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# PCR-BASED SCREENING OF *PLASMODIUM* SPECIES IN MOSQUITO VECTORS OF FAISALABAD DISTRICT, PUNJAB, PAKISTAN

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## ABSTRACT

*Plasmodium (P),* mosquito-borne unicellular parasite, is responsible for "malaria". Pakistan remains at risk of malaria and almost 1.6 million cases of malaria are reported every year. The present study was planned to screen the mosquito vectors for *Plasmodium* sp. in Faisalabad district, Punjab, Pakistan using nested PCR. For this purpose, convenient sampling of adult mosquitoes was done from different places including: animal populated areas, lavatories, water storage tanks, livestock farms and road-side ditches in 70% ethanol. DNA extraction was done after stereomicroscopic identification of the specimens. Species identification of *P. falciparum, P. vivax, P. ovale* and *P. malariae* was done through universal forward and species-specific reverse primers in the nested PCR. Prevalence of *Culex* mosquitoes was higher as compared to *Anopheles*. *Plasmodium falciparum* and *P. vivax* were found higher as compared to other species of *Plasmodium*. The overall prevalence of *Plasmodium* sp. in mosquito vectors was 46% (14 out of 30 pools for *Plasmodium sp.*). Results were analyzed through chi-square analyses. Present study may explore the vectorial capacity of mosquitoes which can be an indicator of *Plasmodium* sp. distribution in an area for large scale metagenomics.

**Key words:** PCR, Mosquito, Plasmodium, Faisalabad, Molecular epidemiology.

## INTRODUCTION

*Plasmodium* (Haemosporida: Plasmodiidae) is responsible for the occurrence of "malaria". Various species of *Plasmodium,* which have been reported to cause malaria, include *P. falciparum, P. hylobati*, *P. cynomolgi*, *P. malariae, P. vivax, P. inui*, *P. jefferyic, P. knowlesi, P. fieldi*, *P. simiovale*, *P. silvaticum, P. youngi, P. eylesi*, *P. coatneyi*, and *P. pitheci* (White 2008; Beignon*et al*. 2014; Indra *et* al. 2014; Maeno*et al*. 2015). In most parts of the world (Asia, Africa and America), it is considered as an endemic disease. Every year, more than 200 million cases and a million of those ending as death have been reported resulting in economic losses in the form of macroeconomics (like depletion of foreign investments and human capital) and microeconomics (like direct cost invested in treatment and prevention and indirect cost due to sickness) leading to increased poverty level and interruption in the economic development (Kenneth *et al*. 2004; Anonymous, 2014, 2017). According to a survey, about 216 million cases and 0.445 million deaths due to malaria were reported in 2016, globally (Anonymous, 2017). Endemic reports of malaria have been reported from different countries of the Indian subcontinent like Pakistan, India, Bangladesh and Sri Lanka. According to World Health Organization (WHO), Pakistan contributes 31% of estimated malaria cases in 2016 and falls among the countries which showed increase in case incidence from 2010 to 2016 (Anonymous, 2017). The prevalence of different species of mosquitoes depends upon the environmental conditions of the area under study, for example from rural areas; *Anopheles* (*An.) culicifacies* has been reported (Pervez and Shah 1989; Rasheed *et al*. 2013) while *An. stephensi* is mostly reported in urban areas (Regmi*et al*. 2016). Similarly, *An. fluviaitlis* and *An. pulcherrimus* have been found in the mountainous areas of Punjab and Khyber Pakhtunkhwa (KPK) provinces of Pakistan (Suleman *et al*. 1993). There are different diagnostic techniques (conventional and molecular) available for the detection of malaria. The gold standard and the older one conventional method used for the diagnostic purpose is the blood smear. It is cost effective and applicable on large scale (Hanscheid 1999). The disadvantages of conventional diagnosis are (a) time consuming and (b) unable to detect low levels of parasitemia (Wongsrichanalai *et al*. 2007). While nucleic acid and proteins are being used in molecular diagnosis of malaria which is less time consuming but, expensive as compared to conventional diagnostic techniques (Wilson 2012). Based on the above mentioned facts, there was a need to optimize a molecular study on the epidemiology of *Plasmodium* species prevalent in mosquitoes. The study

was aimed to extract DNA of the collected specimens, their identification and vectorial capacity of *Plasmodium* species through nested PCR in the selected study district (Faisalabad, Punjab, Pakistan).

## MATERIALS AND METHODS

**Study area:** Faisalabad district (73°74 E; 30°31.5 N) is about 604 feet above sea level with maximum and minimum temperature ranges in summer 39 ºC and 27 ºC, respectively. Whereas, in winter the temperature ranges from 21 °C (70 °F) and 6 °C (43 °F).

**Sampling and taxonomy of mosquitoes:** Convenient sampling was done twice a day for collection of mosquitoes from the Faisalabad district during September 2015 till May 2016. General collecting nets were used for mosquito capturing and insect killer for indoor collection. Different localities of the study district were searched including human and animal populated areas, lavatories, clogged sewage drains, temporary road side ditches, stagnant stream side pools, household water storage tanks, cemented open water storage tanks, cemented temporary pools as these are the suitable habitats and favorable mosquito breeding sites (Fig. 1). During sampling, collection bottles containing 70% ethanol were used to ensure the safety of organism's morphological structures from the transportation damage. The samples for further processing were transported to the Molecular Parasitology Laboratory, Department of Parasitology, University of Agriculture Faisalabad, Pakistan. Mosquito identification was performed using standard taxonomic keys (Soulsby 1982; Schaffner *et al*. 2001).

**Molecular detection of *Plasmodium* species in vectors:** Processing of mosquitoes was done after the pooling of female *Anopheles* mosquitoes. Each Eppendorf tube contained 15 female mosquitoes to attain the weight of 25 mg after grinding. Extraction of DNA was done using commercially available ThermoFisher Scientific Gene JET Genomic DNA purification kit (cat. # K0722, ThermoScientific, USA) as per the manufacturer's recommendations. The DNA yield was determined with Nanodrop spectrophotometer 2000 (ThermoFisher Scientific, USA). Samples were stored at -20 °C till further use.

**Nested PCR:** The 18S rRNA genes at species-specific nucleotide sequences were replicated for *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum* following the primers designed by Han *et al.* (2007). In first round of amplification, a 25 µL reaction volume consisting of 2 µL (100 ng) of template DNA, 12.5 µL of PCR master mix consisted of (25 mM MgCl2, 0.25 U AmpliTaq Gold DNA polymerase, 1 x PCR Gold Buffer II (50 mMKCl, 15 mMTris-HCl, pH 8.0), and 200 µM each deoxynucleoside triphosphate), 0.5 µM each *Plasmodium* universal forward (P1) and universal reverse primers (P2) and 9.5 µL PCR grade water. PCR cyclic protocol for first round of amplification was; initial denaturation at 94 °C for 10 minutes and 92 °C for 30 s, annealing at 60 °C for 1.5 minutes, and extension at 72 °C for 1 minute (35 cycles) with a final extension at 72 °C for 5 minutes.

For second round of amplification, amplified DNA of first round was used as template DNA and diluted 20 folds in PCR grade water and reaction volume was the same as that for first round except 1 µL DNA, universal forward *Plasmodium* genus-specific primers (P1) and each species specific reverse primers (of four species) for single PCR reaction (Table I). Cyclic conditions were also the same as that for first round except number of cycles which were 20 (Schaffner *et al*. 2001). PCR products of each round were subjected to 2% agarose gel. The positive control was the DNA extracted from the blood of malaria positive patients (courtesy: Pakistan Health Research Council, Research Center, Fatima Jinnah Medical University, Lahore, Pakistan)

**Statistical analysis:** The variable species of mosquito with respect to sex (categorical variables) were explored using chi-square test. Data was analyzed by chi-square test of independence using SPSS 20.0 software. P values of less than 0.05 were considered statistically significant (Schork and Remington 2010).

## RESULTS

Conventional taxonomy of mosquitoes revealed the higher (82.6%) prevalence of *Culex* mosquitoes as compared to *Anopheles* (17.30%). However, the number of females *Culex* was found less (12.99 %) as compared to female *Anopheles* (72.5%). The higher prevalence of female *Anopheles* indicates the prevalence of *Plasmodium* vector in the study area. The total number of *Culex* and *Anopheles* mosquitoes identified using stereoscope from study district has been shown in Figure 2.

The results of nested PCR have been shown in Figures 3 and 4. Of 30 pools, 14 were infected with *Plasmodium.* The positive samples for *Plasmodium* genus have been shown at 160 base pair (bp). *Plasmodium* species show positive bands at 110 base pairs and *P. falciparum* (09/14) and *P. vivax* (05/14) were identified (Figure 4).

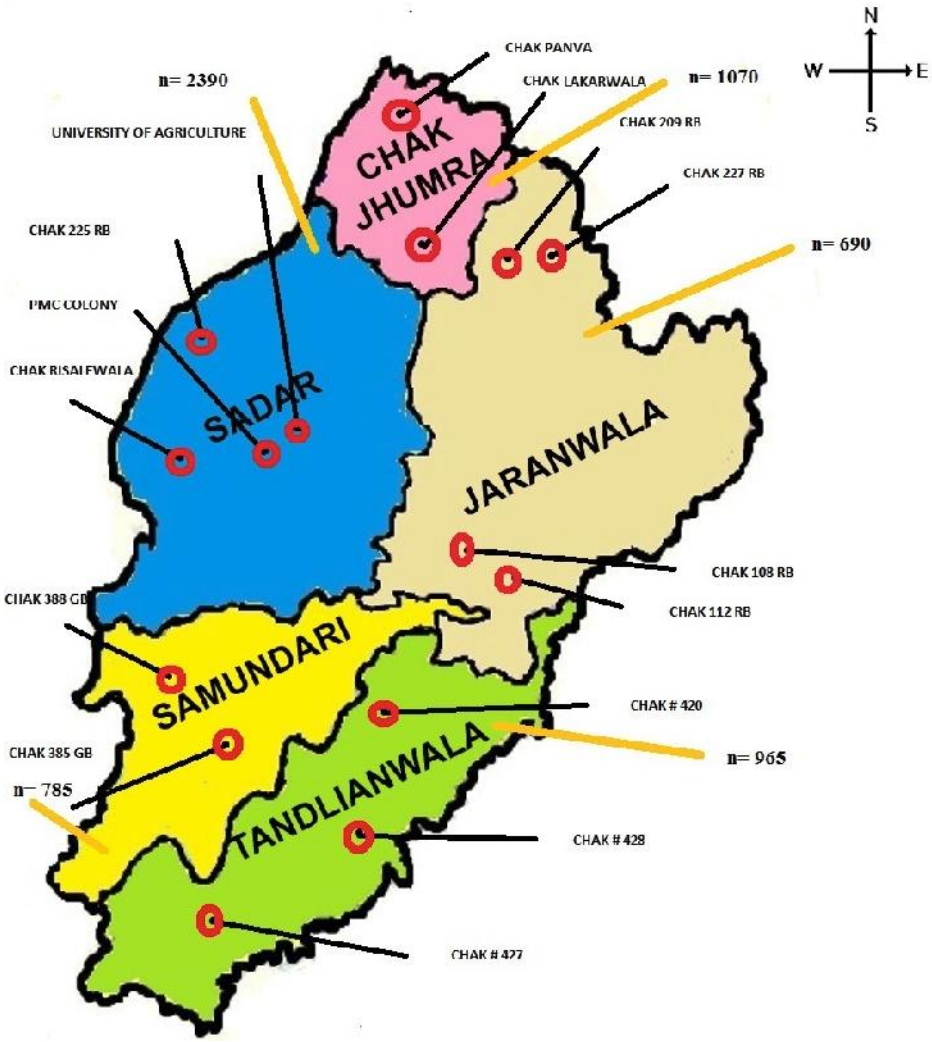


**Figure 1. Physical map of Faisalabad district showing areas of sampling of mosquitoes.**

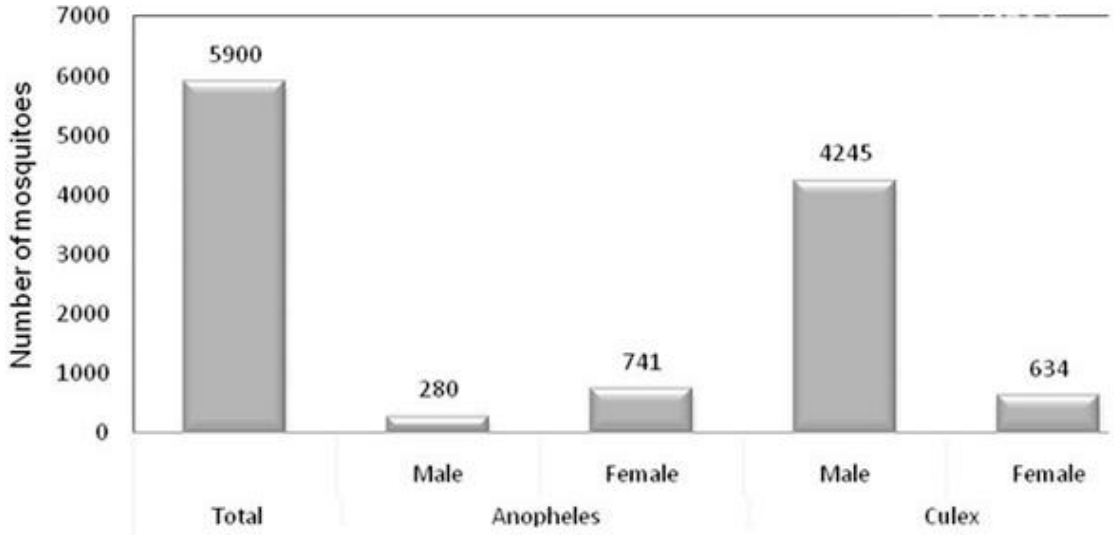


**Figure (2). Overall Prevalence of mosquito species during September 2015 to May 2016 in Faisalabad district.**

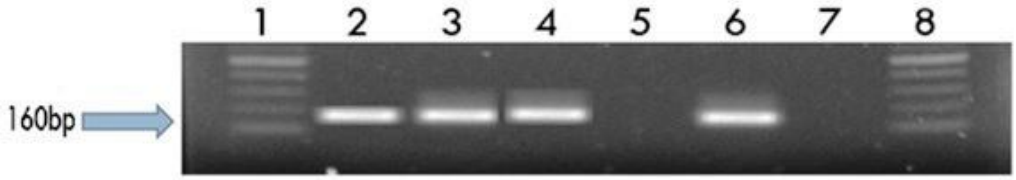


**Figure (3). DNA fragments amplified by genus specific primers of Plasmodium; Lane 1 & 8 are 100 bp plus DNA marker, Lane 2 is positive control, Lane 3,4 and 6 shows positive samples, Lane 7 is negative control.**

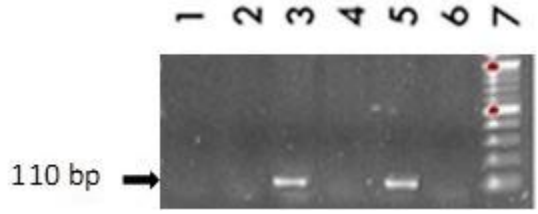


**Figure (4). DNA fragments amplified by species specific primers of Plasmodium; Lane 7 is 100 bp plus DNA marker. Lanes 1 and 6 are negative control, Lane 2 is *P. malariae*, Lane 3 is *P. falciparum*, Lane 4 is *P. ovale*, Lane 5 is *P. vivax* PCR products, respectively.**

**Table 1. List of Primers used for genus and species specific amplification of *Plasmodium* sp. from mosquitoes collected from district Faisalabad, Punjab, Pakistan** (Han *et al.* 2007).

| Primers | Sequence | Tm (ºC) | PCR product size (bp) |
|---|---|---|---|
| Universal forward primer *Plasmodium* genus specific (P1) | 5'-ACGATCAGATACCGTCGTAATCTT-3' | 55.4 | 160 |
| Reverse Primer (P2) | 5'-GAACCCAAAGACTTTGATTTCTCAT-3' | 53.8 | 160 |
| Species-specific reverse primers for *P. falciparum* | 5'-CAATCTAAAAGTCACCTCGAAAGATG-3' | 54 | 110 |
| Species-specific reverse primers for *P. vivax* | 5'-CAATCTAAGAATAAACTCCGAAGAGAAA-3' | 53.3 | 110 |
| Species-specific reverse primers for *P. malariae* | 5'-GGAAGCTATCTAAAAGAAACACTCATAT-3' | 53.2 | 110 |
| Species-specific reverse primers for *P. ovale* | 5'-ACTGAAGGAAGCAATCTAAGAAATTT-3' | 53.2 | 110 |

## DISCUSSION

Mosquitoes are distributed all around the world with around 3,537 described species and 112 genera reported so far (Harbach 2014). Mosquitoes are responsible for many vector-borne diseases/pathogens such as malaria, Dengue fever virus, West Nile virus *Francisella tularensis, Wuchereria bancrofti* etc.. Almost 700 million people around the globe are being encountered by mosquitoes on yearly basis (Caraballo and King 2014). The pregnant women are at higher risk of malaria due to lack of awareness to the disease prevention (Okiring *et al*. 2019). The study was planned to find out the prevalence of mosquito species in district Faisalabad, Punjab, Pakistan. The major impact of malaria disease in Faisalabad was reported by Anonymous (2012). In this investigation, mosquitoes were collected through convenient sampling from different subdivisions of study area. The prevalence of *Culex* mosquitoes was higher as compared to *Anopheles* mosquitoes which could be due to non-probability (convenient) sampling. It may have many positive and negative impacts on research as it provides convenient method of sampling and samples can be collected from different regions easily but it can provide false positive results as abundancy of *Culex* or *Anopheles* mosquitoes can be different at different regions (Teddlie and Yu 2007).

The general collecting net was used for collection of samples but other methods for collection have also been used elsewhere which contains dry ice traps. The collected samples were then preserved in 70% ethanol as the preservation rate of tissue in ethanol is higher as compared to 10% formalin (Bressan *et al*. 2014). The samples were processed for DNA extraction through DNA purification Thermofisher Scientific kit as it has more purification rate and reliable than conventional method (Ruiz*et al*. 2015). DNA was extracted from the guts of mosquitoes as *Plasmodium* is present in midgut of mosquitoes (Aly*et al*. 2009).

In past investigations, comparison has been done between two techniques which included nested PCR and LAMP (loop mediated isothermal amplification of DNA) for identification of *Plasmodium* species (Han *et al*. 2007). The efficiency of both techniques was almost equal in results. The method requires only two steps before running the samples on an agarose gel and is also having a practical value for large scale field based studies where reliable species identification is important. The study has been conducted in which very low parasitemia level of *Plasmodium* infection was detected as 0.1 *Plasmodium* per 50 µL of blood with the real time quantitative nucleic acid sequence based amplification (real-time QT-NASBA) assays to identify human malaria parasite. The sensitivity and specificity of this test makes it a diagnostic tool and more suitable for drug studies as compared to nested PCR (Mens *et al*. 2006).

Another study has been conducted which indicated that the examination of thick and thin blood smears by microscopy was insufficient for the diagnosis of malaria in Turkey. As the number of parasites in blood goes down from 100 parasites per mL, microscopic methods present decreased sensitivity as well as false negative results (Payne 1988; Snounou *et al*. 1993; Kain *et al*. 1993; Anonymous, 1999; Safeukui *et al*. 2008; Fuehrer *et al*. 2011; Joanny *et al*. 2014; Li *et al*. 2014). Nested PCR has more sensitivity and specificity than conventional microscopic methods as 100 % sensitivity, 73.3 % positive predictive values and 97.2 % specificity of nested PCR were reported elsewhere (Snounou *et al*. 1993; Fuehrer *et al*. 2011). In another study, out of the positive *Plasmodium* samples, 93.33 % were *P. vivax* (Doni *et al*. 2016).

The results were based on many factors as mentioned above which can also include seasonal modification. In Autumn and Winter, the prevalence of mosquitoes was higher as compared to Spring and early Summer, due to optimum temperature required for the growth and propagation of the mosquitos. Mosquitoes are being found in peri-domestic and domestic environments. Use of land cultivation, open sewerage drainage etc. by human is playing a vital role in changing the composition of mosquito population and their pattern of transmission of different diseases. Mosquitoes are greatly influenced by different factors including environmental, socioeconomic and climate change. A variety of factors e.g. rapid changes in land use, trade globalization and social upheaval have been documented to affect the vectoral role of mosquitoes in transmission of diseases (Chaves *et al*. 2012). Environmental factors are firmly associated with the interactions between host and vector. These vectors are playing an imperious role in spread of vector-borne diseases to humans (Rochlin *et al*. 2016). Based on the hypothesis and objectives of this investigation, following conclusions have been made: (a) the diversity of *Plasmodium* sp. in mosquitoes *viz*; *P. falciparum* and *P. vivax* were identified through nested PCR from mosquito vectors which confirmed that mosquito is a potential vector for *Plasmodium* infectivity in the study district (b) the prevalence of *Culex* mosquitoes was higher as compared to that of *Anopheles* (vector of malaria) in Faisalabad district and (c) infectivity burden of mosquito vectors was 46% in Faisalabad; 14 of 30 pools for *Plasmodium* species. In the present study, higher prevalence of mosquitoes in Sadar and Chak Jhumra tehsils might be attributable to increased population of humans in this area. Two species of *Plasmodium* were identified: *P. falciparum* and *P. vivax* while infectivity burden of *Plasmodium* was 46% in district Faisalabad. In literature, 2320 cases of malaria were reported in the *Nation News* (Anonymous, 2012) in 20 districts including Faisalabad. However, the infectivity rate of malaria was lower in Faisalabad as compared to other districts. The reason behind this could be the prevention and control strategies for disease which were used in past. Recently, Abbas *et al.* (2019) published on segmentation of Plasmodium on region growing and dynamic convolution based filtering algorithm from thin blood semear points and classified four species viz; *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae.* They found 96.75% of sensitivity for malaria parasitemia and 94.59% of specificity. Two species viz; *P.vivax* and *P. falciparum* have been reported in a baseline survey of the three districts (Bannu, Dera Ismail Khan and Lakki Marwat) of KPK (Qureshi *et al.* 2019). Another recent study compared the blood smear, specie-specific PCR and rapid diagnostic test for detection of malaria in human blood samples from district Bannu, KPK and found P. vivax as the dominating species followed by P. falciparum and mixed infections. Various social/ environmental determinants were also found statistically associated with the cases of malaria in this study (Jahan *et al.* 2019). Among the malaria free regions, Europe and Italy were also included since 1970s but in 2017, malaria due to *P. falciparum* was reported in the Italy which indicates the recurrence of this fatal disease (Tagliapietra *et al*. 2019). Most prevalent type of malaria in Nigeria is *falciparum* while malaria due to *P. malariae* and *P. ovale* are present but infrequently (Oboh *et al*. 2018).

Keeping in mind the above mentioned conclusions, following recommendations have been placed in front of policy makers to plan about the different strategies against the vector accordingly: (a) application of DNA barcoding technique for species of mosquito from other regions of Pakistan. (b) metagenomics of *Plasmodium* sp. in the vector and hosts to estimate the risk analysis, (c) need-based preventive management policy in various areas of the province/ country. Determination of vectorial capacity of mosquitoes can be an indicator of distribution of *Plasmodium* in an area for large scale metagenomics. Alternative control of vector can be planned accordingly

by knowing the species composition status in specified area.

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