquitoes. DNA sample extracted from member of the *A. stephensi* mosquitoes which was used as DNA template for nested PCR reactions. Nested PCR was carried out using universal primers to detect genus and species specific primers.

(i) PCR Amplification for Genus Specific

The Nest 1 primer sequence was rPlu1.(TCA AAG AAT AAG CCA TGC AAG TGA CCT GTT GTT GCC TTG AAC TCC) and rPlu5. (CGT GTT GTT GCC TTA AAC TCC) (Snounou et al, 1993). The Nest 2 primer was rPlu3. (TTT TTA TAA GGA TAA CTA CGA AAA AGCTGT) and rPlu 4. (TAC CCG TCA TAG CCA TGT TAG GCC AAT ACCA) (Singh et al, 1999). Eppendorf thermal cycler was used for PCR amplification. 20µl reaction mixture consisted of 9.6µl of H2O, 2µl of 10x reaction buffer, 2.4 µl of 25mM MgCl2, 1µl deoxynucleotides triphosphate (dNTPs) to a final concentration of 200 µm and 10 pmol of each primer. DNA template was added about 3 micro liters, after denaturation for 5 minutes at 100ºC chilled on ice then Taq polymerase was added, after completion of 30-40 cycles denaturation at 94ºC for 1 minute, annealing at 56ºC for 1 minute and extension at 72ºC for 1 minute. A similar PCR mixture was prepared for Nest 2 with Nest 2 primers. Three µl of template of nest 1 was added and PCR amplification was followed the procedure described above, except annealing temperature was 62ºC [16]. For getting proper size target fragment, 10 micro liters of PCR product was loaded on 2% agarose gel in 1X Tris acetate buffer and gel stain and electrophoreses. Ultra violet (UV) light illumination was used to visualize the DNA bands.

(ii) PCR Amplification for Species Specific

The positive samples for genus *Plasmodium* were then analyzed with species specific primers. Three micro liters of Nested 1 product was mixed with PCR mixture. The specific primer for *P. falciparum* was 5' TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT 3'; 5'ACA CAA TGA ACT CAA TCA TGA CTA TGA CTA CCC GTC 3' and for *P. vivax* was 5'CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC3'; 5' ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA; (Snounou et al. 1993). The PCR amplification was carried out for 45 cycles. The annealing temperature for was 58ºC and 60ºC for *P. falciparum* and *P. vivax,* respectively. 10 micro liters of the product again electrophoreses for the detection of exact band sizes. Infected human blood was used as positive control whereas male mosquitoes were used as a negative control.

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RESULTS

Total 610 pools of (each with single) female *Anopheles* mosquitoes were examined for malarial sporozoites species specific genes, in which 3.27% pools were found positive with *P. falciparum* and 0.8% with *P. vivax* and mixed infection was 2.13%. (Table1). Nested PCR was therefore significantly high sensitive and specific technique for the detection and identification of species specific mosquitoes based on sporozoites gene based primers (Snounou et al. 1993). Thus present investigation revealed that *A. stephensi* involved in transmission of *P. falciparum* [See Fig-2] and *P. vivax* [see Fig-3] in Karachi-Pakistan.

Map of Karachi and its suburb where the present investigation was carried out

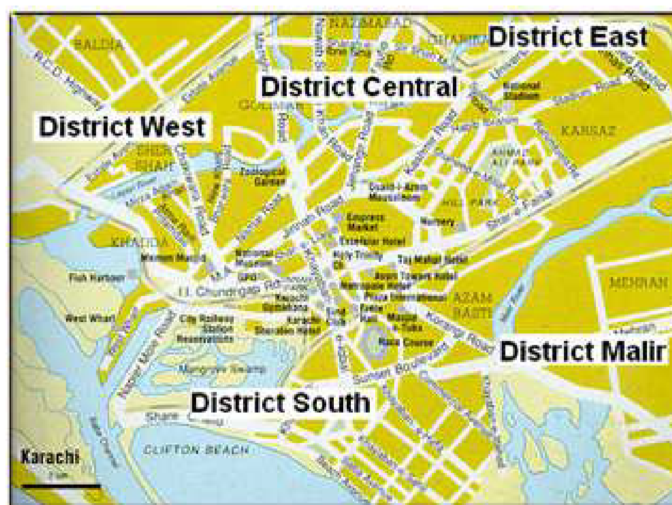

Figure 1

Plasmodium falciparum Fragments obtained from DNA amplification by PCR

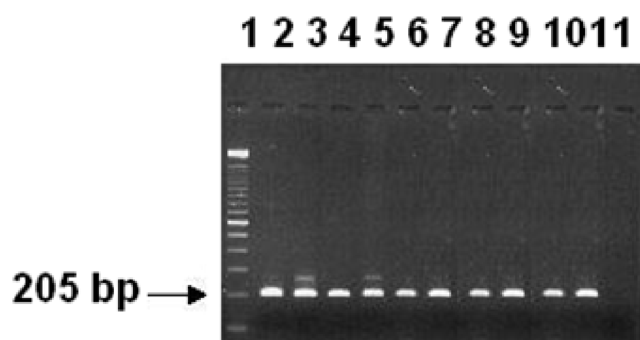

Figure 2

Lane 1 Marker, Lane 2 Positive control, Lane 3 -11 P. falciparum samples bands from positive samples of A.stephensi

Plasmodium vivax Fragments obtained from DNA amplification by PCR

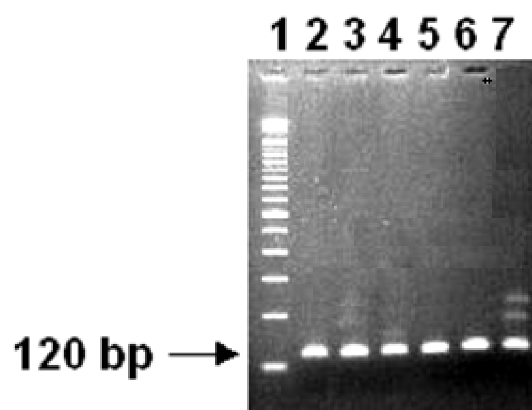

Figure 3
Lane 1 Marker, Lane 2 Positive control, Lane 3 - 6 P. vivax samples from positive samples of A.stephensi

Table 1
Malarial Sporozoites in *Anopheles stephensi* mosquitoes

Species	Total # of mosquitoes pools	Positive pools	Positive pools for *P. falciparum*. % in parenthesis	Positive pools for *P. vivax* % in parenthesis	Positive pools For Mixed infection % in parenthesis	Total Rate of Sporozoites %
A. stephensi	610	38	20 (3.27%)	05 (0.81%)	13 (2.13%)	6.22%

DISCUSSION

Detection and identification of different *Plasmodium* species in mosquitoes is a fundamental key in the epidemiological study of malaria occurrence and also for the estimation of malaria control program, In cases where low density of parasites found in mosquitoes a sensitive, specific and rapid assay is needed. The PCR method has been used to detect parasites in blood samples but very few studies have been reported on the amplification of parasite-specific DNA in mosquito vectors. It is because mosquito extracts contain strong inhibitors of PCR that prohibit direct amplification of *Plasmodium*-specific DNA in vectors. [12]. By dissecting and extracting oocysts or sporozoites from infected mosquitoes before amplification, some scientists reported that the PCR procedure could detect as few as 10 sporozoites in salivary glands or one single oocysts on the midgut [1,3, 15] But this method is impractical in the field surveys. Every female mosquito has the chance to feed on a blood of patient suffering from malaria. It is not necessary that *Plasmodium* develop in mature sporozoites in all species of mosquitoes but gametocytes may develop only in oocysts stage, especially in endemic areas mostly mosquitoes carry dead and degenerative stages of *Plasmodium* in their midguts so, extraction of whole mosquito give false positive result [8]. In this present report, we inspected only head and thorax of mosquitoes without abdomen to minimize the interferences from blood and oocysts stages of *Plasmodium vivax* and *P. falciparum.* According to Snounou et al, (1993), nested PCR is a sensitive technique. Singh et al. (1999) designed internal primers for the genus species specific *Plasmodium* for nest 1 and nest 2 amplifications and they reused the nest 1 amplification products of

positive samples for species specific nest 2 amplifications.[13] Vythilingham et al. (1999) also successfully used these techniques. The same technique was applied in the present research. In this study, nested PCR was carried out for the determination of genus as well as species using single mosquito for pool preparation. In some studies DNA has been extracted from a single mosquito or pools of mosquitoes. Vythilingham et al. (1999) extracted DNA from pools of 4 mosquitoes. The technique used in current study is sensitive and specific, and can recommend for use in field surveys where ever sophisticated laboratory facilities are present. The technique is especially useful for confirmation of vector status regarding its exposure of *Plasmodium* parasites in field caught mosquitoes. By way of evaluation and estimation of infection status of mosquitoes the technique can be a highly useful tool for adopting control measures in malaria eradication program. The investigation revealed that the nested PCR described is highly specific because other human *Plasmodium* species does not show 120 bp specific bands for *Plasmodium vivax* and 205 bp specific bands for *Plasmodium falciparum* which is very important in those areas where malaria is prevalent.

CONCLUSION

This investigation has shown that *A. stephensi* as the main vector for *P. falciparum* and *P. vivax* in Karachi and its suburbs, Pakistan. The abundance of anopheline species was found to be dependent on the ecotype studied, with *A .stephensi* being predominantly found in this city. These findings will be useful for the planning of control strategies for malaria vectors.

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