Phylogenetic analysis of *Plasmodium vivax* from clinical isolates of Rawalpindi, Islamabad and Azad Kashmir

By

Nargis Shaheen

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

2015

Phylogenetic analysis of *Plasmodium vivax* from clinical isolates of Rawalpindi, Islamabad and Azad Kashmir

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTERS OF PHILOSOPHY

IN

PARASITOLOGY

By

Nargis Shaheen

Department of Animal Sciences

Faculty of Biological Science

Quaid-i-Azam University

Islamabad

2015

DECLARATION

I hereby declare that the work accomplished in this thesis is the result of my own research carried out in Parasitology lab, Department of Animal Sciences, Quaid-i-Azam University, Islamabad.

This thesis has neither published previously nor does it contain any material from the published resources that can be considered as the violation of the international copy right law.

I also declare that I am aware of the term ‘copy right’ and ‘plagiarism’. I will be responsible for the consequences of any violation to these rules (if any) found in this thesis. This thesis has been checked for plagiarism.

Nargis Shaheen

CERTIFICATE

This dissertation "Phylogenetic analysis of *Plasmodium vivax* from clinical isolates of Rawalpindi, Islamabad and Azad Jammu and Kashmir" submitted by Ms. Nargis Shaheen is accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Masters of Philosophy in Parasitology.

Internal Examiner: _____

(Supervisor)

External Examiner: _____

Chairman: _____


بسم الله الرحمن الرحيم

DEDICATION

I dedicate this humble effort

To

My Grand Father and Parents Who have prayed for my fruition ceaselessly and have demanded nothing for their endless efforts.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENT

First and foremost, my thanks goes to ***Allah Almighty****, Who is most Gracious and Merciful.* ***Allah*** *created the universe with the idea of beauty, symmetry and harmony. He gave the abilities to human to discover what He did. All praises to Allah for awarding me with patience, health and knowledge in completing this thesis. I praise to my everlasting source of guidance* ***Prophet Muhammad (PBUH).***

ALLAH vastly bless me through people, who always contribute to my studies. I would like to present my thanks and appreciations to my honorable supervisor ***Dr. Naveeda Akhter Qureshi,*** *Assistant Professor, Department of Animal Sciences for affectionate supervision. I have been infact lucky to have a supervisor who care about my work and who answer back to my queries and demands so punctually. Her invaluable guidance, encouragement, patience, and financial support will always be appreciated. Her positive attitude and confidence in my research work inspired me and gave me self-confidence. She provided me the vision and motivation to be successful in life like her. I would like to extend my thanks to* ***Dr. Irfan Zia Qureshi*** *Chairman, Department of Animal Sciences, for providing me all the privileges during my research work.*

I present bundle of thanks to ***Dr. Waseem,*** *who provided me the facilitation at his biochemistry lab for doing my molecular level work. Special thanks to* ***Dr. Irfan*** *and* ***Farooq*** *from biochemistry lab.*

I am indeed humbly grateful to ***Irfan Hussain*** *for his cooperation, valuable suggestion, most cooperative affectionate behavior, inspiring and impetuous guidance. His experimental, written and moral help for the completion of this task. I wish to regard my deep sense of gratitude and sincere thanks to my lab fellows,* ***Irfan Hussain, M. Afzal, Asma Ashraf, Sumbal Haleem, Faiza Naqvi, Abid Ali, Naseer Ullah, Atiya Iqbal, Huma Fatima, Fahmi, Alamgeir, Imtayaz, Rabia Afridi, Farah Obaid, Naseeha, Wajeeha, Amina*** *and* ***Hameed Ur Rahman*** *for their cooperation.*

*The cooperation of lab assistant **Yasir Sultan** in the lab is also noteworthy to be acknowledged. I am obliged to **Naeem Masih** and **Sami Ullah** for their help, support and motivation during my research. I appreciate the moral support and encouragement of my friends **Maria Afrique, Sidra Khalid, Anam Hammid, Saddaf Razzaq, Sana Sarfraz, Almas Basharat, Faryal Basharat, Nazia Afrique, Farzana Rahim, Hifsa Shafahat, Fozia Rsaheed** and **Hafsah Imama-Tul-Kubra.***

*I can never forget the prayers and untiring efforts of my grandfather, **Captain M. Said Khan**, my father **M. Razaq Khan** and my mother **Khadija Begam.** They encourage me always and they sacrificed their comfort for my brilliant future.*

*Thanks to my uncle **Basharat Hussain**, whose company always helped me to relieve from the hectic and tiring moments during my study. His immaterial love and sincere devotion have made me strong both physically and mentally.*

*I submit my earnest thanks to my special relations **M. Irshad, M. Rahim, M. Rashid, M. Naheem** and **M. Afrique** who always positively respond at the real time of need. My handicap words can never express my sentiment, attachment and indebtedness to all of them.*

*Last but not the least I am greatly indebted to my brothers **Amir Sohail, Umer Razaq** and **Khalid Rahim** for their love, immense orison and mellifluous affections, which hearten me to achieve success in every sphere of life. How can I forget my little cute younger brother **Danial Irshad** in whom I can see the innocence of nature.*

Nargis Shaheen

ABSTRACT

Malaria eradication program efforts have a significant impact on the epidemiology and parasite population dynamics. In countries aiming for malaria elimination, malaria transmission may be restricted to limited transmission hot spots, where parasite populations may be isolated from each other and experience different selection forces. No detailed studies have yet been performed to reveal the actual situation of malaria in the local population, due to which, despite of malaria being common, the epidemiological data is insufficient to elucidate the actual incidence of malaria in Pakistan and Azad Kashmir. Here we aim to examine the *Plasmodium vivax* population divergence in geographically isolated transmission zones in Pakistan and Kashmir. A malariometric population survey was conducted in 2013 and 2014 using whole blood samples collected from 1500 patients of all ages and genders from different localities of Pakistan and Azad Jammu Kashmir. Microscopically confirmed positive species were subjected to nested PCR for reconfirmation and detection of four species of *Plasmodium* causing human malaria. For characterizing *P. vivax* populations based on the extensive diversity. We employed the *P. vivax* merozoite surface protein 3β (*PvMSP*3β) and 3α (P*vMSP*3α) as a molecular marker. To examine parasite populations with different transmission levels Kashmir and Pakistan, we obtained 142 *P. vivax* isolates and 72 *P. falciparum* from Kashmir, and 184 *P.vivax* and 69 *P. falciparum* from Islamabad. From Rawalpindi, we obtained 168 *P.vivax* and 83 *P. falciparum* Positive isolates where the annual parasite incidence (API) was more than 25%. We sequenced the *Pvmsp*3β and *Pvmsp*3α gene and examined its genetic diversity and molecular evolution between the parasite populations. Using the *Pvmsp*3α molecular marker we characterized, 12 different genotypes of *P.vivax* circulating in Islamabad, 14 in Rawalpindi and 14 Predominant in Kashmir. In the case of *Pvmsp*3β we characterized 15 different genotypes of *P.vivax* circulating in Islamabad, 12 in Rawalpindi and 15 in Kashmir. The phylogenetic analyses of both the marker show a clear and distinct relation with the isolates from India, China and Brazil. We have more confirmed that *Pvmsp*3β and *Pvmsp*3α sequencing deals significantly higher power for defining genetic diversity of parasite linked with the PCR-RFLP simple method. From the current study, we concluded that malaria parasite populations in a given region may vary significantly in genetic diversity, which may be the result of control and influenced

by the magnitude of malaria transmission intensity. This is an issue that should be taken into an account for the implementation of *P. vivax* control measures such as drug policy and vaccine development.

INTRODUCTION

1.1: Malaria

Malaria is a multifactorial disease, caused by a protozoan parasite belong to genus *Plasmodium*. The term malaria comes from two Italian words mal means "bad" and aria means "air". It was believed that people become infected in association with bad air (Tuteja, 2007) . The malaria is an ancient disease. Its symptoms were described in the Chinese medical. Various characteristic symptoms were described and later they were named as malaria in BC 2700. Malaria was broadly known in Greece and was responsible for city population damage (Cox, 2010). The disease was known by various names like swamp fever, jungle fever and ague. These all were classified as an intermitted fever (Frenkel & Western, 1988).

Qinghao is a plant, which is used for medical treatment in China. This plant is recognized such as annual wormwood. This plant had anti-fever activity. The artemisinin was recognized as constituent of Qinghao. In 1971, it was isolated by the Chinese scientists. Plant extract derivatives are collectively known as artemisinin. Now a days it is very operative antimalarial drug, mostly with additional medicine combination (Hsu, 2006).

Jesuit missionaries well-versed from local populations of Indian about remedial bark. Fever was cure by this bark. So it was used for, the Countess of Chinchón (the wife of the Viceroy of Peru) treated of her fever. The tree was called Cinchona and the bark was named *Peruvian bark*. Andean Cinchona is a name of tree, from its bark Quinine is made. Quinine is presented today with artemisinins (Sattabongkot *et al*., 2004).

1.2: Discovery of the Malarial Parasite

Charles Louis Alphonse Laveran (1881) was handling the malarial patients. Laveran saw crescentic forms in the blood of malarial patients. Although these forms were absent in non-malarial patients. Laveran thought, malaria is caused by a parasite. For this discovery, Laveran was presented by Nobel Prize in 1907 (Cox, 2010).

1.3: Malarial Species Differentiation

The disease had two forms. One is tertian periodicity and other is quartan periodicity. Temperature arises every other day in tertian periodicity while every third day in quartan periodicity. Upon maturity, different numbers of new parasites (merozoites) produced from this form. Temperature concurred by the merozoites discharge and rupture (Batchelor *et al*., 2014).

1.4: Human Malarial Parasite Name

The Italian investigators (1890) was first introduce the names of *Plasmodium malariae* and *Plasmodium vivax* that affect human. Laveran had believed that *Oscillaria malariae* was the only species. In (1897) the malignant tertian malaria parasite *P. falciparum* was reported. There were various point of views against the use of this name. The use was so wide in the literature that a change back to the name given by Laveran was no longer thought possible. John William Watson Stephens (1922) defined the *P. ovale.* As the 4th human malaria parasite. Stephen saw this species 1st time in the soldier's blood. In 1931 *P. knowlesi* was first described by in (long-tailed) macaque. It is recognized that *P. knowlesi* also cause human infection in 1965 (Garnham, 1988).

1.5: A Discovery that Mosquitoes Transmit Malarial Parasite

Ronald Ross (1897) showed that, malarial parasites might be passed on from diseased persons to mosquitoes. Ross also showed that mosquitoes might pass on malarial parasites from one bird to another. So the sporogonic cycle is required. The sporogonic cycle is the time period in which malarial parasite developed in the mosquitoes. Ross was awarded with the Nobel Prize in 1902 (Garnham, 1988).

1.6: Discovery of the Transmission of the Human Malarial Parasite *Plasmodium*

The Italian professor collected local mosquitoes called *Anopheles claviger* and allowed them to feed on malarial patients. Infected mosquitoes were sent in London to Manson (1898). He fed the mosquitoes on his son and his own technician. Both developed tertian malaria. It was demonstrated that only *Anopheles* mosquitoes transmit human

malaria. By this experiment a complete sporogonic cycle of *P. vivax,* P. *falciparum* and *P. malariae* was demonstrated (Tuteja, 2007).

1.7: The Parasite (*Plasmodium*)

Malaria is caused by a protozoan. Which belongs to the phylum Apicomplexa, order Haemosporidia and genus *Plasmodium*. Marchiafava and Celli were first introduced generic name of *Plasmodium* in 1885. This has not changed since today (Seman *et al*., 2008). About one hundred species of *Plasmodium* infected several animals, birds, reptiles, numerous mammals and human (Sattabongko *et al*., 2004). In the case of human, four species cause malaria named as *P. vivax*, *P. falciparum, P. malaria* and *P. ovale. P. falciparum* causes the highest mortality worldwide. *P. vivax* is widely distributed and most prevalent (Sahar *et al*., 2010).

1.8: Life cycle of *Plasmodium*

The female *anopheline* mosquito serves as primary host for malarial parasite, whereas other vertebrates are secondary host (Ekpuka *et al*., 2014). The life cycle of the parasite begins through mosquito bite. The *Plasmodium* in the form of sporozoites is injected into the capillaries of the skin of the vertebrate. The sporozoites then travels quickly to the liver cells and invade the hepatic cells. The maturation process results into shizont and then it differentiates into thousands of merozoites. They (merozoites) released into the blood stream and invades the erythrocyte (Bannister & Sherman, 2009).

Inside the erythrocyte merozoites again replicate and divide (asexually). About 6 to 32 daughter merozoites are produced by each merozoite in 24 to 72 hours, but it varies according to species. Finally the infected RBCs lyse and then merozoites are again, releasing into the bloodstream. They invade other cells. This cycle carries on till the patient's death occurs. Sometimes the parasite reduce by the immune system of the host (Anstey *et al*., 2009).

In the other hand the merozoites are differentiated into microgametocytes and macrogametocytes. They do not invade the erythrocyte although they live in the (human) host. *Anopheline* mosquitoes ingest them and grow into female and male gametocytes.

Inside the gut of the mosquito the gametes meet and fuse into diploid zygotes (the ookinete). This is the single diploid stage during the *Plasmodium* parasite life time. Ookinetes pierce the mosquito's gut wall, and oocyst are formed. The oocyst are transfered into sporozoites. They move toward mosquitoes' salivary gland. The motile sporozoites are ready for the transmission into another vertebrate host (Bannister & Sherman, 2009).

In mosquito the time frame necessary for the complete sporogonic cycle is different and are influenced by the *Plasmodium* species and external temperature. *P. vivax* required 27^{O}C for 8 to 13 days, 20^{O}C for 20 days, and 18^{O}C for 30 days while *P. falciparum* required 20^{O}C for 30 days. For both species at temperatures below the range of 16 to 18^{O}C cycle cannot complete while the upper limit for the completion is 33^{O}C (Zucker, 1996).

1.9: Seasons of Transmission

The Pakistan is semi-arid or arid, it depends on snow feed river and irrigation, so rainfall and snow melt provide habitats for larvae and increase transmission of malaria. The major seasons are from June-November (Safi *et al*., 2010). The main transmission period for *P. vivax* topping from June- September, again in April-June. In the case of *P. falciparum* is from August-December (Khattak *et al*., 2013). The short transmission period of *Plasmodium* occurs during March-April (Khatoon *et al*., 2010).

1.10: Molecular Epidemiology

Malaria is mainly a disease of poverty that is more prevalent in the rural areas, resulting in large financial loss (Kakar *et al*., 2010). The host, vector, parasite and socioeconomic factors greatly affect the epidemiology of malaria (Zakeri *et al*., 2010). Epidemiology varies because of species differences, the presence of malarial parasite in different areas, mosquito distribution and exposure of anti-malarial drugs to *Plasmodium* and the immunity of the population (Alemu *et al*., 2014). It is estimated that malaria is responsible for about 1 million deaths yearly worldwide (Haque *et al*., 2009). In 1979 malaria had been eliminated in Korea, but in 1993 an incident of malaria was reported and after this, increase was observed in the rate of malaria yearly (Kim *et al*., 2009).

P. vivax is the dominant species (except Africa) in endemic areas, one hundred million cases are recorded every year (Bozdech *et al*., 2008)**.** Infants and young children are getting an infection of *P. vivax* in prevalent areas of *P. falciparum* and *P. vivax* (Poespoprodjo *et al*., 2009). In Pakistan *P. falciparum* and *P. vivax* is responsible for malaria. Round about 36% of infection is due to *P. falciparum* and 64% of infection caused by *P. vivax* (Khattak *et al*., 2013). In all provinces of Pakistan high rate of endemicity were observed (Raza & Beg, 2013).

Presence of malaria is different at different ages. After the age of 5 years, the infection rate of *P. falciparum* is very rare in highly endemic areas (Dondorp *et al*., 2008). The age of infants <6 months are safe from clinical malaria due to the presence of maternal antibodies and foetal haemoglobin (Larru *et al*., 2009). In area with high transmission, severe malaria does not affect the adults, young children are affected in moderate transmission areas, while all age groups are affected in areas with low transmission (Dondorp *et al*., 2008). According to the Schellenberg in 1999 among hospitalized children <1 year of age, 54% death occurs due to malaria in prevalent areas of Africa. Carneiro in 2010 observed that the splitting of the middle age of malaria death between seasonal, highly endemic areas and moderate endemic areas, at 12 and 28 months of age, could be recognized to immunity increased in area with high transmission and in case of middle age similar trend is seen in clinical malaria. Out the areas have high transmission median ages were low, but in case of 28 months of median age reveal age dependence nevertheless the intensity of transmission. These differences are may be due to the complication in access neurological status in early stage (Aponte *et al*., 2007).

1.11: Phylogeny of Human *Plasmodium vivax*

The monophyletic clade of *Plasmodium* parasite have been formed with the *P. vivax* and Asian macaque (Tanabe *et al*., 2007). The species of Asian macaque is believed to ancestral strains of *P. vivax* whereas the derivation of *P. simius* is from *P. vivax* (Lim *et al*., 2005) and in the Asia *P. vivax* is originated , central and west Africa can't provide a proof about its origin and another way should be used for its explanation (Escalante *et al*., 2005). *P. vivax* fit in the plasmodia branch which is specific for Asian macaques,

significantly distinct to the group of Lavernia , by the help of several genes it has been confirmed that the phylogenetic location of *P. vivax* is close to Asian species of *P. knowlesi* and *P. cynomologi* (Leclerc *et al*., 2004; Mitsui *et al*., 2010) .The ancestral position was proposed on the basis of high polymorphism for macaque *Plasmodium* species than *P.vivax* case (Escalante *et al*., 2005). Asian origin of *P.vivax* had proposed by a hypothesis (Cornejo & Escalante, 2006; Mu *et al*., 2005). The vital facts had not been discovered about *P.vivax* so the hypothesis had not been yet challenged. The origin of *P.vivax* is very complicated because the closest counterpart of *P. simium* (monkey parasite) is genetically undistinguished from *P. vivax* (Rich *et al*., 2009). An accepted view is, that after the host switch event one of *P.vivax* and *P. simium* species emerged in South America (Lim *et al*., 2005). The Lavernia group appears to be more host specific as compare to simian group. The monkeys and humans are not only the source of *Plasmodium*, which related to *P. vivax*, however also from chimpanzees and gorillas (Liu *et al*., 2010).

1.12: Clinical Manifestation

During *P.vivax* infection the prepetant period is ≥8 and the periodicity of febrile attacks is 48 hours. For *P.vivax* the incubation periods is 13 days range from 12-16 days. The longest incubation period recorded is 30 years because of the presence of hypnozoites in the liver (Trampuz *et al*., 2003). Clinical symptoms are caused by the RBCs repeated cycle of rupture and invasion (Batchelor *et al*., 2014). After the bitten, 6-8 days required for the development of malaria symptoms. Sometimes it may take several months after leaving from an endemic area (Sattabongkot *et al*., 2004). After infection, malaria takes 8 to 30 days after initiation with a common symptom of a high fever (Rasheed *et al*., 2009). The patients have complained of fever is >92% of cases, headaches 70%, chills 79% and diaphoresis 64%. Consistency of fever is not always essential. The absence of temperature is not a signal of minor disease. In addition to fever, chills, sweating, headache, vomiting, diarrhea, abdominal pain, cough, distension, hepatomegaly and splenomegaly also occur (Rasheed *et al*., 2009).

Infection of *P. vivax* cause severe lethal disease similar to *P. falciparum* including cerebral malaria, splenic rupture, thrombocytes deficiency, trauma, anemia (Kochar *et al*.,

2009). During pregnancy, *P. falciparum* cause severe clinical manifestations, including maternal anaemia, low birth weight and pre- maturity. *P. vivax* also cause the same problems (Binello *et al.*, 2014).

Malaria is the major cause of hematological and neurological infections (Dzeing-Ella *et al*., 2005). In the case of hematological infection, anemia occurs (Wickramasinghe & Abdalla, 2000) by damage of uninfected and infected erythrocytes, deficient erythropoiesis and low proportion of RBCs in circulation (Kumar, 2009). Perkins *et al (*2011) stated that in case of *P. falciparum* the vital reason of severe anemia is erythropoiesis destruction. Prevalence of severe *P. falciparum* malaria was studied through multicenter and it was observed that severe anemia occurs in 54.1% while in 17.75% severe malaria was associated with kidney failure, metabolic acidosis and increasing lactate levels.

In the case of neurological infection, cerebral malaria is observe. Cerebral malaria is a clinical syndrome where the patient has reduced consciousness that can develop into coma or death (Kumar, 2009). Cerebral malaria has highest mortality rate, symptoms range from confusion to coma, long term coma causes cortical blindness (Medana & Turner, 2006). The disease severity may rise in short period, where the conditions can get worse in 60 minutes. Acute respiratory distress syndrome is also observed in *P. vivax* infection (Anstey *et al*., 2009).

1.14: Diagnosis

To diagnose malaria, thick and thin films are used for all suspected cases and thick film is very sensitive for parasites. The samples are stain by using Giemsa and the thin film is the easiest way to differentiate the *Plasmodium* species. Parasite count can be found by counting RBC's and WBC's in the thin film (Bailey *et al*., 2013). This process needs a skill to discriminate malarial parasites and non-parasitic components (Tek *et al*., 2009). The microscopic examination is laborious and need well trained experts (Khairnar *et al*., 2009).

The modern technique used for the detection of *Plasmodium* is immunodiagnostic tests and these test are based on antigens. The antigens are released from infected erythrocytes. One specific test used for *falciparum* is para HITf test, it detects histidine rich protein11 (Taviad *et al*., 2011). The antigen lactate dehydrogenase are produced by all four

species of genus *Plasmodium* (Panchal & Desai, 2012). RDT's test are also available for *P. falciparum* species in many develop countries. The RDT performance varies with disease prevalence, parasite specie, geographical location and environmental occupation, the sensitivity of RDT decrease with low parasitic densities. The comparison of RDTs performance test give different results of laboratory test and field test (Kim *et al*., 2013). This method is easy, fast but have less specificity and sensitivity than microscopy and it gives results within 15 to 20 minutes. RDTs needs less skill as compare to other diagnostic methods (Okiro *et al.*, 2009).

Molecular techniques are used to distinguish and classify malarial parasites. Molecular means are used for allelic discrimination and in an endemic area various concurrent infections are generally present in blood samples (Falk *et al*., 2006). The nested PCR are used for detecting *Plasmodium* in low level parasitaemia. It is amplifying the 18s ribosomal DNA region of the parasite for indentification of species specific *Plasmodial* DNA (Kebede *et al*., 2014).

Real-time PCR is used to identify low levels of parasitemia, detect mixed infections and also used for species diffferciation (Mangold *et al*., 2005). Real-time PCR shows high specificity and sensitivity. This procedure prevent cryptic results because it does not need agarose gel, minimizes pipetting errors, reduces manual work and performs well. ELISA is also used for malaria test (Batista *et al.*, 2012).

1.13: Genetic Diversity

Genetic variation of a parasite can observe, when various *Plasmodium* strains flow in a human host (Cui *et al*., 2003). *Plasmodium* species manifests diversity in innumerable parameters e.g. symptoms, morphology, period of infection, resistance to drugs and transmissibility by vector *anopheles*. Genetic diversity of a parasite is lower in low transmission areas while it is higher in areas with high endemicity. Genetic diversity is affected by rate of transmission, immune pressure and natural selection of the parasite (Khatoon *et al*., 2013). A parasite's diversity is the main trouble in development of a vaccine (Gomez *et al*., 2002). The characteristics of genetic resistant were observed for the first time before half a century (Phimpraphi *et al*., 2008). False and cheap drugs not only

induce resistance development and decreased treatment efficacy, but also result in extreme complications and occasionally cause patient's death (He *et al*., 2014).

Genetic makeup of *P. falciparum* is studied by using *msp*1, *msp*2 and glur (Kim *et al*., 2006). The antigen *msp*-1 is abundant and best studied for malaria. The *Pvmsp*1 has an identical biology and function as *Pfmsp*1. *Pfmsp*-1 has 3 distinct allelic familes RO33, MAD20 and K (Salem *et al*., 2014).

Pfmsp-2 has 2 separate allelic families 3D7/IC and FC27 (Khatoon *et al*., 2010). In case of *P. vivax* genetic makeup of genes *Pvmsp*1, *Pvmsp*3-alpha and *Pvcsp* are well studies. Other markers used for *P. vivax* are PvAma 1, gam 1 and *msp*3β (Kim *et al*., 2006). Mostly merozoite surface protein-1 and circumsporozoite protein are studied in *P. vivax*. As compare to CSP gene, *msp*1 gene give better information about genetic variation (Cui *et al*., 2003). They produce various allelic forms which have potency of surviving in host and environment. Allelic forms provide us details about the genesis and solidity of different parasitic genotypes. They also help us to understand their role in host response, drug resistance and mechanism of control. Size polymorphisms is detected by using PCR in the particular blocks (Raza & Beg, 2013).

Merozoites are covered by merozoite surface proteins layers ordered into a complex coat. Gene *msp*3 is a family of *P. vivax (*Khatoon *et al*., 2010). It contains 11 members. These genes are located as head to tail. Studies of gene expression revealed that all genes are expressed in blood infection phase. The family members discovered first are *Pvmsp*-3α, *Pvmsp*-3β and *Pvmsp*-3$^{\gamma}$. In pair-wise comparisons they show 48 to 53% resemblance and 35 to 38% identity. The *Pvmsp*3β and *Pvmsp*3α renamed as *Pvmsp*3.3 and *Pvmsp*3.10 respectively. Among all 11 gene family members they are oriented on their chromosomal placement. Other related members also needs future enquiry. The members *Pvmsp*3α and *Pvmsp*3β are varied and show different point mutations including insertions and deletions (Rollinson *et al*., 2013).

Pvmsp-3α is expressed during schizogony (Raza *et al.,* 2013). It appears to become closely linked with the surface of the merozoite. *Pvmsp*3α shows high polymorphism due to intragenic recombinations. In *Pvmsp*3β sequences polymorphism is observed because of large deletion and insertion in the central alanine rich region (Chuquiyauri *et al*., 2013).

According to structure, these proteins lack a GPI-lipid and transmembrane domain modification to anchor them in the external membrane of the merozoite. The main part of these proteins is an alanine rich central domain comprising a chain of heptad repeats expected to make a coiled coil tertiary peptide structure. This might protect them on the merozoite surface by interaction with other surface proteins. Because of the significant diversity, the *Pvmsp*-3α gene sequence has turn into highly polymorphic marker studies on population based. The hydrophilic N-terminus and C-terminal domain (acidic) are comparatively conserved. The central domain is highly polymorphic (Rollinson *et al.*, 2013). Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis is used for rapid genotyping of *Pvmsp*3α and *Pvmsp*-3β gene of *P. vivax* (Cui *et al.*, 2003).

Aims and Objectives

- To undertake situation analysis with respect to Parasitological, Epidemiological, serological and molecular aspects of Malaria in study areas,
- Development of predictors and determinates of malaria in Pakistan and Azad Jammu Kashmir
- To study mechanism of Malaria in study areas in context to the role of genomic changes and determination of regional risk factors of Malaria and to reveal the phylogenetic relationship of the variant form and isolate of the parasite according to localities and time period.
- To identify polymorphic markers suitable for genotypic characterization of *P. vivax* parasites.
- Evaluation of the genetic diversity of *P. vivax* parasites by these markers, locally as well as globally by available *P. vivax* samples.

MATERIALS AND METHODS

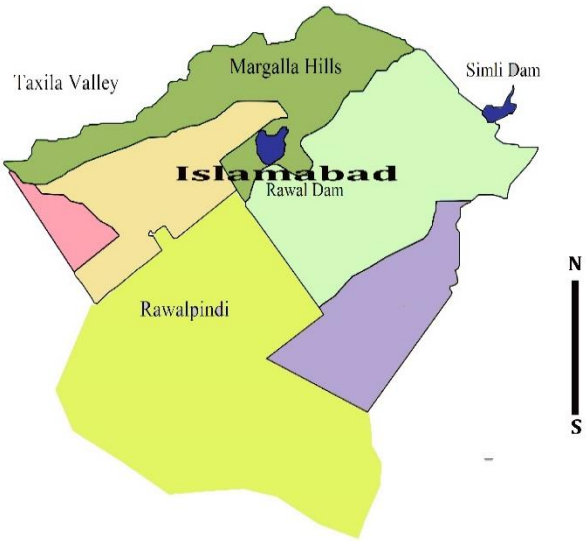
2.1: Study Area

Azad Jammu and Kashmir is a hilly area. Climate fluctuates with height. The summer temperature is nearly 20 to 38°C and in winter is 2 to 0°C. Snowfall occurs from end of December to February and rainfall from July to September. The Longitude of AJK is 73° 41′ 9″ E and Latitude is 33° 42′ 54″ N. The Elevation is 5374 feet. It is linked with Islamabad and Rawalpindi via Azad Pattan road. Study areas included are Pallandari, Poonch, Bagh and Kotli. The bank of rivers and streams provide habitats for malarial vector growth.

The population of **Islamabad** and **Rawalpindi** is over 4.5 million. It has hot and long summer while wet and short winter. The maximum temperature recorded is 42°C during summer and -3.9°C in winter. The Latitude and Longitude for twin cities is 33°37'N and 73°5'E. The Altitude of Islamabad and Rawalpindi is 508 m (1667 ft) and 497 meters (1633ft). Following figure: 1 showing map of Azad Kashmir, Islamabad and Rawalpindi.

Map of Azad Jammu and Kashmir

Map of Islamabad and Rawalpindi



2.2: Blood Sampling

1500 blood samples were collected from patients infected with *Plasmodium* species from hospitals and labs of Rawalpindi, Islamabad and Azad Jammu and Kashmir.

Venipuncture technique was used for blood collection as follow; Patient was positioned on a chair at sitting and tourniquet was placed to the upper arm of the patient, suitable site was selected for venipuncture. Methylated spirit was used to clean the site of collection in a circular motion, the area was air dried, with the help of needle attached to a syringe (5ml) venipuncture was made and the tourniquet was removed after the last tube filled. By immediate backward motion the needle was removed from the patient's arm, the blood sample was transferred into an EDTA tube and cotton was placed on the punctured site (Kim *et al.*, 2006).

2.3: Microscopy

Microscopy was used for the detection of *Plasmodium* and for the species identification. Thick and thin films were used for malaria diagnosis. Because of storage of ethylenediaminetetra-acetic acid (EDTA) morphological changes of parasites occur so films were made immediately. Thin film was exposed to acetone for one minute, air dried, methanol fixed for 1 minute and then stained by Giemsa. Thick film was dried at room temperature for 15 minutes, exposed to acetone for 10 minutes and stained using Giemsa. Prepared slide was examined under (10x100) oil emersion (Tek *et al*., 2009).

2.4: DNA Extraction

Plasmodium DNA was extracted from blood samples by using K5121 SPINKLEAN GENOMIC DNA EXTRACTION KIT with some modifications of the provided manual. For extraction of DNA, 200µl blood sample was taken in a 1.5ml micro centrifuge tube. Protein-K (20µl) and 220µl solution of MS was added to the sample and mixed thoroughly. Black homogeneous solution was produced by incubation for 10-15 minutes at 65°C. Ethanol (220µl) was added to the sample and mixed carefully by inverting, as a result precipitates were formed. The mixture was transferred into the spin column which was in turn placed in a 2ml collection tube. Centrifugation was carried out for 1 minute at 12000rpm. Flow was discarded completely. PS washing buffer (500µl) was

added to the mixture and was centrifuged at 12000rpm for 1 min, again flow was discarded. Another wash buffer PE 500µl was added and centrifuge at 12000rpm for 1 minute, discarded the flow. Above step was repeated. The column membrane was dried by centrifugation at 12000rpm for 3 minutes, flow and collection tube was discarded. The spin column was removed carefully and it was placed in a 1.5ml micro centrifuge tube. At the center of column 30-100µl elution buffer was added. Incubation was carried out at room temperature for 3 minutes. The tube was centrifuged at 12000rpm for 1 minute, purified DNA was obtained and stored at -20°C.

2.5: Gel Electrophoresis

For 1% gel preparation, (0.5g) agarose gel was weighted. Mixed in 10X TBE buffer (5ml) and distilled water (45ml). In a microwave, mixture was heated for 1 minute. Ethidium bromide (5µl) was added to the mixture. Mixture was discharged into the electrophoresis tray and comb was placed on it and left until it was solidified. The tray with solidified gel was placed in the chamber with the wells fronting in the direction of the negative end of chamber. The sample of DNA and loading dye were miscellaneous separately and laden in the wells. At the end chamber was closed. The electrophoresis run at 120v. After 40 minutes agarose gel was observed in gel doc apparatus and the image was recorded.

For all the PCR amplifications, for each reaction 50µl volume was used. A master mix was prepared containing all reagents except the DNA. All the reagents were vortexed before use. The DNA template was added at the end. To avoid contamination only one tube was opened at one time. Primers used for PCR amplification are shown in table no. 1.

Table 1: Showing list of primers

Primer	Sequence	Size
18SSRNAF	GGCCTAACATGGCTATGACG	700bp
18SSRNAR	GCCTTCCTTAGATGTGGTAGC	700bp
*VIV*18SRNAF	CGAGGAATGCCTGGTAAGC	700bp
*VIV*18SRNAR	CGGATCCATTACGTGTGTCG	700bp
*Fal*18SRNAF	CGTATTCAGATGTCAGAGGTGA	500bp
*Fal*18SRNAR	TGCTGGCACCACACTTGC	500bp
*Pvmsp*3α1F	CAGCAGACACCATTTAAGG	700bp
*Pvmsp*3α1R	CCGTTTGTTGATTAGTTGC	700bp
*Pvmsp*3α2F	GACCAGTGTGATACCATTAACC	300bp
*Pvmsp*3α2R	ATACTGGTTCTTCGTCTTCAGG	300bp

In the labeled master mix tube, volume of reagents were added in the following order: PCR water 33.3µl, PB 5µl, $MgCl_2$ 4µl, dNTPs 1µl, forward primer 2µl, reverse primer 2µl and *Taq*. 0.7µl. These contents were mixed by vortexing and short spin at 6000rpm for 30 seconds. In the every PCR tube 48µl master mix and 2µl DNA template was added. New tip was used for every sample.

Following is the PCR cyclic conditions for *Plasmodium.*

Stage	Cycle	Setup	Temperature	Time
1	1	Initial Denaturation	$94^{O}C$	4min.
2	30	Denaturation	$94^{O}C$	1min.
		Annealing	$55^{O}C$	2 min.
		Final Extension	$72^{O}C$	2min.
3	1	Hold	$4^{O}C$	5min.

Nested cycle for *Plasmodium* species identification.

Stage	Cycle	Setup	Temperature	Time
1	1	Initial Denaturation	94^{O}C	4 min.
2	35	Denaturation	94^{O}C	40 sec.
		Annealing	58^{O}C	1 min.
		Final Extension	72^{O}C	2 min.
3	1	Hold	4^{O}C	4 min.

2.6: Gel Electrophoresis

For the electrophoresis, agarose gel was prepared by dissolving 1g agarose in 5ml 10X TBE buffer and 45ml distilled water. Mixture was boiled for 2 minutes and 7µl ethidium bromide was added than poured into gel tray. Combs were placed, till the gel was solidified and then combs were removed. Gel tray was put into gel tank and 7µl PCR product mixed with 5µl loading dye and loaded into the wells. In one well of each row 100bp ladder was loaded. The gel was run for 40 minutes. At last it was examined under UV light. The image was recorded (Zakeri *et al*., 2010).

2.7: Purification of Amplified Product

Gene Jet PCR purification kit was used to purify amplified products. The binding buffer (100µl) was added in the amplified PCR products, vortex and centrifuge for 30 seconds at 8000rpm. The mixture was poured into the Gene Jet purification column. Centrifugation was carried out at 13000rpm for 1 minute and the flow through was wasted. The column was washed by adding washing buffer (700µl). Centrifugation was carried out at 13000rpm for 1 minute. Discard the flow through. To remove the remaining washing buffer, the tube was set for a vacant rotation at 13000rpm for 2 to 3 minutes. The collection tube was thrown away. The column shifted to a (1.5ml) micro centrifuge tube. At the center of the column 25µl elution buffer was added and centrifuge at 13000rpm for 1 minute.

Discard the Gene Jet purification column. The purified product was checked on 2% agarose gel by placing the gel under UV light. The picture was taken and saved on computer.

2.8: Amplification of *P. vivax msp*3α gene

Pvmsp-3α was amplified via procedure of Bruce *et al* (1999) with some modification. The PCR for *msp*-3α amplification was performed by using these primers: Forward 5′CAGCAGACACCATTTAAGG3′ and reverse 5′CCGTTTGTTGATTAGTTGC3′. In the labeled master mix tube, volume of reagents were added in the following order: PCR water 33.3µl, PB 5µl, $MgCl_2$ 4µl, dNTPs 1µl, forward primer 2µl, reverse primer 2µl and *Taq*. 0.7µl. These contents were mixed by vertaxing and short spin at 6000rpm for 30 seconds. In the every PCR tube 48µl master mix and 2µl DNA template was added. The conditions for PCR are as: 95°C used for 3 minutes, 94°C for 30 seconds, 58°C for 30 seconds, 68°C for 10 minutes (30 cycles) and 72°C for 5 minutes (Khan *et al*., 2014). Gel (2%) was stained by ethidium bromide to envision PCR products in UV illumination (Khatoon *et al*., 2010).

2.9: RFLP/PCR Analysis of *Pvmsp*-3α genes

Restriction enzyme Alu1 was used for RFLP analysis of *Pvmsp*-3α genes. The volume of each reaction was 20µl. Following components were used:

Alul buffer (1.2µl), PCR water (9.8µl), Alul enzyme (1µl) and PCR product (8µl). Samples were incubated at 37°C for 1 hour (Khan *et al.*, 2014). Agarose gel (2%) was used to envision the product in UV light and the image was recorded (Khatoon *et al*., 2010).

2.10: Amplification of *Pvmsp*-3β

Pvmsp-3β gene was amplified using a procedure described by Yang *et al*. in 2006 with little modifications. Following primers were used for PCR:

Forward 5′GACCAGTGTGATACCATTAACC3′

Reverse 5′ATACTGGTTCTTCGTCTTCAGG3′.

In the labeled master mix tube, volume of reagents were added in the following order: PCR water 33.3µl, PB 5µl, $MgCl_2$ 4µl, dNTPs 1µl, forward primer 2µl, reverse primer 2µl and *Taq*. 0.7µl. These contents were mixed by vortexing and short spin at 6000rpm for 30

seconds. In the every PCR tube 48µl master mix and 2µl DNA template was added. The conditions for PCR are as: 95°C for 3 min, 94°C for 30 sec, 58°C for 30 sec, 68°C for 10 minutes (30 cycles) and 72°C for 5 min (Khan *et al*., 2014). Agarose gel was used for envision of the PCR product in UV light. The image was saved (Khatoon *et al*., 2010).

2.11: RFLP/PCR Analysis of *Pvmsp*-3β gene

Pest 1 restriction enzyme was used for RFLP analysis that was defined by Yang *et al*. in 2006 with little modifications. Volume (20µl) of the reaction was prepared. The following components were used. 1.2µl Pest 1 buffer, 9.8µl PCR water and 1.0µl Pest 1 enzyme. PCR product (8.0µl) was added at the end. The samples were incubated at 37^{O}C for 1 hour (Khan *et al*., 2014). Agarose gel (2%) was used to observe the product under UV light and the image was recorded.

2.12: Sequencing of DNA

Sanger sequencing method (1977) was used to perform the sequencing. Recombinant plasmids were used to perform sequence reactions. By using the pGEM-T Easy vector-based primers for the T7 and SP6 RNA polymerase promoter sequences or directly on the purified PCR products, using internal primers corresponding to the particular DNA fragment. Sequence reactions were set up. Precipitated according to the protocol provided ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v.3.

Following reagents were used in the sequencing reactions:

BigDye reagents (2µl), Tris-Cl (2µl), 10X reaction buffer (10 mM $MgCl_2$), 1µL SP6 or T7 primer (3.2 pmol/µl), and dH_2O (20µl) and template DNA was used according to its concentration. About 50ng of PCR product was used while in the case of plasmid DNA 200ng was used. The following conditions were used for PCR: 45 cycles: 96^{O}C for 30 Sec, 55^{O}C for 15 sec. and 60^{O}C for 4 min. and 5 cycles: 96^{O}C for 30 sec., 60^{O}C for 4 minutes and 25^{O}C for 1 minute. The sequence reactions precipitation was carried out by using the method which was delivered with the BigDye reagent with little modifications.

Sequencing reactions (20µl) were mixed with dH_2O (20µl), sodium acetate (6µl)

and 100% ethanol (80µl). The mixture was vortex and incubated at room temperature for 15 minutes. The mixture was centrifuged for 30 seconds at 14000rpm. The supernatant was discarded. DNA was risen by adding 70% ethanol (1L) to the pellet. Centrifuge for 10 minutes at 14000rpm to pellet risen it. ABI 373A automated sequencer was used to analyze the samples (Fakruddin *et al*., 2012).

2.13: Sequence Data Analysis

The Bio navigator interface of the Australian National Genomic Information Service was used to analyze and edit the sequence data. Following programs were used to analyze the data. The particular input file was arranged in **Edit.** Nucleotide query sequence compared to a nucleotide sequence database by **blasting.** The six frame conceptual translation products of a nucleotide query sequence was used to compare a protein sequence database by **BlastX**. Two complete sequence alignment was found by **Gap**. The default conditions included a gap extension penalty of 2.0 and gap creation penalty of 8.0. The levels of sequence characteristics and protein resemblances was obtained from those results. Multiple sequence alignments were performed by using **ClustalW**. The default conditions of a gap extension penalty of 0.1 and gap opening penalty of 10.0 were used. The areas of homology among the numerous sequences were created by an output.

2.14: Evolutionary Genetic Analyses

Three alignments were constructed to analyze genetic diversity and infer phylogenetic relationships. MUSCLE algorithm in SeaView4 was used to perform *P.vivax* alignment of translating sequences by manual editing. Bayesian methods were used to estimate phylogenetic relationships as carried out in MrBayes v3.1.2. Neighbor joining method was used as applied in MEGA5 v5.05. The neighbor joining tree based distance was built by the Kimura 2-parameter model of nucleotide substitution to determine a pairwise nucleotide distance matrix. Through invariant rates between sites and 1000 bootstrap imitates to determine the assurance of clades. Pairs of further similar sequences were identified by using pairwise distance matrix separately. A GTR + G + I (general time reversible + gamma + evolutionarily invariable) model was used for the Bayesian method. Bayesian support for nodes were conditional in

MrBayes using 42 x 106 MCMC (Markov Chain Monte Carlo) phases, with each 100 generations sampling. About 50% of the samples were wasted. Convergence was achieved when the average standard deviation of the posterior possibility is below 0.01 while the rate of the potential scale reduction factor is between 1.00 and 1.02. Genetic diversity among isolates were assessed by using the normal number of pairwise nucleotide differences per site (overall mean distance, d) in MEGA5. The Jukes Cantor method was used to correct the multiple mutations during distance calculation. Standard error was determined by 1000 bootstrap imitates. Neighbor joining process was used to calculate the dS (diversity at synonymous) and dN (nonsynonymous sites). To determine whether the estimated difference between dS and dN deviated significantly from the null expectation, the standard error of dN and dS was calculated through 1000 bootstrap replicates. Two-tailed Z test was used to test (evolutionary genetic test). A null assumption of neutrality was expected, with the expectation that dS = dN (Similar levels of diversity). FST statistic was used to estimate the genetic differentiation among subpopulation as it was calculated in DnaSP. Tajima's test, the McDonald and Kreitman test were also used for neutrality in DnaSP.

2.15: Simulated PCR-RFLP Analysis

The PCR-RFLP analysis of Bruce *et al* (1977) has been currently used to genotype of *P. vivax* clinical isolates for *msp*-3α was computer-generated in order to match PCR-RFLP haplotype diversity to the nucleotide diversity pattern. The genotype of *msp*-3α was distinguished by insertion-deletion mutations and sequence variation at restriction enzyme cut sites. In the 1st step, alleles were separated by means of variations in the size of the product. Nested PCR was used to amplify the indel rich region of the gene. Sequences of the same length were more separated at the base of the banding patterns resulting from nested PCR product digestion distinctly through the restriction enzymes Alu1. PCR product was simulated in silico used for the 45 *P. vivax* sequences. Perl script was used to perform silico digestion on the sequences. The positions of enzyme cut sites were noted and according to the size the resultant restriction digestion bands were arranged. For the identification of PCR-RFLP haplotypes, it was supposed that bands less than ±30bp would be indistinguishable during electrophoresis. A fine scale detection limit for agarose gels

with a standard DNA ladder. Every sequence within a size class was assigned to the corresponding haplotype. According to protocol bands less than 100bp in length were ignored when determine haplotypes (Rice *et al*., 2013).

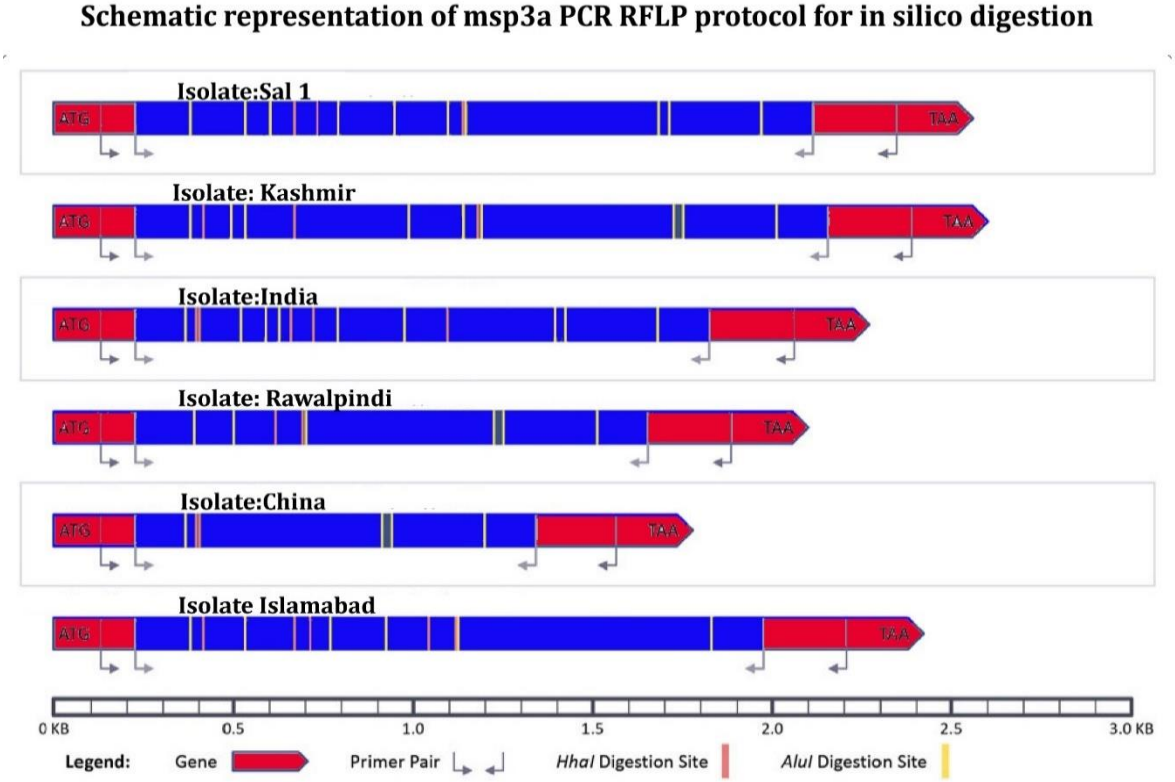


Figure 2: Showing Silico digestion of *Pvmsp*3

2.16: Statistical Analysis

Data was analyzed using SPSS 19.00 using Chi square distribution and correlation. Pairwise genetic differences (*F*ST) was calculated using Mantel test correlating pairwise genetic and geographic distances between populations.

RESULTS

3.1: Prevalence and Distribution of Malarial Parasite

A total number of 500 samples were considered as suspected cases from Azad Jammu & Kashmir. Out of them 311 (62.2%) were positive by using microscopy, identified as (n=207) 41% were *P. vivax,* (n=103) 20.6% were *P. falciparum,* (n=1) 0.2% were mixed species containing both *P.vivax* and *P. falciparum* and (n=189) 37.8% were identified as negative. By using nested PCR for amplification so these 500 samples along with positive and negative control were run. The PCR identified as (n=142) 28.4% *P.vivax,* (n=72) 14.4% were *P. falciparum,* (n=5) 1% were mixed containing both *P.vivax* and *P. falciparum* and (n= 281) 56.2% were identified as negative. *P. ovale* and *P. malariae* were not found in microscopy and PCR identification (Table 2).

From Islamabad total number of 500 samples were considered as suspected cases. Out of them 384 (76.8%) samples were positive by using microscopy, they were identified as (n=247) 49.4% were *P. vivax,* (n=133) 26.6% were *P. falciparum and* (n=40) 0.8% were mixed species containing both *P.vivax* and *P. falciparum* while (n=116) 23.2% were negative. Amplification was carried out by using positive and negative control by nested PCR. The PCR identified 272 (54.4%) samples positive as (n=184) 36.8% *P.vivax,* (n=69) 13.8% were *P. falciparum* and (n=19) 3.8% were mixed species whereas (n=228) 45.6% were negative. *P. ovale* and *P. malariae* were not identified by microscopy or by PCR (Table 3).

From Rawalpindi total number of cases were 500. Out of them 372 (74.4%) were microscopically positive as (n=228) 45.6% were *P.vivax,* (n=142) 28.4% were *P. falciparum* and (n=2) 0.4% were mixed species while (n=128) 25.6% were negative. Nested PCR amplification was carried by run both positive and negative controls. The PCR identified samples 259 (51.8%) were positive, out of them (n=168) 33.6% were *P.vivax,* (n=83) 16.6% were *P. falciparum,* (n=8) 1.6% were mixed species and (n=241) 48.2% were negative. *P. ovale* and *P. malariae* were not identified during microscopy or PCR identification (Table 4).

Table 2: Showing comparison of microscopy and PCR based diagnosis of isolates collected from Azad Kashmir

Species	Microscopy	PCR analysis
P. vivax	207 (41%)	142 (28.4%)
P. falciparum	103 (20.6%)	72 (14.4%)
P. ovale	0	0
P. malaraie	0	0
Mixed	1 (0.2%)	5 (1%)
Negative	189 (37.8%)	281 (56.2%)

Table 3: Showing comparison of microscopy and PCR based diagnosis of isolates collected from Islamabad

Species	Microscopy	PCR analysis
P. vivax	247 (49.4%)	184 (36.8%)
P. falciparum	133 (26.6%)	69 (13.8%)
P. ovale	0	0
P. malariae	0	0
Mixed	4 (0.8%)	19 (3.8%)
Negative	116 (23.2%)	228 (45.6%)

Table 4: Showing Comparison of microscopy and PCR based diagnosis of isolates collected from Rawalpindi

Species	Microscopy	PCR analysis
P. vivax	228 (45.6%)	168 (33.6%)
P. falciparum	142 (28.4%)	83 (16.6%)
P. ovale	0	0
P. malariae	0	0
Mixed	2 (0.4%)	8 (1.6%)
Negative	128 (25.6%)	241 (48.2%)

For molecular identification of *Plasmodium* collected from different areas of Islamabad, Rawalpindi and AJK nested PCR was used. For the amplification of 18SSRNA gene, in the primary reaction the included primers were as follow:

Forward 5′GGCCTAACATGGCTATGACG3′

Reverse 5′GCCTTCCTTAGATGTGGTAGC3′. The agarose gel was used for visualize the samples under UV light. For the species identification secondary reaction carried and the included primers for *Pviv*18SRNA and for *Fal*18SRNAF were:

Forward: 5′CGAGGAATGCCTGGTAAGC3′

Reverse: 5′CGGATCCATTACGTGTGTCG3′

Forward: 5′CGTATTCAGATGTCAGAGGTGA3′

Reverse: 5′TGCTGGCACCACACTTGC3′. Agarose gel was used to visualize the bands. In the secondary round, species of *P. vivax* were 142 from Azad Kashmir, 184 from Islamabad and 168 from Rawalpindi. The species of *P. falciparum* were 72 from Azad Kashmir, 69 from Islamabad and 83 from Rawalpindi. The mixed infections were 5 from Azad Kashmir, 19 from Islamabad and 8 from Rawalpindi. PCRs were repeated twice for all samples and same results were obtained. 281, 228 and 241 were negative from Azad Kashmir, Islamabad and Rawalpindi respectively. Bands of *P.vivax* are seen in figure 2.

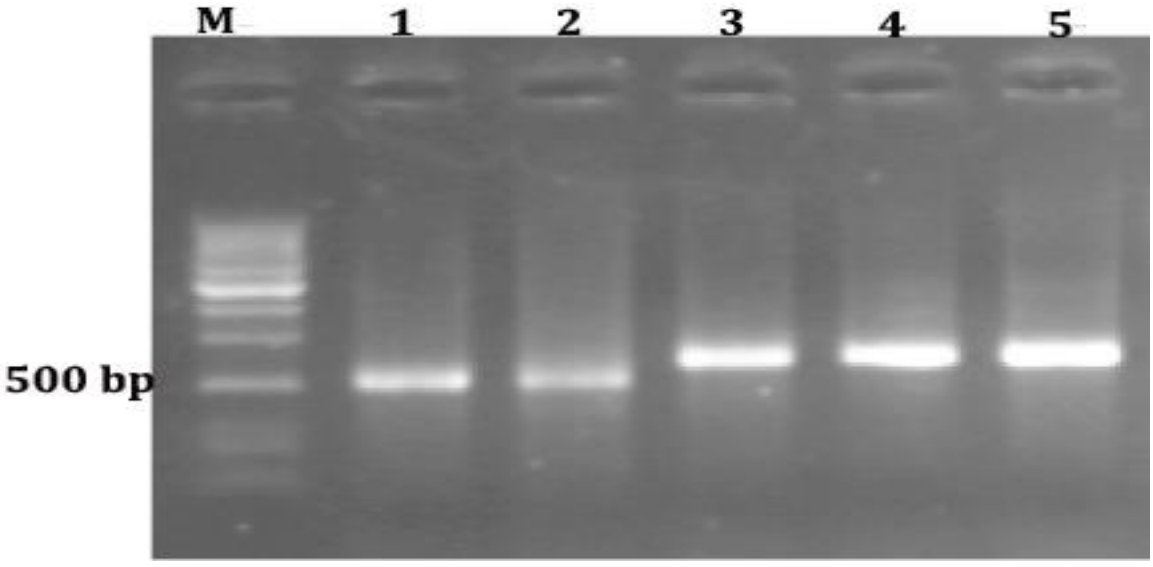


Figure 3: Showing Gel electrophoresis results of PCR Product

Table 5: Showing overall prevalence of *Plasmodium* in Azad Kashmir

Hospital names	Total	Male	Female	Age Range
MH Palandari	138	56 (40.5%)	82(59.4%)	1-10
MH Kotli	71	32(45.0%)	39 (54.9%)	11-20
CMH Poonch	107	52 (48.5%)	55 (51.4%)	21-30
MH Bagh	184	97 (52.7%)	87 (47.2%)	31-50
Total	500	237 (47.45)	263 (52.6%)	

In the study from Azad Jammu and Kashmir, all the subjects participated were both males and females had age between 1 to 50y. They were residents of their native's localities. The prevalence of *Plasmodium* in male population was 47.45% and in the case of female was 52.6%. The prevalence of *Plasmodium* in females were high while males had a low prevalence (Table 5).

Table 6: Showing overall prevalence of *Plasmodium* in Islamabad

Hospital names	Total	Male	Female	Age Range
Pims	135	83 (61.4%)	52 (38.5%)	1-10
Polyclinic	130	70 (53.8%)	60 (46.1%)	11-20
Islamabad Diagnostic Center	108	69 (63.8%)	39 (36.1%)	21-30
Kulsoom hospital	127	66 (51.9%)	61 (48.0%)	31-50
Total	500	288 (57.6%)	212 (42.4%)	

In the study from Islamabad, both males and females were participated and they had age between 1 to 50y. They were citizen of their native's areas. The prevalence of *Plasmodium* in male population was 57.6% while in the case of females was 42.4%. The prevalence of males were high while females had low prevalence (Table 6).

Table 7: Showing overall prevalence of *Plasmodium* in Rawalpindi

Hospital names	Total	Male	Female	Age Range
Holy family	145	59 (40.6%)	86 (59.3%)	1-10
Central hospital	128	66 (51.5%)	62 (48.4%)	11-21
Razi hospital	100	55 (55%)	45 (45%)	21-30
Jinnah hospital	127	71 (55.9%)	56 (44.0%)	31-60
Total	500	251 (50.2%)	249 (49.8%)	

In the study from Rawalpindi, both males and females were participated and they had age between 1-60y. They were citizen of their local areas. The prevalence of *Plasmodium* in male population was 50.2% while in the case of females the population was 49.8%. Males were more affected than females (Table 7).

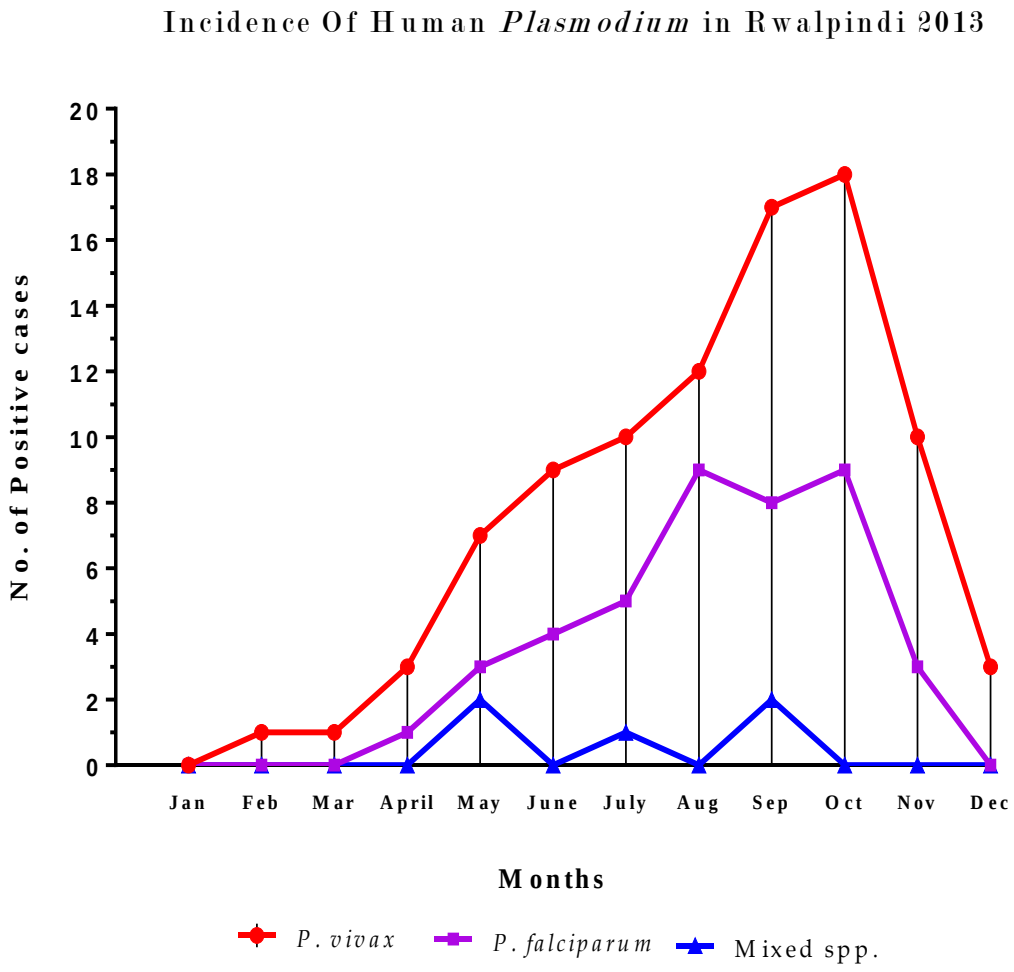


Figure 4: Showing Incidence of *Plasmodium* in Rawalpindi 2013

The graph in figure 4 is from Jan. 2013 to Dec. 2013. Very brief transmission of *P. vivax* occurs from Jan. to March. *P. vivax* shows two peaks, 1st peak in June and second is in Oct. From end of Oct it starts decrease up to December. From Jan to Feb. the prevalence of *P. falciparum* is zero. From end of March it is increasing and it shows a peak in August and a second peak in Oct. than it decreasing up to Dec. Mixed species infections shows variable peaks of transmission from April to Oct.

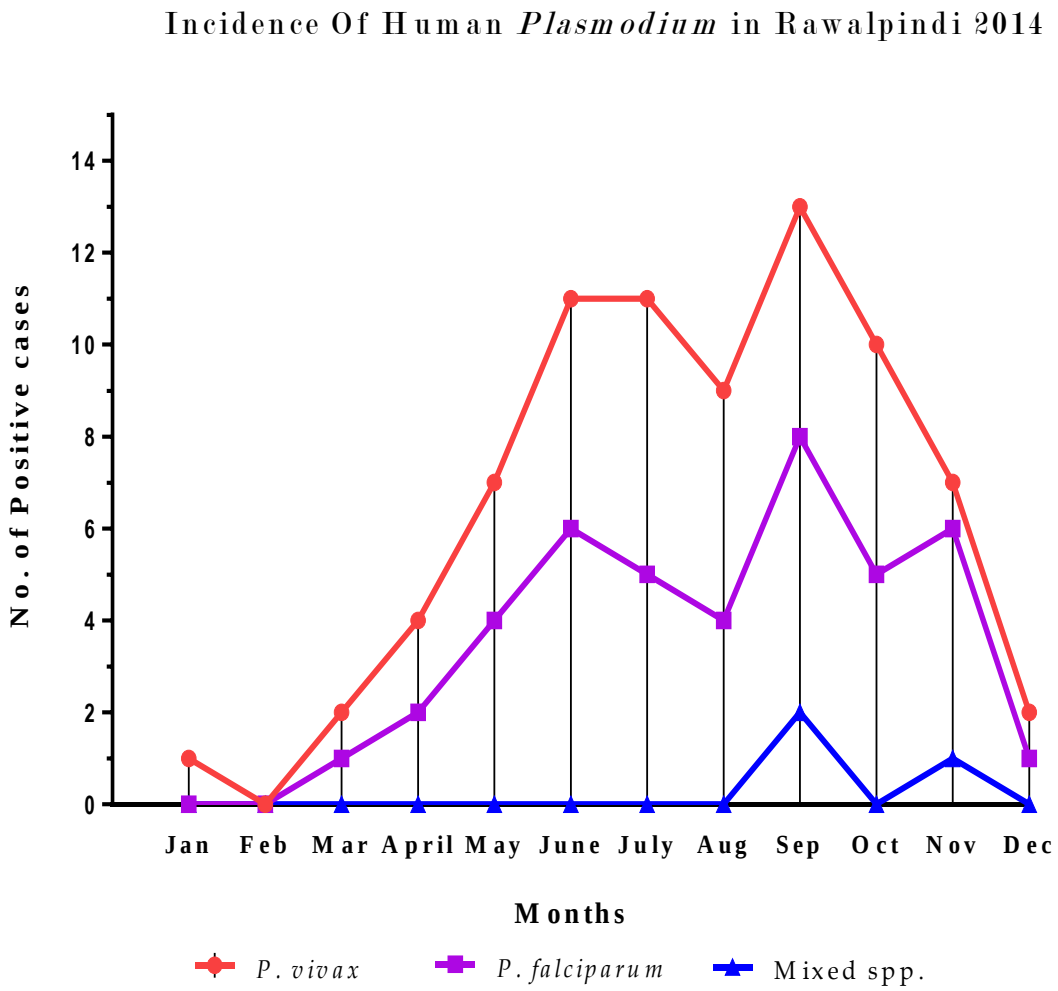


Figure 5: Showing Incidence of *Plasmodium* in Rawalpindi 2014

The graph in figure 5: Represents the prevalence of *Plasmodium* in Rawalpindi from Jan. 2014 to Dec. 2014. No transmission occurs in January. At the end of Feb. it's going to increase up to September. Highest prevalence of *P.vivax* occurs in June and September. From end of September it starts decreasing up to Dec. In case of *P. falciparum* transmission occurs from the end of March up to September by showing discontinuous peaks. It decline and once again increase in November and then drop in Dec. Mixed species infections also occurs from August to December.

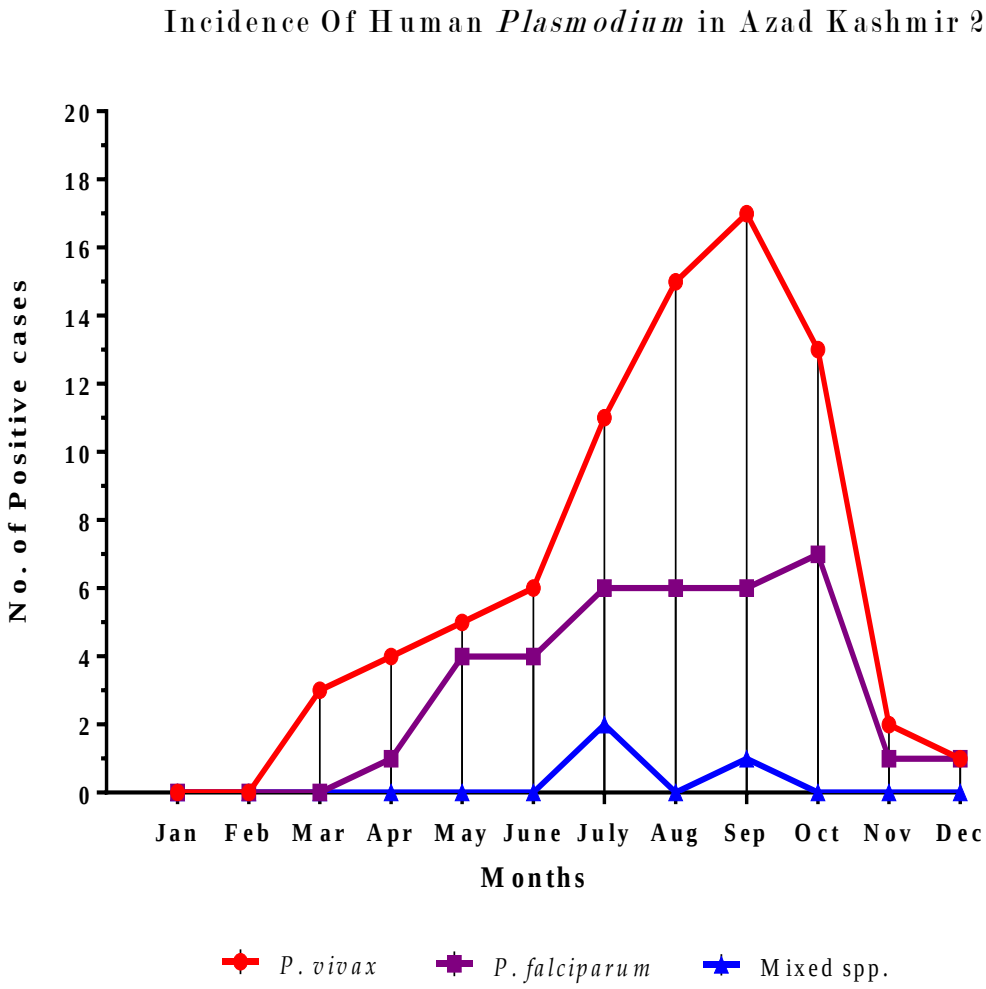


Figure 6: Showing Incidence of *Plasmodium* in Azad Kashmir 2013

Graph in figure 6: Shows rate of infection of *Plasmodium* in AJK in year 2013. The graph indicates most prevalent species is *P. vivax*. The rate of infection is zero from January to February. In the March it starts increasing up to September. It starts decreasing from end of Sep. to Dec. The *P. falciparum* shows no infection during Jan. to March. From April it starts increasing up to Oct. by showing discontinuous peaks. At the end of Oct it starts decreasing up to December. Mixed infections occurs from June to Oct.

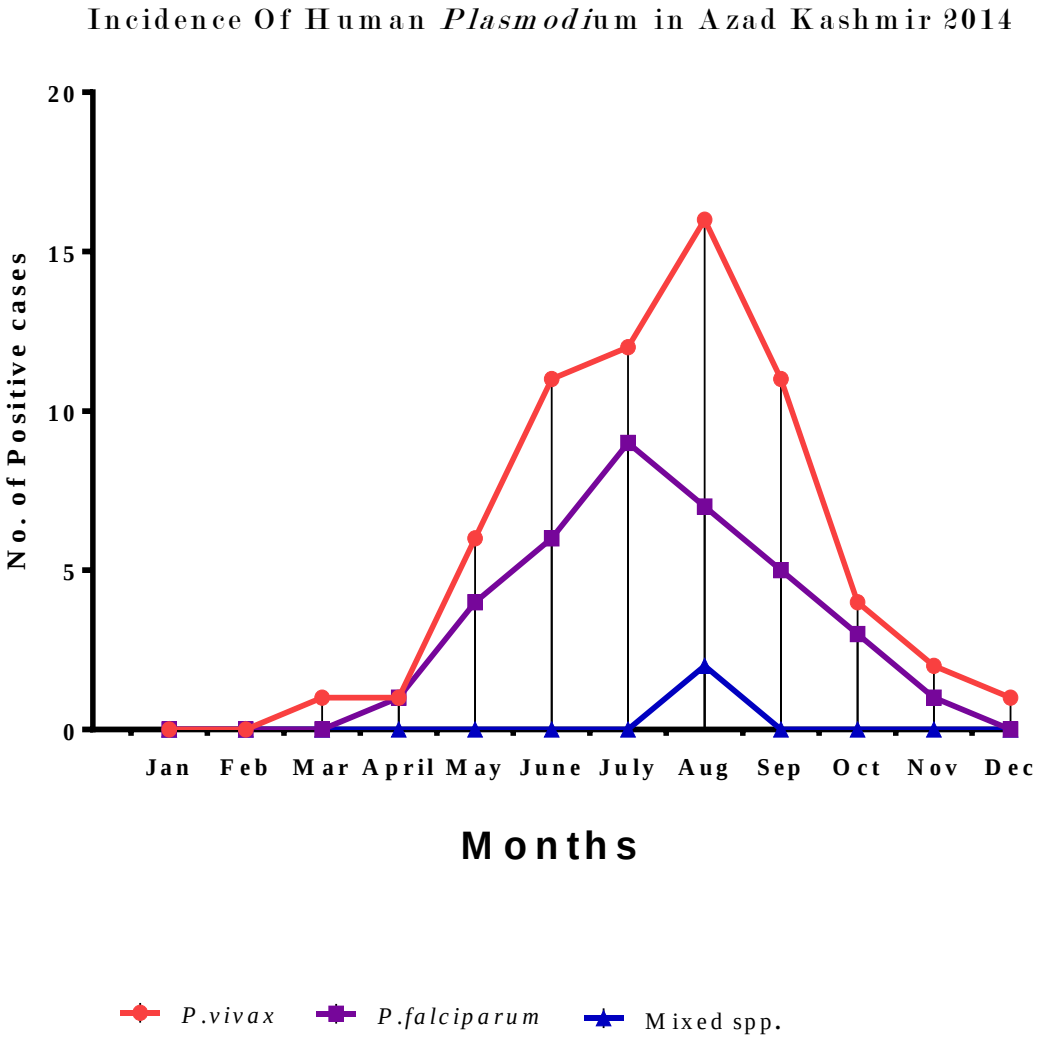


Figure 7: Showing Incidence of *Plasmodium* in Azad Kashmir 2014

Graph in figure 7: Shows transmission of *Plasmodium* in AJK in the year 2014. From Jan. to March transmission of the *P.vivax* is very brief. Highest transmission occurs in June and August. After August it starts decreasing up to Decmber. The transmission of *P. falciparum* does not occur in Jan and Feb. At the end of March it starts increasing up to July and then it starts decrease up to December. Mixed species transmission occurs only from July to September.

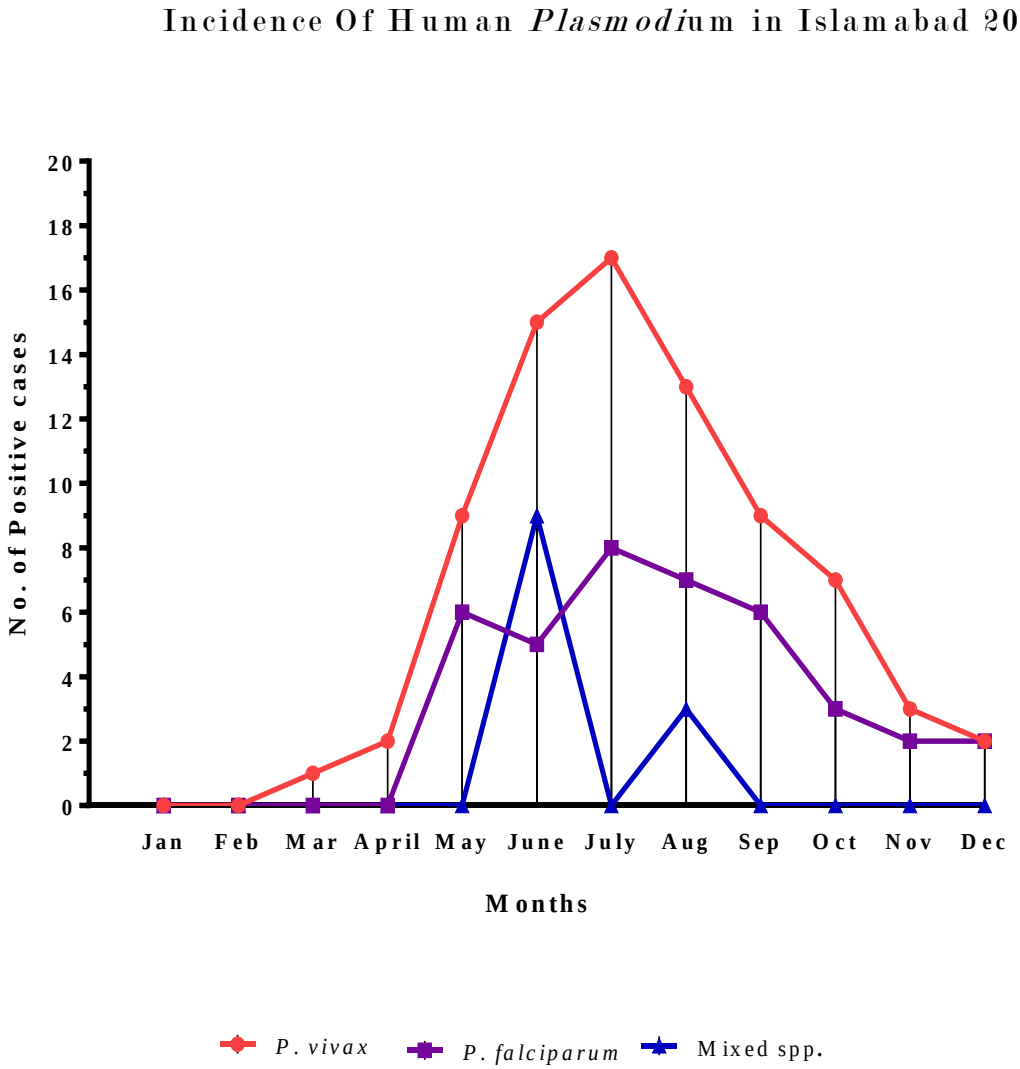


Figure 8: Showing Incidence of *Plasmodium* in Islamabad 2013

Graph in figure 8: Shows the incidence of *Plasmodium* in Islamabad in year 2013. Little transmission occurs in January to March. Incidence starts increasing from April to July and then it starts dropping till December. The transmission of *P. falciparum* starts increase from the end of April to July and then it starts decrease till December. Mixed species transmission occurs only from May to September.

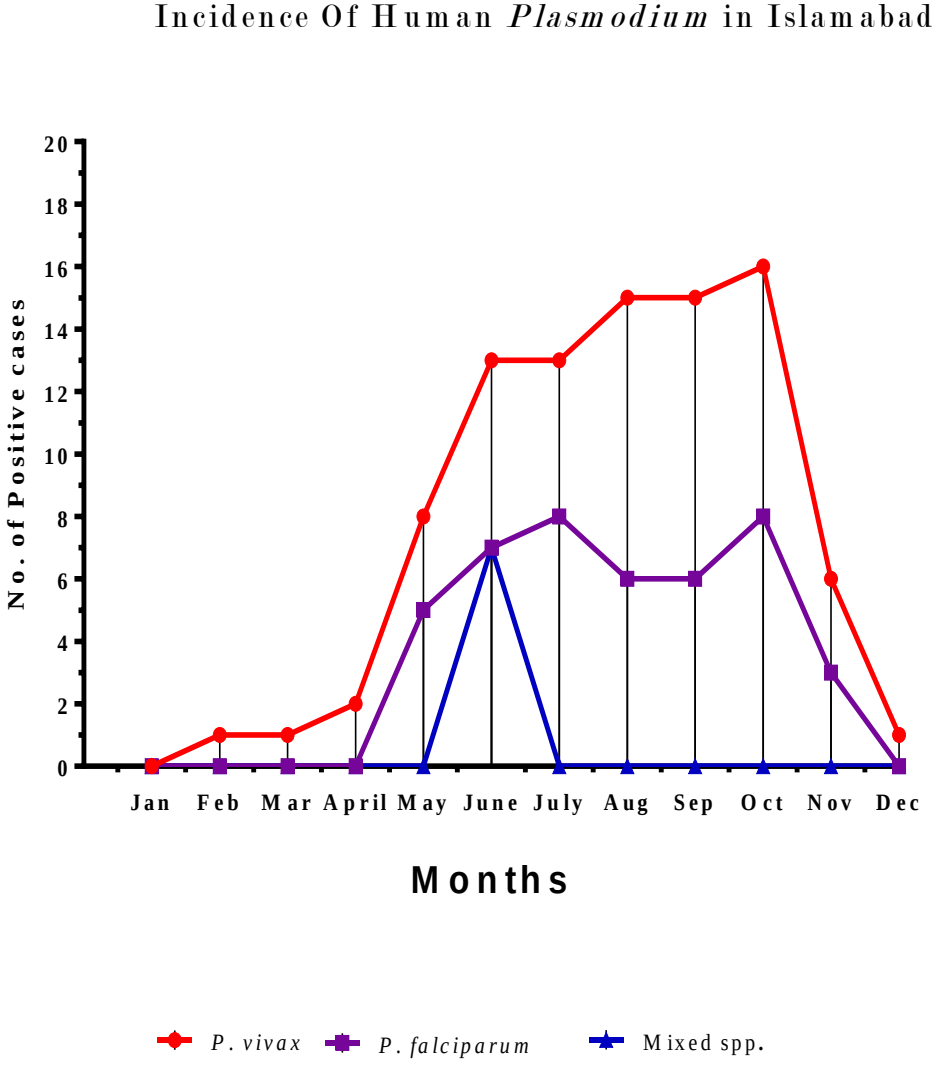


Figure 9: Showing Incidence of *Plasmodium* in Islamabad 2014

Graph in figure 9: Shows the prevalence of *Plasmodium* in Islamabad in year 2014. From January to March brief transmission occurs. From April it starts increase till Oct. and then it starts decrease up to Dec. The highest incidence occurs during June, Aug. and Oct. The incidences of *P. falciparum* start increasing from the end of April to Oct. and then it starts decreasing. The incidence of mixed infection occurs from May to July.

3.2: PCR Amplification of *msp*-3α and Allelic Frequency

For the analysis of allelic frequency 15 Vivax isolates from each locality i.e. Islamabad, Rawalpindi and Kashmir were subjected for the PCR amplification of *msp*-3α gene. The resultant products revealed four kinds of allelic variants (G1, G2, G3, G4) both for Islamabad and Rawalpindi with a size of about 2.0kb, 1.8kb, 1.4kb and 1kb respectively. While in Kashmir we found only three kinds of allelic variants i.e. G1, G2, and G3.

Overall, frequency distribution of G1, G2, G3, and G4 in Islamabad is 5/15, 3/15, 4/15 and 2/15. In Rawalpindi it is 5/15, 4/15, 4/15 and 1/15. In Kashmir the frequency distribution for G1, G2, G3 is 5/15, 3/15 and 4/15 respectively.

3.3: Restriction Fragment Length Polymorphism (RFLP)

The resulted 15 samples from each locality were analyzed for RFLP using Alu1 enzyme and the RFLP pattern of individual sample ad their genotypic distribution for each location is shown it Table 8,9,10 and figure 10,11,12,13. In RFLP 12 patterns were observed in Islamabad, 14 in Rawalpindi and 14 in Kashmir. RFLP analysis also show the differential level of diversity among *msp*-3 alpha alleles and variant G1 is more diverse in field isolates compared to variants G2, G3 and G4. Digestion with these enzymes yielded fragment sizes that were highly polymorphic. The sum of the fragment sizes did not always equal the size of the complete PCR product, indicating non-resolvable variation in size of the uncut amplification products but never more than the total size, thereby indicating a number of mixed genotypes in all study areas.

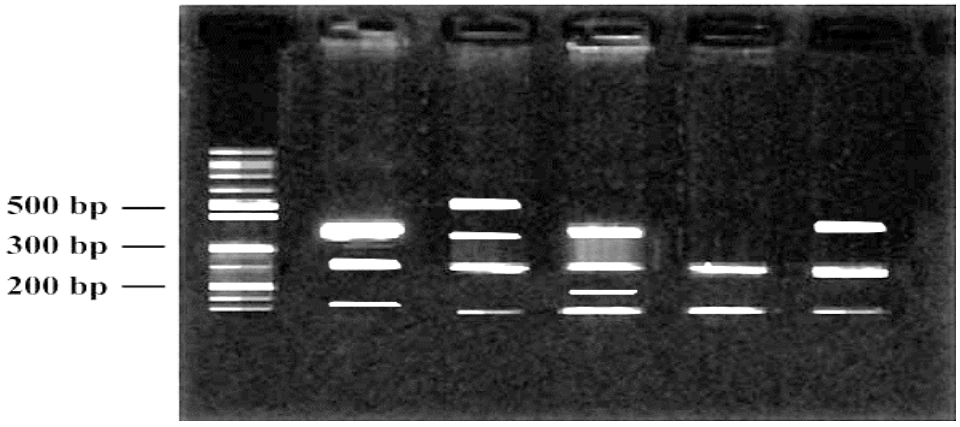


Figure 10: Showing RFLP pattern of *Pvmsp3α* gene

Table 8: Showing Size and RFLP patterns and frequency of *Pvmsp*3α alleles collected from Islamabad

Sample ID	Allelic Variant Size		Fragments Size in Base Pair					Genotype
			A	B	C	D	E	
I-1	2.0	**G1**	500	400	250	350	200	1
I-2	1.8	**G2**	500	300	250	150	-	2
I-3	1.8	**G2**	500	350	250	150	-	3
I-4	1.0	**M**	500	300	200	150	-	4**
I-5	1.4	**G3**	500	250	200	-	-	5
I-6	1.4	**G3**	500	300	200	-	-	6
I-7	2.0	**G1**	500	200	150	-	-	7
I-8	1.0	**G4**	500	250	200	-		5
I-9	2.0	**G1**	500	350	250	200	-	8
I-10	2.0	**G1**	500	300	200	-	-	3
I-11	2.0	**G1**	500	400	190	150	-	9
I-12	1.8	**G2**	500	450	300	200	160	10
I-13	1.4	**G3**	500	350	250	200	-	8
I-14	1.4	**G3**	500	250	210	170	-	11
I-15	1.8	**G4**	500	400	300	150	220	12

The RFLP analysis of sample from Islamabad region revealed 6.6% mixed alleles of the allelic variants. Out of the total 15 amplified samples, 5 (33.33%) isolates for Type-G1, 3 (20%) for Type-G2, 4(26.6%) for Type-G3 and 2(13%) for type G4 were observed. Among these isolates Type-G1 and G3 was found to have highest per cent frequency and Type- G4 was found the lowest in percent frequency (Table 8).

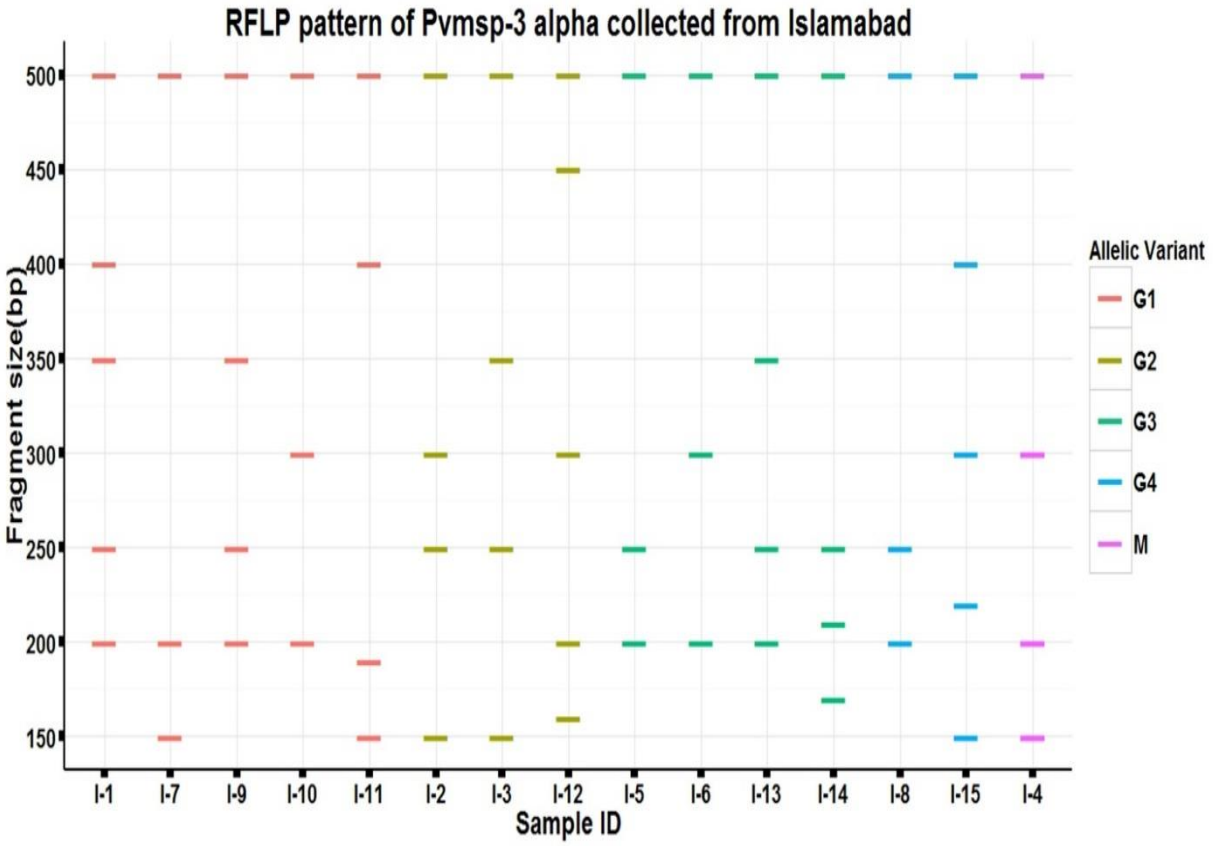


Figure 11: Showing RFLP patterns and frequency of *Pvmsp3α* alleles collected from Islamabad

The RFLP analysis of sample from Islamabad region revealed 6.6% mixed alleles of the allelic variants. Out of the total 15 amplified samples, 5 (33.33%) isolates for Type-G1, 3 (20%) for Type-G2, 4(26.6%) for Type-G3 and 2(13%) for Type G4 were observed.

Table 9: Showing Size and RFLP patterns and frequency of *Pvmsp*3α alleles collected from Rawalpindi

Sample ID	Allelic Variant Size		Fragments Size in Base Pair					Genotype
			A	B	C	D	E	
R-1	2.0	**G1**	500	400	300	350	-	1
R-2	1.8	**G2**	500	350	150	160	-	2
R-3	1.8	**G2**	500	350	250	160	-	3
R-4	1.8	**G2**	500	250	200	150	-	4
R-5	1.4	**G3**	500	250	210	190	150	5
R-6	1.4	**G3**	500	300	200	160	-	6
R-7	2.0	**G1**	500	200	150	200	170	7
R-8	1.8	**M**	500	450	350	300	250	8**
R-9	2.0	**G1**	500	350	250	200	160	9
R-10	2.0	**G1**	500	300	210	180	-	10
R-11	2.0	**G1**	500	300	210	180	-	10
R-12	1.8	**G2**	500	410	350	270	160	11
R-13	1.4	**G3**	500	350	250	-	-	12
R-14	1.4	**G3**	500	250	210	-	-	13
R-15	1.0	**G4**	500	350	350	-	-	14

The RFLP analysis of samples from Rawalpindi is somewhat different from Islamabad. Out of the total 15 amplified samples, 5 (33.33%) isolates for Type-G1, 4 (26.66%) for Type-G2, 4 (26.66%) for Type-G3 and 1(6.6%) for type G4 were observed. Among these isolates Type-G1 was found to have a highest per cent frequency and Type-G4 was found the lowest in per cent frequency (Table 9). Here the frequency of mixed alleles (M) is 6.6% of the allelic variants.

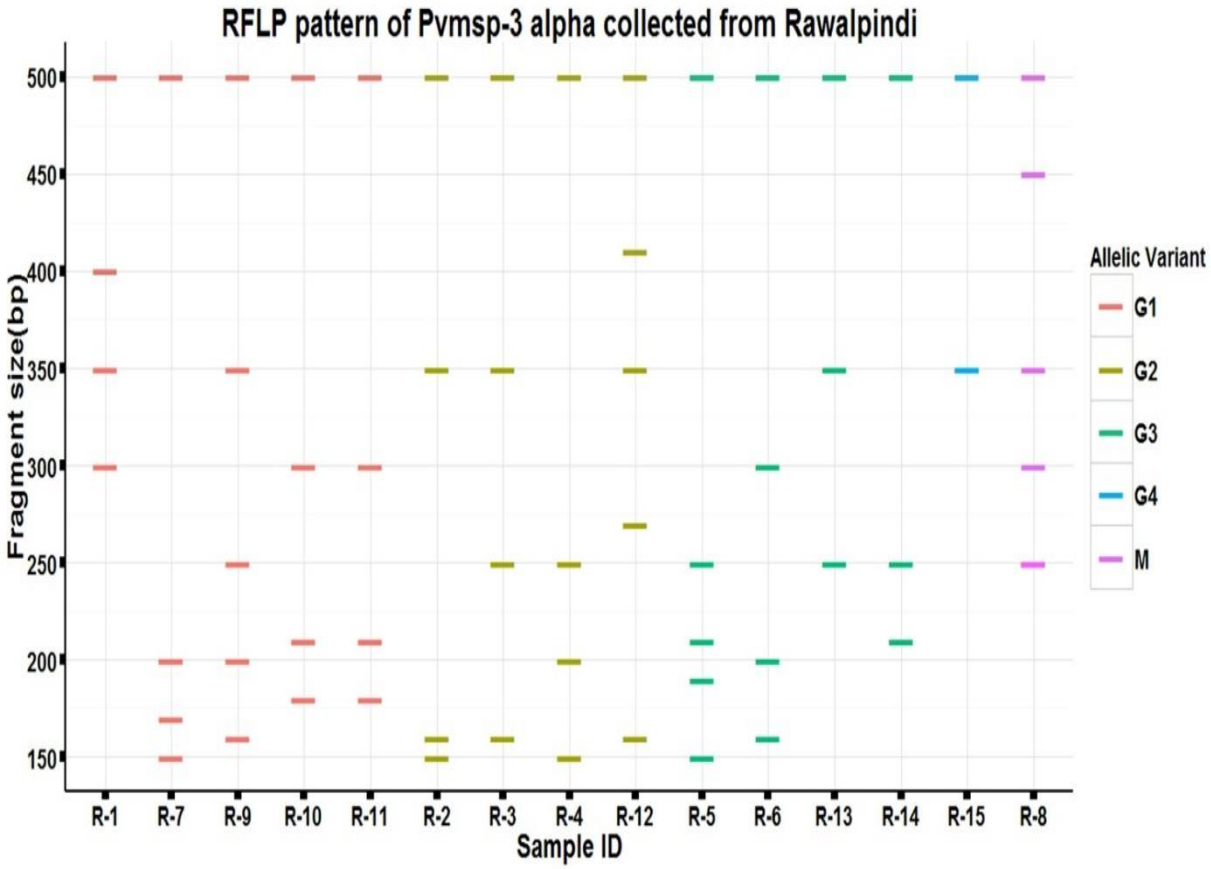


Figure 12: Showing RFLP patterns and frequency of *Pvmsp3α* alleles collected from Rawalpindi

The RFLP analysis of sample from Rawalpindi is somewhat different from Islamabad. Out of the total 15 amplified samples, 5 (33.33%) isolates for Type-G1, 4 (26.66%) for Type-G2 (26.66%) for Type-G3 and 1(6.6%) for type G4 were observed. 1 (6.6%) isolates were observed for M.

Table 10: Showing Size and RFLP patterns and frequency of *Pvmsp3α* alleles collected from Kashmir

Sample ID	Allelic variant Size		Fragments Size in Base Pair					Genotype
			A	B	C	D	E	
K-1	2.0	**G1**	500	300	250	350	-	1
K-2	1.8	**G2**	500	320	150	160	-	2
K-3	1.8	**M**	500	450	350	300	200	3**
K-4	2.0	**M**	500	300	200	160	-	4**
K-5	1.4	**G3**	500	400	300	250	200	5
K-6	1.4	**G3**	500	300	200	-	-	6
K-7	2.0	**G1**	500	250	150	-	-	7
K-8	1.8	**G2**	500	250	200	150	-	8
K-9	2.0	**G1**	500	350	250	200	-	9
K-10	2.0	**G1**	500	350	250	160	-	10
K-11	2.0	**G1**	500	420	190	180	-	11
K-12	1.8	**G2**	500	400	300	200	-	12
K-13	1.4	**G3**	500	350	250	200	-	9
K-14	1.4	**G3**	500	250	200	-	-	13
K-15	1.4	**M**	500	450	300	250	200	14**

Out of the total 15 amplified samples for Kashmir region 5 (33.33%) were isolates for Type-G1, 3(20%) for Type-G2, 4(26.66%) for Type-G3 and 0(0%) for G4 were observed. Among these isolates Type-G1 was found to have highest per cent frequency beside the mixed type which were observed to b 26% of the allelic variants. In Kashmir no allelic variant of G4 found.

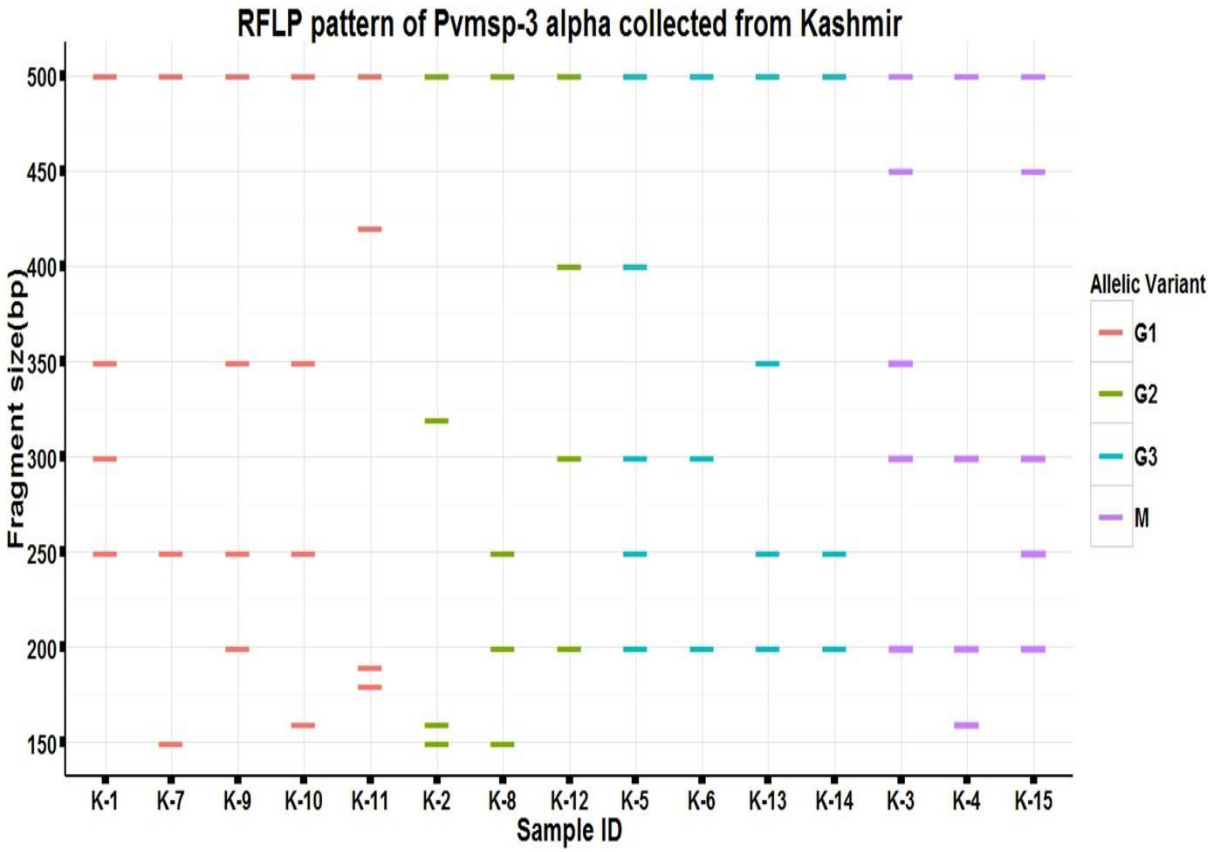


Figure 13: Showing RFLP patterns and frequency of *Pvmsp3α* alleles collected from Kashmir

Out of the total 15 amplified samples for Kashmir region 5(33.33%) were isolates for Type-G1, 3(20%) for Type-G2, 4(26.66%) for Type-G3 and 0(0%) for G4 were observed. 3 (20%) isolates had Type M.

3.4: RFLP Analysis *Pvmsp*-3ß gene

In the present study 15 samples of blood from each localities had been successfully amplified for the analysis *Pvmsp*-3ß gene. A high frequency of polymorphism in Islamabad, Rawalpindi and AJK among their different allelic variants were observed. The amplified DNA fragments for these alleles were classified according to their size variation. Based on variation in size of the fragments, the amplified samples could be differentiated into four allele sizes: for Type G1 (2.2kb), Type G2 (1.7) and Type G3 (1.5 kb) and type G4 (1.2 kb). Two alleles were simultaneously found in a number of samples, showing that some infections contained mixed genotypes. If the sum of individual alleles is greater than the size of respective allelic variant it will be considered as mixed genotype. The exploration of the sequences revealed that SNPs dispersed throughout the gene, as well as small or large insertions and deletions that are the key basis of variations in the size among the sequences. Based on the results of the sequence analysis, *Pvmsp*-3ß sequences of all isolates were classified as five different subtypes. A number of samples reveal more than one band of different sizes in all the resulted fragments. The RFLP pattern of all these allelic variants yield further sub-allelic variants of different size i.e. A, B, C, D. on the basis of these sub allelic variant genotypes were assigned to each and every sample.

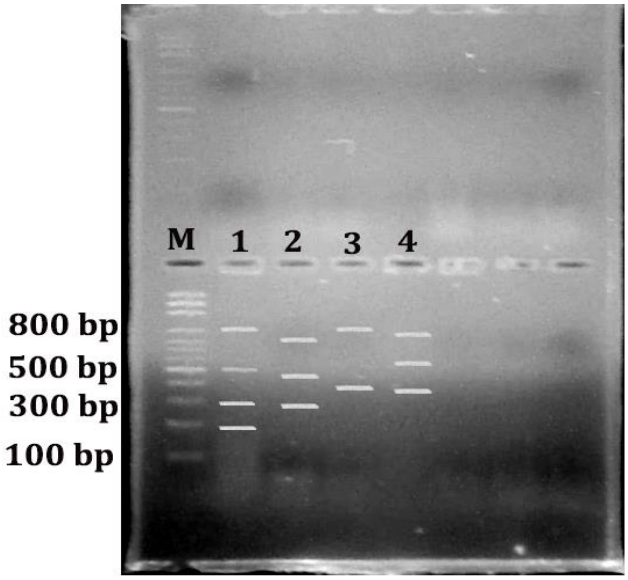


Figure 14: Showing RFLP pattern of *Pvmsp*3β gene

Table 11: Showing Size and RFLP patterns and frequency of *Pvmsp*3β alleles collected from Islamabad

ID	Allelic variant Size		A	B	C	D	Genotype
I-1	2.2	**G1**	750	400	150	-	1
I-2	1.7	**G2**	800	500	350	-	2
I-3	1.7	**G2**	800	500	-	-	3
I-4	1.2	**G4**	700	200	-	-	4
I-5	1.5	**M**	800	450	350	-	5**
I-6	1.5	**G3**	700	500	-	-	6
I-7	2.2	**G1**	800	400	300	-	7
I-8	1.2	**G4**	700	150			8
I-9	2.2	**G1**	800	450	350	-	9
I-10	2.2	**G1**	800	500	400	-	10
I-11	2.2	**G1**	800	400			11
I-12	1.7	**M**	800	500	400	300	12**
I-13	1.5	**G3**	750	450	-	-	13
I-14	1.5	**G3**	750	400	200		14
I-15	1.7	**G4**	700	400	300	-	15

The analysis of sample through RFLP from Islamabad region revealed 13.3 % mixed alleles of the allelic variants. Out of the total 15 amplified samples, 5(33.33%) isolates for Type-G1, 2(13%) for Type-G2, 3(20%) for Type-G3 and 3(20%) for type G4 were observed. Among these isolates Type-G1 was found to have highest per cent frequency and Type- G2 was found the lowest in per cent frequency (Table 11).

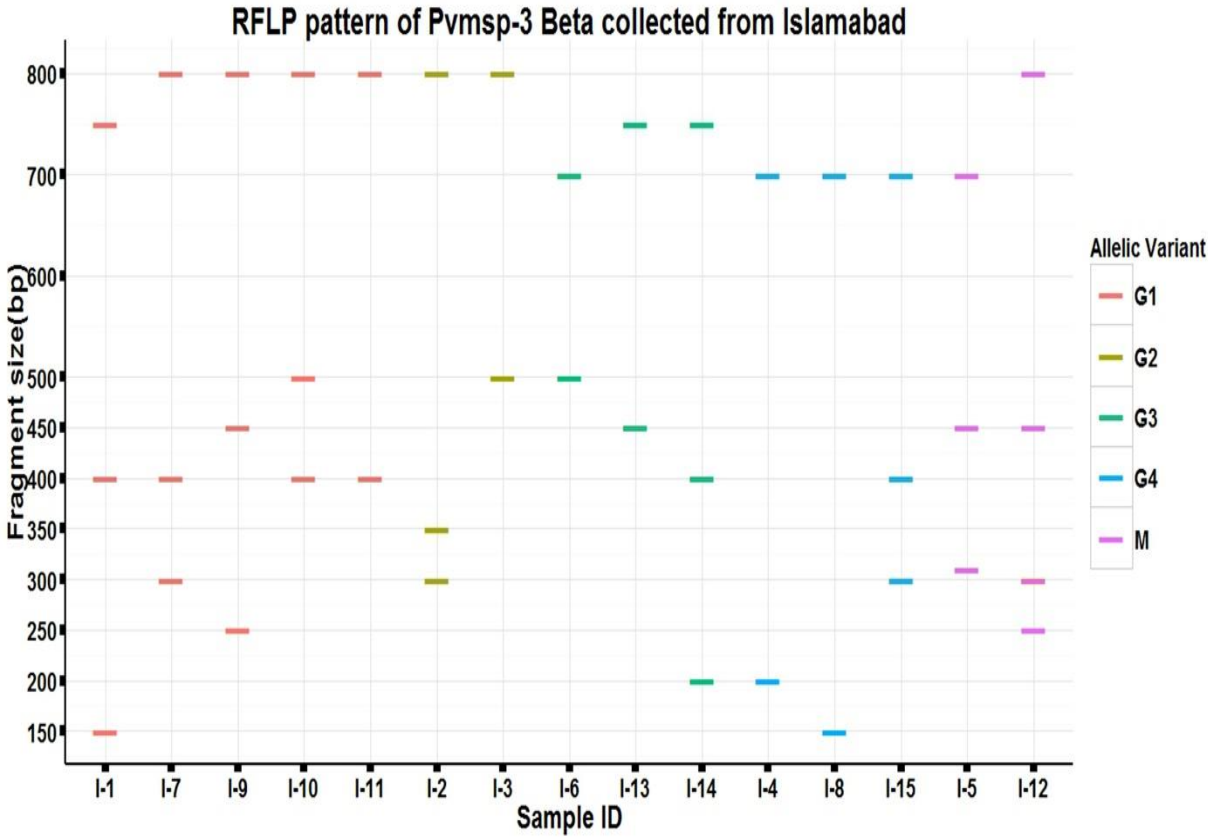


Figure 15: Showing RFLP patterns and frequency of *Pvmsp*3β alleles collected from Islamabad

The analysis of sample through RFLP from Islamabad region revealed 13.3% mixed alleles of the allelic variants. Out of the total 15 amplified samples, 5(33.33%) isolates for Type-G1, 2(13%) for Type-G2, 3(20%) for Type-G3 and 3(20%) for type G4 were observed.

Table 12: Size and RFLP patterns and frequency of *Pvmsp*3β alleles collected from Rawalpindi

ID	Allelic variant Size		A	B	C	D	Genotype
R-1	1.7	**G2**	750	300	150	-	1
R-2	2.2	**G1**	800	400	200	-	2
R-3	1.7	**G2**	700	500	300	-	3
R-4	1.2	**M**	700	400	300	-	4**
R-5	1.5	**M**	800	500	300	-	5**
R-6	2.2	**G1**	800	400	-	-	6
R-7	1.5	**G3**	750	400	350	-	7
R-8	1.2	**G4**	700	150	-	-	8
R-9	1.2	**G4**	800	350	300	-	9
R-10	2.2	**G1**	800	500	400	-	10
R-11	1.5	**G3**	700	400		-	11
R-12	1.5	**G2**	600	400	250	-	12
R-13	1.5	**G3**	800	400	-	-	13
R-14	2.2	**G1**	750	400	200	-	14
R-15	2.2	**G1**	600	400	250	-	12

The RFLP analysis of sample from Rawalpindi is somewhat different from Islamabad. Out of the total 15 amplified samples, 5(33.33%) isolates for Type-G1, 3(20%) for Type-G2, 3(20%) for Type-G3 and 2(13%) for type G4 were observed. Among these isolates Type-G1 was found to have highest per cent frequency and Type- G4 was found the lowest in per cent frequency (Table 12). Here the frequency of mixed alleles (M) is 13.3% of the allelic variants.

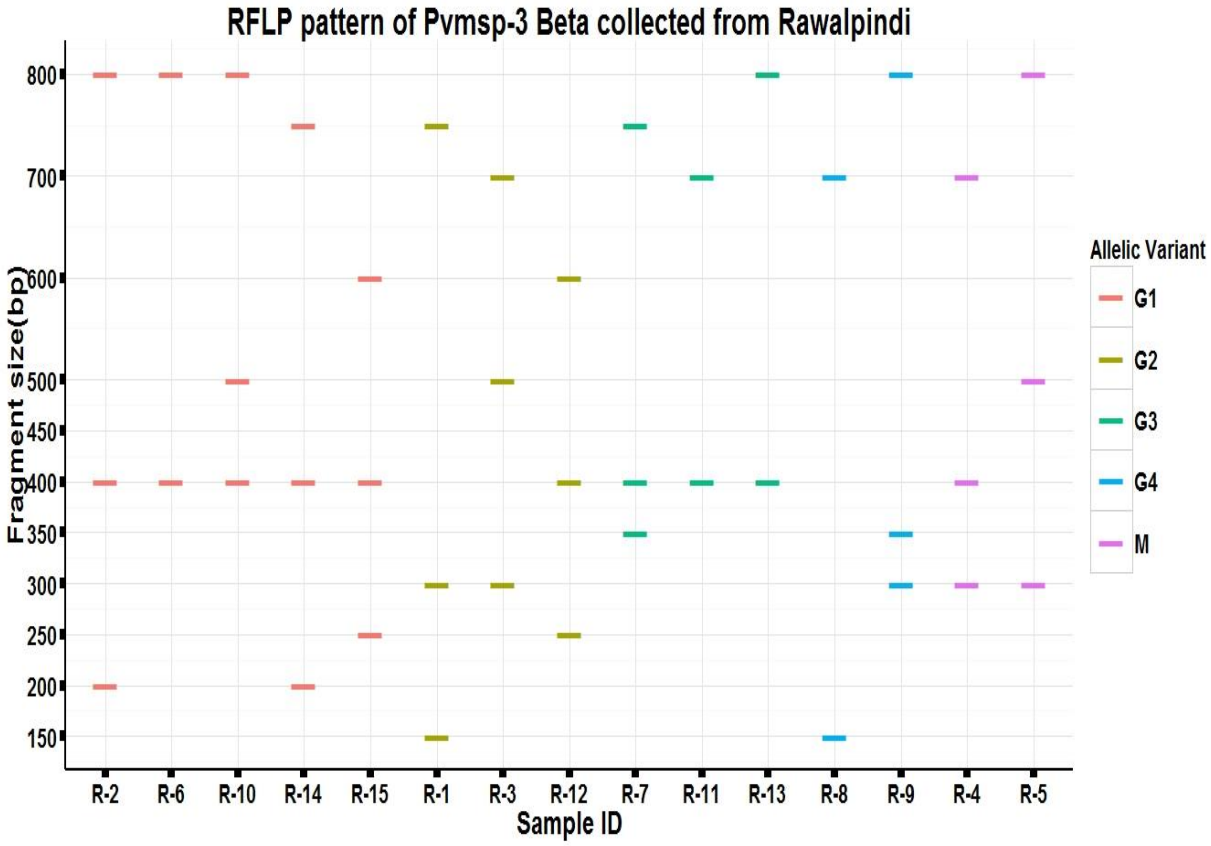


Figure 16: Showing RFLP patterns and frequency of *Pvmsp*β alleles collected from Rawalpindi

The RFLP analysis of sample from Rawalpindi is somewhat different from Islamabad. Out of the total 15 amplified samples, 5(33.33%) isolates for Type-G1, 3(20%) for Type-G2, 3(20%) for Type-G3 and 2(13%) for type G4 were observed.

Table 13: Size and RFLP patterns and frequency of *Pvmsp*3β alleles collected from Kashmir

ID	Allelic variant Size		A	B	C	D	Genotype
K-1	1.2	**G3**	800	450	300	-	1
K-2	1.7	**M**	800	500	450	300	2**
K-3	1.5	**G3**	700	450	200	-	3
K-4	1.2	**G4**	700	150	-	-	4
K-5	1.5	**G3**	700	450	310	-	5
K-6	1.7	**G2**	800	500	-	-	6
K-7	2.2	**M**	800	500	400	300	7**
K-8	1.2	**M**	700	450	400	-	8**
K-9	1.7	**G2**	800	450	250	-	9
K-10	1.5	**G3**	800	500	400	-	10
K-11	2.2	**M**	800	750	600	-	11**
K-12	1.2	**G4**	800	450	300	-	12
K-13	2.2	**G1**	750	450	-	-	13
K-14	2.2	**G1**	750	400	200	-	14
K-15	2.2	**G1**	700	200	150	-	15

Out of the total 15 amplified samples for Kashmir region 3(20%) were isolates for Type-G1, 2(13%) for Type-G2, 4(26%) for Type-G3 and 2(13%) for G4 were observed. Among these isolates Type-G3 was found to have high per cent frequency beside the mixed type which were observed 20% of the allelic variants.

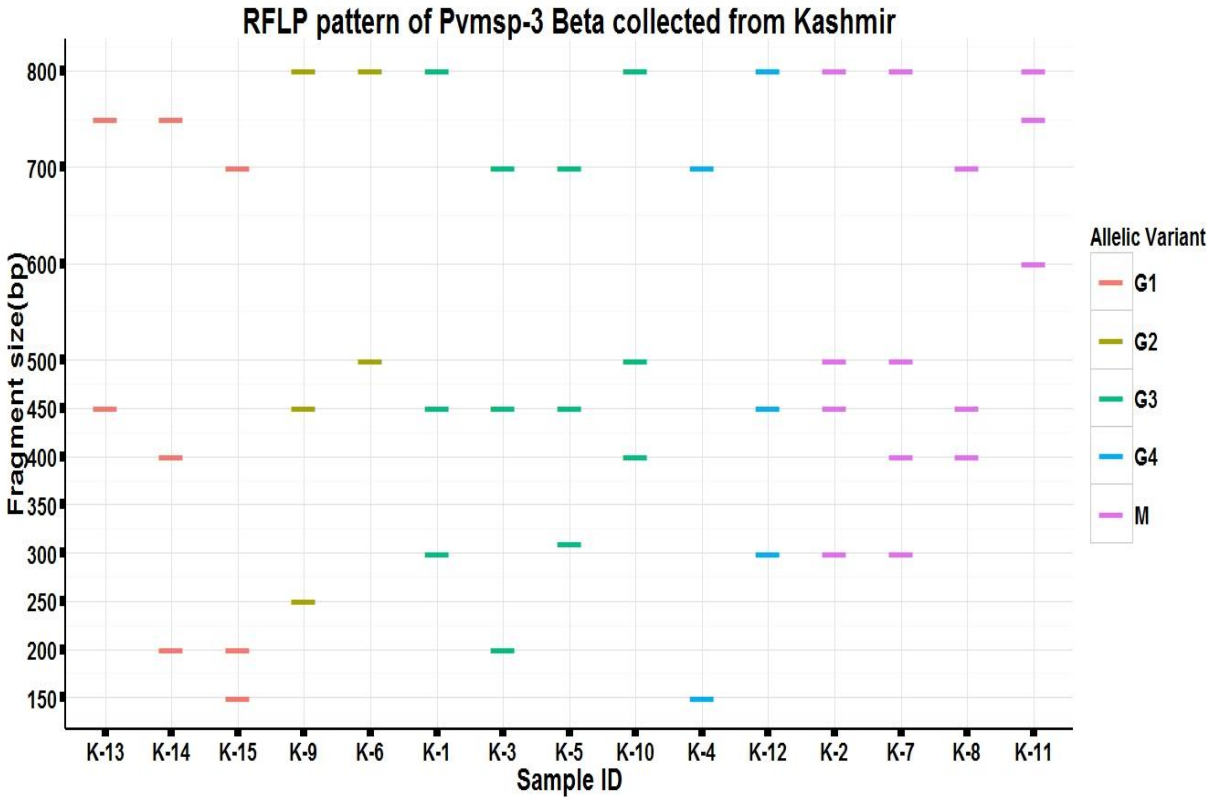


Figure 17: Showing RFLP patterns and frequency of Pvmspβ alleles collected from Kashmir

Out of the total 15 amplified samples for Kashmir region 3(20%) were isolates for Type-G1, 2 (13%) for Type-G2, 4(26%) for Type-G3 and 2(13%) for G4 were observed.

3.5: Allelic Diversity of *P. vivax* Population

It is suggested that meiotic recombination is the main source of allelic diversity and recombination can be inferred by evaluating the level of linkage dis-equilibrium between polymorphic sites of the alleles. Allelic diversity of the *Pvmsp*3α, between *P. vivax* populations, estimated by pairwise fixation index, *F*ST is shown in the table (15, 16). However, the number of alleles differed remarkably between populations, although most likely skewed by the differences in sample size. For both markers, the Asian *P. vivax* populations were highly polymorphic (except for the Korean *P. vivax* populations), whereas the Salvador *P. vivax* populations were less diverse. The diversity by the *Pvmsp*3α was highest in Pakistan with significant pairwise comparison with the other samples. Although the sample sizes differed from 3-13 samples per site and with that in mind Brazil also seemed to be the most polymorphic.

The average pairwise nucleotide diversity per site, was used to measure diversity across the *Pvmsp*-3ß gene. We calculated pairwise fixation index for all isolates and compare with the data base sequence from the gene bank. Insert A, insert B, and the C-terminal domain following the insert that is shared by all isolates. The allelic diversity of most of the Korean and Asian populations was found to be significantly different from each other by pairwise *F*, whereas the China and Indian population generally could not be distinguished statistically from most of the isolate from Pakistan and Kashmir, most likely due to the limited sample size. Pairwise genetic differences (*F*ST) ranged from 0.002-0.63 and 0.03-0.128 and a Mantel test correlating pairwise genetic and geographic distances between populations. However, this correlation result might be influenced by various parameters within population characteristics i.e. level of endemicity, study designs, diverging time spans of collection and random and systematic errors.

Table 14: Showing Sequences Submitted to Gene bank

S.NO	Nucleotide Seq. ID	Sequence name	Protein ID	Accession Number
1	IH-140611	Kashmir alpha	IH-140611	KP720660
2	IH-140613	Kashmir beta	IH-140613	KP730713
3	IH-140615	Islamabad alpha	IH-140615	KP730714
4	IH-140616	Islamabad beta	IH-140616	KP730715
5	IH-140617	Rawalpindi alpha	IH-140617	KP730716
6	IH-140618	Rawalpindi beta	IH-140618	KP730717

Table 15: Showing Allelic diversity of the *Pvmsp*3*α*, between 12 *P. vivax* populations, estimated by pairwise fixation index, F_{ST}.

***Pvmsp*3a**	China	Ecuador	Salvador	Indian	Korea	Africa	Brazil	Pakistan	Sri Lanka	Thailand	Iran	Kashmir
China (8)		***	***	***	***	**	***	***	***	***	NS	NS
Ecuador (7)	0.0202		NS	***	**	*	***	***	***	***	*	**
Salvador(13)	0.0347	0.1349		**	**	***	***	***	***	***	NS	*
Indian (8)	0.0123	0.0371	0.1395		NS	***	NS	**	***	***	NS	NS
Korea (8)	0.0146	0.0944	0.0709	0.7119		**	***	***	***	***	**	***
Africa (11)	0.0766	0.3137	0.0100	0.0533	0.2290		*	***	***	NS	NS	NS
Brazil (5)	0.0912	0.3049	0.0981	0.0151	0.2497	0.0158		NS	***	NS	NS	NS
Pakistan (3)	0.0857	0.2796	0.0924	0.0192	0.2229	0.0301	0.0010		***	NS	NS	NS
Sri Lanka (5)	0.1766	0.3494	0.1473	0.0511	0.3071	0.1119	0.0666	0.0588		***	NS	NS
Thailand (9)	0.0789	0.2820	0.0766	0.0326	0.2182	-0.0005	0.0037	0.0116	0.0680		NS	NS
Iran (4)	0.1528	0.6276	0.1940	0.0219	0.4522	0.0557	0.0099	0.0435	0.1063	0.0536		NS
Kashmir (3)	0.2064	0.6094	0.2384	0.0905	0.4844	0.1029	0.0809	0.0697	0.1605	0.0840	0.1767	

In brackets are mentioned number of positive Isolates. The pairwise significance after standard Bonferroni corrections are listed as: "***"Highly significant, "**" significance at moderate level "*"significant, while "NS" stands for non- significant.

Table 16: Showing Allelic diversity of the *Pvmsp*3β, between 12 *P. vivax* populations, estimated by pairwise fixation index, F_{ST}.

***Pvmsp*3 beta**	China	Ecuador	Salvador	Indian	Korea	Africa	Brazil	Pakistan	Sri Lanka	Thailand	Iran	Kashmir
China (8)		NS	***	NS	***	***	**	***	***	***	NS	NS
Ecuador (5)	0.4429		***	NS	***	NS	*	***	***	***	NS	NS
Salvador (8)	0.0016	0.0152		***	***	***	***	***	***	***	NS	NS
Indian (9)	0.0448	0.6062	0.1463		***	NS	NS	***	***	*	NS	NS
Korea (5)	0.1988	0.2413	0.2129	0.1215		***	***	***	***	***	NS	**
Africa (4)	0.0713	0.0786	0.1604	-0.0066	0.1226		NS	***	***	NS	NS	NS
Brazil (9)	0.0861	0.1411	0.1412	0.0075	0.1060	0.0177		NS	***	NS	NS	NS
Pakistan (3)	0.1141	0.1613	0.0819	0.0311	0.1061	0.0351	0.0154		***	***	NS	NS
Sri Lanka (5)	0.1325	0.2108	0.2838	0.0658	0.1439	0.0745	0.0509	0.1108		***	NS	*
Thailand (13)	0.1034	0.1387	0.1846	0.0256	0.1377	0.0228	0.0323	0.0344	0.0815		NS	NS
Iran (4)	0.6069	0.1921	0.3269	0.1075	0.1292	0.1203	-0.2209	0.2507	-0.1987	0.1207		NS
Kashmir (1)	-0.5039	0.2341	0.2358	-0.1262	0.0464	-0.1092	0.2141	0.4455	0.05118	0.1239	0.1282	

In brackets are mentioned number of positive Isolates. The pairwise significance after standard Bonferroni corrections are listed as: "***"Highly significant, "**" significance at moderate level "*"significant, while "NS" stands for non- significant.

Figure18: Phylogenetic Analysis of *Pvmsp*3 alpha

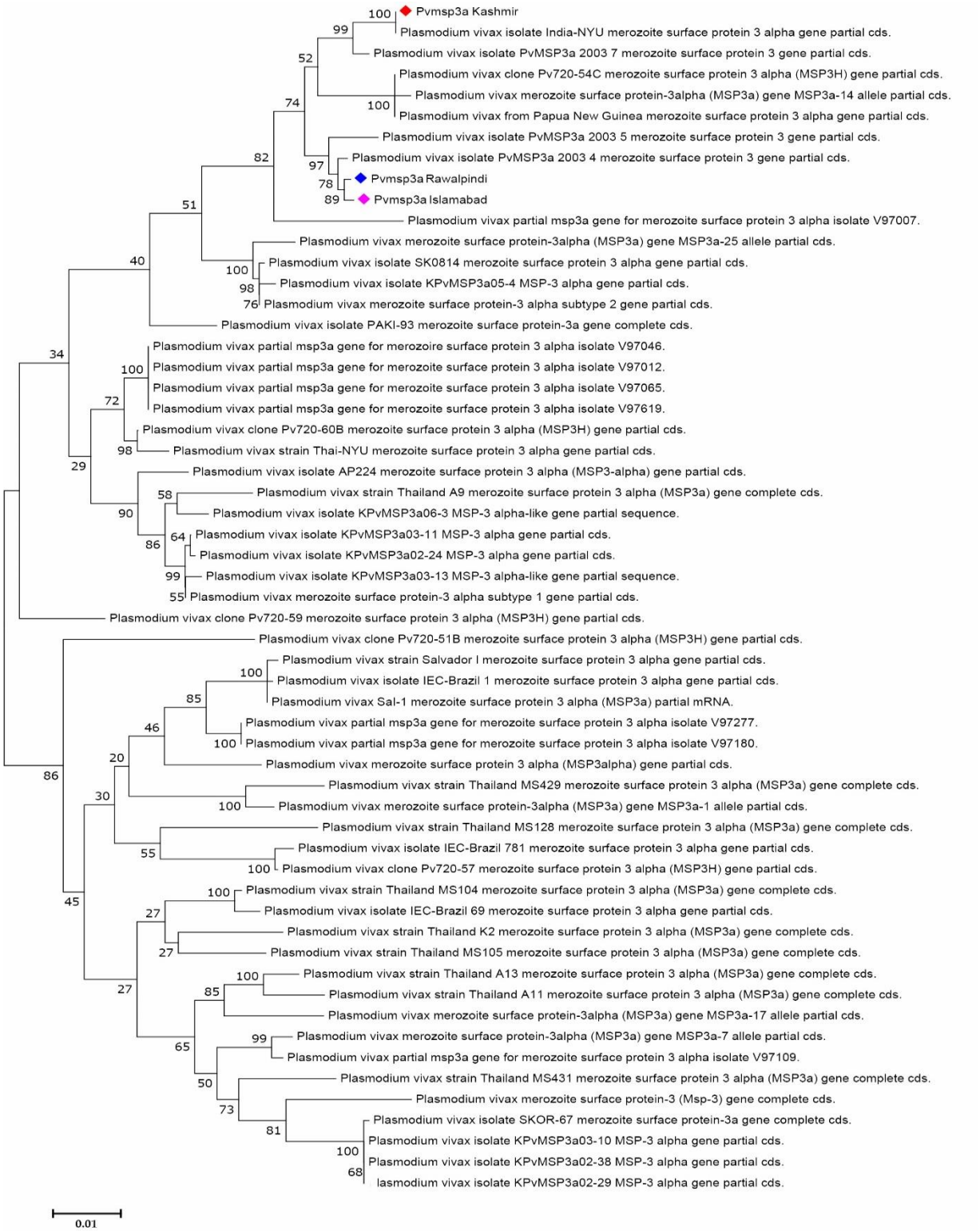


Figure 17: Showing Evolutionary Relationships of *Pvmsp*3 alpha

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.55918034 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 57 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1270 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

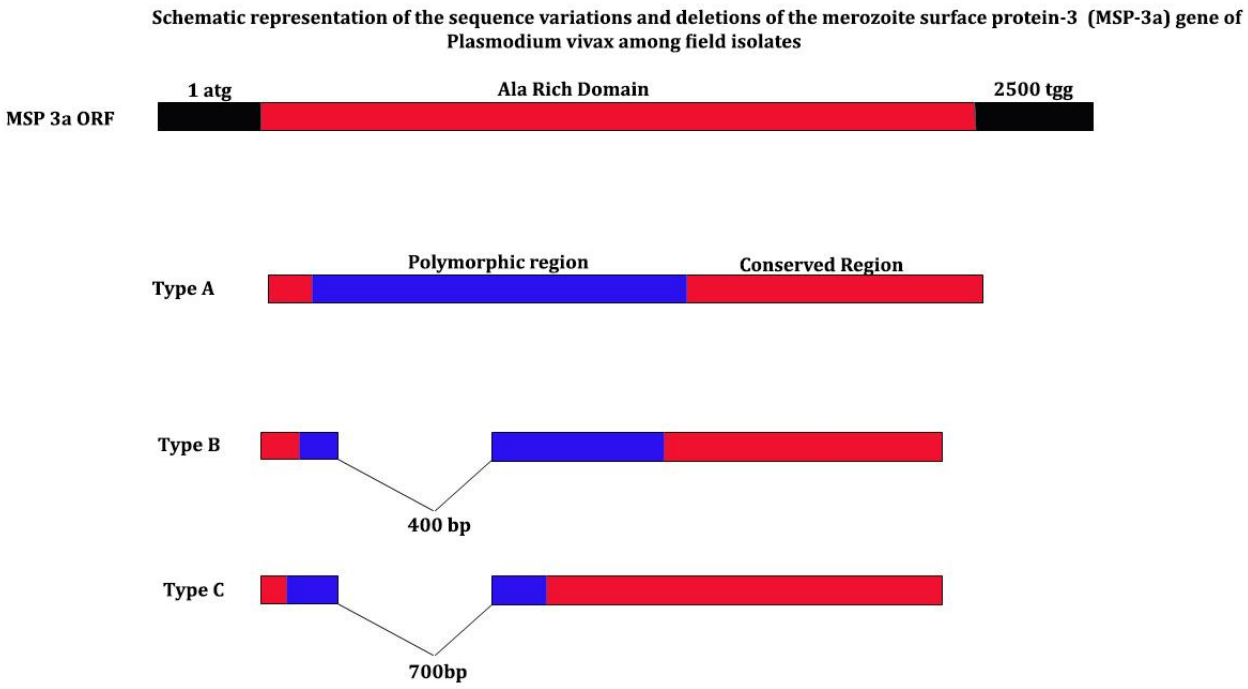


Figure 19: Showing *Pvmsp3α* sequence variations and deletions

Figure 20: Phylogenetic Analysis of *Pvmsp*3 beta

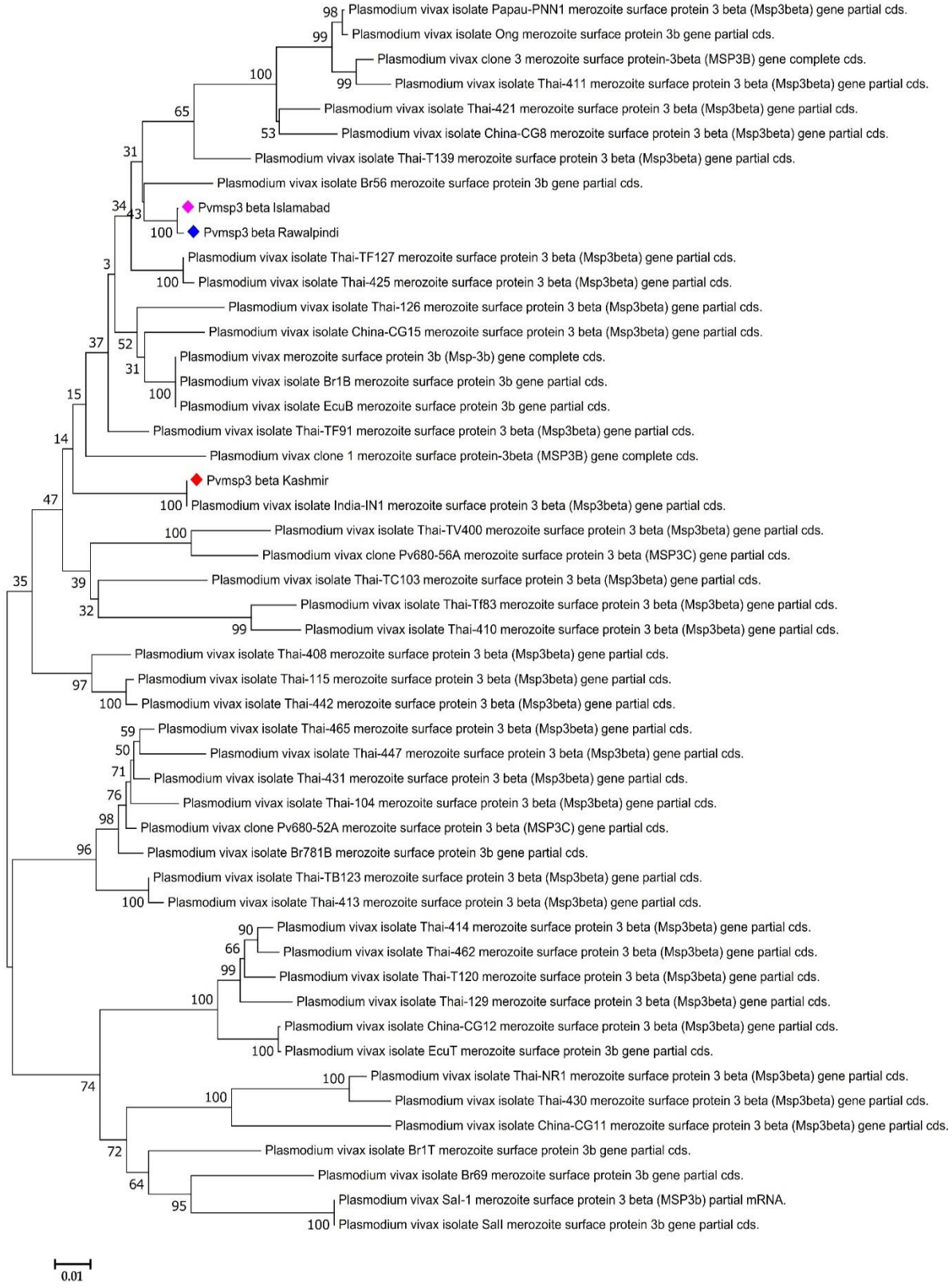


Figure 19: Showing Evolutionary Relationships of *Pvmsp*-3 beta

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.05285495 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 50 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 975 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

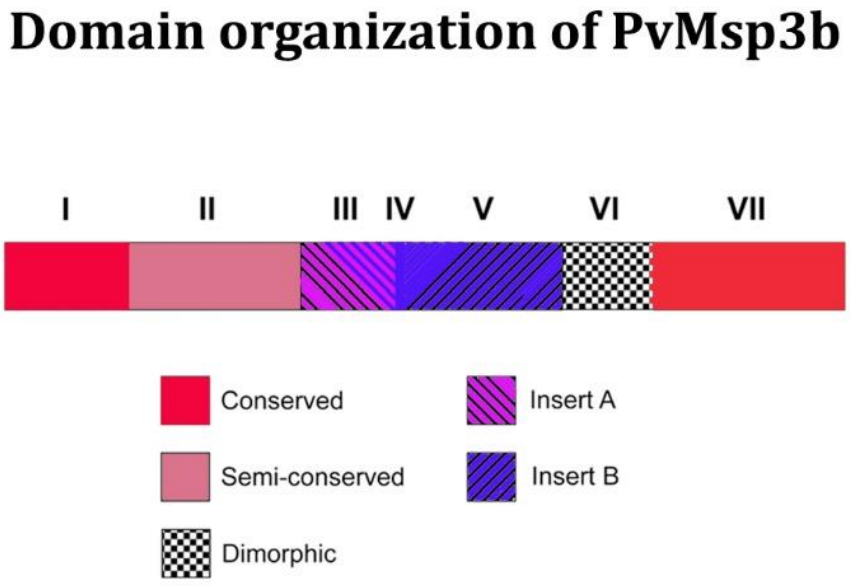


Figure 21: Showing Domain organization of *Pvmsp*3β

Table 17: Analysis and comparasion of conserved and non conserved site across *Pvmsp*3 beta Conserved.

```
#XM001613146-Sal MKQFCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVHSDGSD DVTDKEEDGE VLEGQKGSPK KSAEQKVHAQ EEVNKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#AY454081-Br1T   MKQFCGLAFL ALLLNFLTCD NAATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQNGSPE KSAEPKVHAQ EEVNKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#AY454082-Br69   MKQFCGLAFL ALLLNFLTRD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNSHGSD DVTDKKEDGE VLEGQKGSPK KLAEPKVHAQ EAETKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#AY454092-India  ********** ********** ********** ********** SMNNLSAQNE ENIVNPDGSD DVTDEEGDGE VLEGQNGSPE KSAEPKVHAQ EEETKESLKS KAQNAKEEAE KAAKAAASAK  [ 120]
#AY454091-EcuT   ********** ********** ********** ********** SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQKGSPK KSAEPKVHAQ EEGNKDELKS KATKAKADAT EAVKAAELAK  [ 120]
#Isb-414         MKQFCGLAFL ALLLNFLTCD NVATRGEIVN LKNPYLRNGW SMNNLSAQNE ENIVHSDGSD DVTDKEEDGE VLEGENESPK KSAEPKVHAQ EAESKESLKL KAANAKKDAE EAAKAAQSAK  [ 120]
#Rwp-129         MKQFCGLASL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVHSDGSD DVTDEEGDGE VLEGQNGSPE KSAEPKVHAQ EEETKESLKL KAANAKKDAE EAAKAAQSAK  [ 120]
#Isb-T120        MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNSHGSD DVTDKKEDGE ALEGQNGSPK KSAEPKVHAQ EAETKESLKL KAANAKKDAE EAAKAAQSAK  [ 120]
#Pak-462         MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNTNLRNGW SMKNVSAQNE ENIVHPDGSD DVTDKEEDGE VLEGENESPK RSAEPKVHAQ EAESKESLKL KAANAKKDAE EAAKAAQSAK  [ 120]
#China-CG12      MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNPYLRNGW SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQKGSPK KSAEPKVHAQ EEGNKDELKS KATKAKADAT EAVKAAELAK  [ 120]
#China-CG8       MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMNNLSAQNE ENIVNSHGSD DVTDKEENGE VLEGENGSPK KLAEPKVHAQ EAETKESLKL KATKAKTEAV EAANAAELAK  [ 120]
#AY454084-Bangl  ********** ********** ********** ********** SMKNVSAQNE ENIVHSDGSD DVTDKEEDGE VLEGQKGSPK KSAEPKVHAQ EEGNKDELKS KATKAKADAT EAVKAAELAK  [ 120]
#AY454097-SL57   ********** ********** ********** ********** SMKNVSAQNE ENIVHLDGSD DVTDKEEDGE VLEGQNGSPK KLAEPKVHAQ EEETKDELKS KATKAKTEAV EAAKAAETAK  [ 120]
#AY454096-SL     ********** ********** ********** ********** SMKNVSAQNE ENIVHLDGSD DVTDKEEDGE VLEGQNGSPK KLAEPKVHAQ EEETKDELKS KATKAKTEAV EAAKAAETAK  [ 120]
#Ajk-106         MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVNSHGSD DATDKEEDGE VLEGENESPK KLAEPKVHAQ EVVNKESLKS KATTAKTEAE KAANAADLAK  [ 120]
#Isb-NR1         MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE VLEGQKGSPK KSAEPKVHAQ EEGNKDELKS KATKAKADAT EAVKAAELAK  [ 120]
#Bra-T107        MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMKNVSAQNE ENIVHSDGSD DVTDKEEDGE VLEGQNGSPK KSAEQKVHAQ EEVNKESLKS KATTAKTEAE KAANAADLAK  [ 120]
#Ind-113         MKQLCGLAFL ALLLNLLTCG NVATRGEIVN LKNTNLRNGW SMNNLSAQNE ENIVHSDGSD DATDKEEDGE VLEGQKGSPK KSAEQKVHAQ EEGNKDELKS KATTAKTEAE KAANAADLAK  [ 120]
#Thai-430        MKQFCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVHSDGSD DVTDKEEDGE VLEGQKGSPK KSAEPKVHAQ EEGNKDELKS KATKAKADAT EAVKAAELAK  [ 120]
#China-CG11      MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVNSHGSD DVTDKEENGE VLEGQNGSPK KLAEPKVHAQ EEVNKESLKS KATTAKTEAE KAANAADLAK  [ 120]
#AY454083-Chess  MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNVSAQNE ENIVNSHGSD DVTDKEENGE VLEGENGSPK KLAEPKVHAQ EEETKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#Ajk-TC103       MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMNNLSAQNE ENIVNSHGSD DVTDKEEDGE VLEGQNGSPK KLAEPKVHAQ EEETKDELKS KATKAKTEAV EAAKAAETAK  [ 120]
#Rwp-TV400       MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQKGSPK KSAEPKVHAQ EEGNKDELKS KATKAKADAT EAVKAAELAK  [ 120]
#AY454089-Br781T ********** ********** ********** ********** SMNNLSAQNE ENIVNSHGSD DVTDKEEDGE VLEGQNGSPE KSAEPKVHAQ EEETKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#PVNG01493-NK    MKQFCGLAFL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVHSDGSD DVTDKEEDGE VLEGQNGSPK KLAEPKVHAQ EAESKDELKS KATKAKTEAV EAANAAELAK  [ 120]
#Ecu-411         MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQNGSPE KSAEPKVHAQ EAETKESLKL KATKAKTEAV EAAKAAALAK  [ 120]
#Sri-TF83        MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMNNLSAQNE ENIVNSDGSD DVTDKEENGE VLEGENGSPK KLAEPKVHAQ EAETKESLKL KATKAKTEAV EAAKAAASAK  [ 120]
#CHi-109         MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMKNVSAQNE ENIVHSYGSD DVTDKEEDGE VLEGENGSPE KSAEPKVHAQ GAESKDELKS KATKAKTEAV EAANAAELAK  [ 120]
#Sri-410         MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVNSHGSD DVTDKEENGE VLEGQKGSPK KSAEQKVHAQ EEETKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#AY454098-Thai   ********** ********** ********** ********** SMKNVSAQNE ENIVHSDGSD DVTDKEEDGE VLEGENGSPE KSAEPKVHAQ EAESKDELKS KATKAKTEAV EAANAAELAK  [ 120]
#AF099662-Belem  MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQNGSPE KSAEPKVHAQ EEVNKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#AY454080-Br1B   MKQFCGLAFL ALLLNFLTCD NAATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQNGSPE KSAEPKVHAQ EEVNKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#AY454085-Br56   ********** ********** ********** ********** SMKNLSAQNE ENIVNSHGSD DVTDKKEDGE ALEGQNGSPK KSAEPKVHAQ EAETKESLKL KAANAKKDAE EAAKAAQSAK  [ 120]
#China -115      MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNSHGSD DVTDKKEDGE ALEGENGSPK KLAEPKVHAQ EAESKDELKS KATKAKTEAV EAANAAELAK  [ 120]
#Thil-TF91       MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVHSDGSD DVTDKEEDGE VLEGENGSPK KLAEPKVHAQ EAENKESLKL KATKAKTEAV EAANAAELAK  [ 120]
#Iran-126        MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVNSHGSD DVTDKEEDGE VLEGQNGSPE KSAEPKVHAQ EEVNKESLKS KAQNAKAEAE KAAKAAESAK  [ 120]
#India-442       MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNSHGSD DVTDKKEDGE ALEGENGSPK KLAEPKVHAQ EAESKDELKS KATKAKTEAV EAANAAELAK  [ 120]
#Bra- 408        MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVNPDGSD DVTDKKEDGE ALEGENGSPK KLAEPKVHAQ EAESKDELKS KATKAKTEVV EAANAAELAK  [ 120]
#India-IN1       MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQKGSPK KSAEPKVHAQ EEGNKDELKS KATKAKADAT EAVKAAELAK  [ 120]
#kor -TB123      MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVNSHGSD DVTDKKEDGE ALEGENESPK ESAEPKVHAQ EAENKDELKS KAATAKEEAE KAAKAAASAK  [ 120]
#chin -T139      MKQLCGLAFL ALLLNFLTRD NAATRGEIVN LKNPNLRNGW SMNNLSAQNE ENIVHSDGSD DVTDKEEDGE VLEGENGSPK KLAEPKVHAQ EAETKESLKL KATKAKTEAV EAANAAELAK  [ 120]
#Thai -TF127     MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQNGSPE KSAEPKVHAQ EAETKESLKL KATKAKTEAV EAAKAAALAK  [ 120]
#Iran -104       MEQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNTNLRNGW SMKNPSAQNE ENIVRSDGSD DVTDKEEDGE VLEGENGSPK KLAEPKVHAQ EAESKESLKL KAAHAKKDAE EAAKAAQSAR  [ 120]
#Ajk-413         MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVNSHGSD DVTDKKEDGE ALEGENESPK ESAEPKVHAQ EAENKDELKS KAATAKEEAE KAAKAAASAK  [ 120]
#Sal1 -431       MKRLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNVSAQNE ENIVHSDGSD DVTDKEEDGE VLEGENGSPK KLAEPKVHAQ EAESKESLKL KAANAKKDAE EATKAAQSAK  [ 120]
#Sal1-447        MKQLCGLAFL ALLLNFLTRD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVHSEGGD DVTDKEEDGK VLEGENGSPK RLAEPKAHAQ EAESKESLKL KAANAKKDAE EAAKAAQSAK  [ 120]
#Kor-465         MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNVSAQNE ENIVHSDGSD DVTDKEEDGE VLEGENGSPK KLAEPKVHAQ EAESKESLKL KAANARKDAE EAAKAAQSAK  [ 120]
#Ajk -425        MKQFCGLAFL ALLLNFLTCD NVATRGEVVN LKNPNLRNGW SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQNGSPE KSAEPKVHAQ EAETKESLKL KATKAKTEAV EAAKAAALAK  [ 120]
```

```
#Sri -421          MKQFCGLAFL ALLLNFLTCD NVATRGEIVN LKNPNLRNGW SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE VLEGQNGSPE KSAEPKVHAQ EEETKESLKS KAQNAKAEAV EAANAAELAK  [ 120]
#Papau-PNN1        MKQLCGLAFL ALLLNFLTCD NVATRGEIVN LKNTNLRNGW SMKNLSAQNE ENIVNPDGSD DVTDEEGDGE ALEGQNGSPE KSAEPKVHAQ EAETKESLKL KATKAKTEAV EAAKAAALAK  [ 120]
                                        Conserved                                                  Semi-Conserved
#XM001613146-Sal ENTLDALEKV NVPTELNNEK NFAESAATEA KKQEKISTEA AE----EVKE IEVDGQLEKL KNEEE---KT AKKARKQEIK TEIAEQAAKA QAAK------ -TEAETAQK- ------DATT  [ 240]
#AY454081-Br1T   KNTLDALEKV NVPTELNNVK KFAESAATEA QKQENIATEA EKK---VAEA NGEVVELQKL KDEVD---KA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#AY454082-Br69   KNTLDALEKV NVPTELNNVK KFAESAATEA QKQENIATEA EKKV---AEA NGEVVELQKL KDEVKKAEKA AKKAKKLQIK AQIAEQAIKA QVAK------ -TEAKKAQKD AEKAKTEATT  [ 240]
#AY454092-India  DRTLVALEKV DVPTELDKVK EFAVSAATQA KNQETIATKA AES----AEA IGGDGDLGNL KSEVENAEKA AKKAKQLQIK AEIAEQAVMA QAAK------ -TEAKKAQKD AEKAKTEATT  [ 240]
#AY454091-EcuT   DRTLVALEKV KVPTELNKAK EFAEAAAAEA KNQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#Rwp-414         DSTLNALEKV KVPTELNKAK EFAEAAAAEA KNQENLA-IA AEK---AAQE MEVNGDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQK- ------DATA  [ 240]
#Ajk-129         DGTLNALEKV KVPTELNKAK EFAEAAAAEA KKQENIA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQKAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#Isb-T120        DSTLNALEKV KVPTELNKAK EFAEAAAAEA KKQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQK- ------DATA  [ 240]
#Rwp-462         DSTLNALEKV KVPTELNKAK EFAEAAAAEA KNQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#China-CG12      DRTLVALEKV KVPTELNKAK EFAEAAAAEA KNQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQK- ------DATA  [ 240]
#China-CG8       NNTLDALKKV KVPTELNKAK EFAESAATEA KKQENIATEA EK---KVAEA NGEVVELQKL KDEVKKAEKA AKKAKKLQIK AQIAEQAIKA QVAK------ -TEAETAQT- ------EATA  [ 240]
#AY454084-Bangl  KNTLDALAKV KVPTELSKAN EFAESAVTEA KKQEEIA--- -------AKE IEVDGEFQKL KDEVDKAEKA AKKAKKLQIK VQIVEQAAKA QVAK------ -TEAETAQK- ------EATA  [ 240]
#AY454097-SL57   ENILVALKKV DVPTELDKVK EFAVSAATQA KNQETIATKA AES----AEA IGDDGDLGNL KSEVENAEKA AKKAKQLQIK AEIAEQAVMA QAAK------ -TEAKKAQKD AEKAKTEATT  [ 240]
#AY454096-SL     ENILVALKKV DVPTELDKVK EFAVSAATQA KNQETIATKA AES----AEA IGDDGDLGNL KSEVENAEKA AKKAKQLQIK AEIAEQAVMA QAAK------ -TEAKKAQKD AEKAKTEATT  [ 240]
#China -106      KNTWVALEKV NVTTELDKVK EFAESAVTEA KKQEQIATEV ETKV---AKA TGEDGELQKL KEEVEKAGRA AKKAKKLQIR VQIAEQAANA QVAK------ -TEAETAQK- ------DATA  [ 240]
#Bra -NR1        KNTLDALAKV KVPTELSKAN EFAESAVTEA KKQEEIA--- -------AKE IEVDGEFQKL KDEVDKAEKA AKKAKKLQIK VQIVEQAAKA QVAK------ -TEAETAQK- ------EATA  [ 240]
#Ecu -T107       KNTWVALEKV NVTTELDKVK EFAESAVTEA KKQEQIATEV ETKV---AKA TGEDGELQKL KEEVEKAERA AKKAKKLQIK VQIAEQAANA QVAK------ -TEAETAQK- ------DATA  [ 240]
#Thai -113       KNTWVALEKV NVSTELDKVK EFAESAVTEA KKQEQIATEV ETKV---AEA TGEDGELQKL KEEVEEAERA AKKAKKLQIK VQIAEQAANA QVAK------ -TEAETAQK- ------DATA  [ 240]
#Sri-430         KNTLDALAKV KVPTELSKAN EFAESAVTEA KKQEEIA--- -------AKE IEVDGEFQKL KDEVDKAEKA AKKAKKLQTK VQIVEQAAKA QVAK------ -TEAETAQK- ------EATA  [ 240]
#China-CG11      KNTWVALEKV NVTTELDKVK EFAESAVTEA KKQEQIATEV ETKV---AKA TGEDGELQKL KEEVEKAERA AKKAKKLQIK VQIAEQAANA QVAK------ -TEAETAQK- ------DATA  [ 240]
#AY454083-Chess  DSTLDALKKV KVPTEHDKVK KFAESAATEA KKQENLAIEA EK----AAQA IEDDGQKEKL KTEVDKAEKA AKKAKQLQIK AEIAEQAAKA QLAKTEAEKA KTEAETAQK- ------DATA  [ 240]
#Thai-TC103      ENILVALKKV NESTELNKAK EFAESAATEA KKQETIATEA ETK---VSEG NGDDGELGNL KTEVDKAEKA AKKAKQLQIK AQIAEQAVKA QVAK------ -TEAETAQK- ------DATA  [ 240]
#Thai-TV400      DRTLDALKKV KVPTEHDKVK KFAESAATEA KKQENLAIEA EK----AAQA IEDDGQKEKL KTEVDKAEKA AKKAKQLQIK AEIAEQAAKA QLAKTEAEKA KTEAETAQK- ------DATA  [ 240]
#AY454089-Br781T ENTLDALEKV NVPTELNNVK KFAESAATEA KKQEKISTEA AE----EVKE IEVDGQLEKL KNEEE---KT AKKARKQEIK TEIAEQAAKA QAAK------ -TEAETAQK- ------DATT  [ 240]
#PVNG01493-NK    NNTLVALAKV KVPTEHDKVK KFAESAATEA KKQEKISTEA AE----EVKE IEVDGQLEKL KNEEE---KT AKKARKQEIK TEIAEQAAKA QAAK------ -TEAETAQK- ------DATT  [ 240]
#Sal1 -411       DNTLVALKKV KVPTEHDKVK KFAELAATEA KKQEEIAIAA EKEAAKEAEA IEVDGEFKKL KDEVKKAEKA AKKAKKLQIK AQIAEQAIKA QVAK------ -TEAETAQT- ------EATA  [ 240]
#Pak -TF83       DRTLVALEKV NVPTELNNVK KFAESAATEA KNQETIATKA AE----SAEA IGDDGDLGNL KSEVENAEKA AKKARKQEIK TEIAEQAAKA QAAK------ -TEAETAQK- ------DATT  [ 240]
#chi -109        NNTLVALAKV KVPTGHDKVK KFAESAATEA KKQEKISTEA AE----EVKE IEVDGQLEKL KNEEK---KT AKKARKQEIK TEIAEQAAKA QAAK------ -TEAETAQK- ------DATT  [ 240]
#Tha -410        KNTLDALEKV NVPTELNNVK KFAESAATEA QKQETIATKA AE----SAEA IGDDGDLGNL KSEVENAEKA AKKARKQEIK TEIAEQAAKA QAAK------ -TEAETAQK- ------DATT  [ 240]
#AY454098-Thai   NNTLVALAKV KVPTEHDKVK KFAESAATEA KKQEKISTEA AE----EVKE IEVDGQLEKL KNEEE---KT AKKARKQEIK TEIAEQAAKA QAAK------ -TEAETAQK- ------DATT  [ 240]
#AF099662-Belem  KNTLDALEKV NVPTELNNVK KFAESAATEA QKQENIATEA EKK---VAEA NGEVVELQKL KDEVD---KA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#AY454080-Br1B   KNTLDALEKV NVPTELNNVK KFAESAATEA QKQENIATEA EKK---VAEA NGEVVELQKL KDEVD---KA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#AY454085-Br56   NSTLDALKKV KVPTELNKAK EFAESAATEA KKQETIATEA EKK---VAEA NGEVVELQKL KDEV---DKA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#Bra-115         NNTLDALKKV KVPTELNKAK EFAESAATEA KKQENIATEA EKK---VAEA NGEVVELQKL KDEVKKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#Sri-TF91        NNTLDALKKV KVPTELNKAK EFAESAATEA KKQENIATEA EKK---VAEA NGEVVELQKL KDEVD---KA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#Ind-126         KNTLDALEKV NVPTELNNVK KFAESAATEA QKQENIATEA EKK---VAEA NGEVVELQKL KDEVD---KA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#tha-442         NNTLDALKKV KVPTELNKAK EFAESAATVA KKQENIATEA EKK---VAEA NGEVVELQKL KDEVKKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#Kor-408         NNTLDALKKV KVPTELNKAK EFAEAAAAEA KKQENIATEA EKK---VAEA NGEVVELQKL KDEVKKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#Pap-IN1         DRTLVALEKV KVPTELNKAK EFAEAAAAEA KKQENLAIAA EK----AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#Ecu-TB123       DRTLVALEKV KVPTELNKAK EFAEAAAAEA KNQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#Sri-T139        NNTLDALKKV KVPTELNKAK EFAESAATEA KKQENIATEA EKK---VAEA NGEVVELQKL KDEVKKAEKA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#Iran-TF127      DNTLVALKKV KVPTELNKAK EFAESAATEA KKQENIATEA EKK---VAEA NGEVVELQKL KDEV---DKA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
#Ind -104        DSTLNALEKV KVPTELNKAK EFAEAAAAEA KNQENLATAA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#Ind-413         DRTLVALEKV KVPTELNKAK EFAEAAAAEA KNQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQT- ------DATA  [ 240]
#Tha-431         DSTLNALEKV KVPTELNKAK EFAEAAAAEA KNQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAEKAQK- ------DATA  [ 240]
#Bra-447         DSTLNALEKV KVPTEFYRAK ESAEAATAEA ENQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAKKAQT- ------DATA  [ 240]
#Bra-465         DSTLNALEKV KVPTELNKAK EFAEAAAAEA KNQENLA-IA AEK---AAQE MEVNEDLQNL KTEVEKAEKA AKKAKQLEIK VQIAEQAAKA QVAK------ -TEAKKAQT- ------DATA  [ 240]
#Chi -425        DNTLVALKKV KVPTELNKAK EFAESAATEA KKQENIATEA EKK---VAEA NGEVVELQKL KDEV---NKA AKKAKQLQLK AEIAEHAVKA QVAK------ -TEAEKAQK- ------DATT  [ 240]
```

```
#Thi-421          NNTLDALKKV KVPTELNKAK EFAESAATEA KKQENIATEA EK---KVAEA NGEVVELQKL KDEVKKAEKA AKKAKKLQIK AQIAEQAIKA QVAK------ -TEAETAQT- ------EATA  [ 240]
#Papau-PNN1       DNTLVALKKV KVPTEHDKVK KFAELAATEA KKQEEIAIAA EKEAAKEAEA IEVDGEFKKL KDEVKKAEKA AKKAKKLQIK AQIAEQAIKA QVAK------ -TEAETAQT- ------EATA  [ 240]


                      Insert A ><Conserved>< Insert B
#XM001613146-Sal  IAAEVAKAEK AKIKAENAKL LADTASKAAE NIAKSSKAAK IANNVSTIAA EKSKVATEAA DEAAKALDET ENPESKIAEV TEKATKAVNA AEEAKKEKAK AEVAVEVAHA EVAKEKAQEA  [ 600]
#AY454081-Br1T    ---------- ----AENAKL IADTASKAAE DIAKSSKAAQ IAKNVSAKAE EKSKVATEAA DEAANALNEA ENPESKIDDV KKKATEAVNA AEEAKKEKSK AEIAVEVAKA EEAKKEAGKA  [ 600]
#AY454082-Br69    ---------- ----AENAKL LADTASKAAE NIAKSSKAAK IANNVSTIAA EKSKVATEAA DEAAKALDET ENPESKIAEV TEKATKAVNA AEEAKKEKAK AEVAVEVAHA EVAKEKAQEA  [ 600]
#AY454092-India   ---------- ----AENAKL IADTASKAAE DIAKSSKAAQ IAKNVSAKAE EKSKVATEAA DEAANALNEA ENPESKIDDV RKKATEAVNA AEEAKKEKSK AEIAVEVAKA EEAKKEAGKA  [ 600]
#AY454091-EcuT    ---------- ----AENANL LADTANKEVE RIAKSSKAAQ IANKVSSKAA GKLEVATKAE DEAAKALDET ENSESKIAEV REKATTAFNA AEEAKKEK-- ---------- ----------  [ 600]
#AJk-414          ---------- ----AENANL LADTANKEVE RIAKSSKAAQ IANKVSSKAA GKLEVATKAE DEAAKALDEA ENSESKIAEV REKATTAFNA AEEAKKEK-- ---------- ----------  [ 600]
#Isb-129          ---------- ----AENANL LADAANKEVE RIAKSSNAAQ IANKVSSKAA GKLEVATKAE DEAAKALDET ENSESKIAEV REKATTAFNA AEEAKKEK-- ---------- ----------  [ 600]
#Rwp-T120         ---------- ----AENANL LADTANKEVE RIAKSSKAAQ IANKVSSHAA GKLEVATKAE DEAAKALDET ENSESKIAEV REKATTAFNA AEEAKKEK-- ---------- ----------  [ 600]
#Thai-462         ---------- ----AENANL LADTANKEVE RIAKSSKAAQ IANKLSSKAA GKLEVATKAE DEAAKALDET ENSESKIAEV REKATTAFNA AEEAHKEK-- ---------- ----------  [ 600]
#China-CG12       ---------- ----AENANL LADTANKEVE RIAKSSKAAQ IANKVSSKAA GKLEVATKAE DEAAKALDET ENSESKIAEV REKATTAFNA AEEAKKEK-- ---------- ----------  [ 600]
#China-CG8        ---------- ----AENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#AY454084-Bangl   ---------- ----AENAKL LAETANKEVQ NIQKSSKAAQ IANNVSAKAS EKSKVATQAA DEAAKALEEV SKELEKAENP ESKIAEVKDK ATKAFDAAEV AK--------- ----------  [ 600]
#AY454097-SL57    ---------- ----AENAKL LADTASKAAE NIAKSSKAAK IANNVSTIAA EKSKVATEAA DEAAKALDET ENPESKIAEV TEKATKAVNA AEEAK----- ---------- ----------  [ 600]
#AY454096-SL      ---------- ----AENAKL LADTASKAAE NIAKSSKAAK IANNVSTIAA EKSKVATEAA DEAAKALDET ENPESKIAEV TEKATKAVNA AEEAK----- ---------- ----------  [ 600]
#Thai-106         ---------- ----AENAKL LAETANKEVQ NIQKSSKAAQ IANNVSAKAS EKSKVATQAA DEAAKALEEV SKELEKAENP ESKIAEVKDK TTKAFDAAEV AK--------- ----------  [ 600]
#Thai-NR1         ---------- ----AENAKL LAETANKEVQ NIQKSSKAAQ IANNVSAKAS EKSKVATQAA DEAAKALEEV SKELEKAENP ESKIAEVKDK ATKAFDAAEV AK--------- ----------  [ 600]
#Thai-T107        ---------- ----AENAKL LAETANKEVQ NIQKSSKAAQ IANNVSAKAS EKSKVATQAA DEAAKALEEV SKELEKAENP ESKIAEVKDK ATKAFDAAEV AK--------- ----------  [ 600]
#Thai-113         ---------- ----AENAKL LAETANKEVQ NIQKSSKAAQ IANNVSAKAS EKSKVATQAA DEAAKALEEV SKELEKAENP ESKIAEVKDK ATKAFDAAEV AK--------- ----------  [ 600]
#Thai-430         ---------- ----AENAKL LAETANKEVQ NIQKSSKAAQ IAKNVSAKAE EKSKVATQAA DEAANALNEA ENPESKIDDV KKKATEAVNA AEEAK----- ---------- ----------  [ 600]
#China-CG11       ---------- ----AENAKL LAETANKEVQ NIQKSSKAAQ IANNVSAKAS EKSKVATQAA DEAAKALEEV SKELEKAENP ESKIAEVKDK ATKAFDAAEV AK--------- ----------  [ 600]
#AY454083-Chess   IAAEVAKAGK AKIKAENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-TC103       IAAEVAKAEK AKIKAENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-TV400       IAAEVAKAGK AKIKAENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#AY454089-Br781T  IAAEVAKAEK AKIKAENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#PVNG01493-NK     IAAEVAKAEK AKIKAENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Ajk-411          ---------- ----AENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Isb-TF83         IAAEVAKAEK AKIKAENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Rwp-109          IAAEVAKAEK AKIKAENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Ajk-410          IAAEVAKAEK AKIKAENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#AY454098-Thai    IAAEVAKAEK AKIKAENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#AF099662-Belem   ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#AY454080-Br1B    ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#AY454085-Br56    ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-115         ---------- ----AENANL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-TF91        ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-126         ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-442         ---------- ----AENANL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-408         ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#India-IN1        ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-TB123       ---------- ----AENANL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-T139        ---------- ----AENAKL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-TF127       ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-104         ---------- ----AENANF LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-413         ---------- ----AENANL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-431         ---------- ----AENANL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
#Thai-447         ---------- ----AENANL LA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
```

3.9: Analysis and comparasion of conserved and non conserved site across *Pvmsp*3 alpha

Conserved

```
#-Br69  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#-India HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#-EcuT  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Isb-4  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Rwp-1  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Isb-2  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Pak-6  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#China2 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#China8 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#-Bangl HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#-SL57  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#A-SL   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Ajk-10 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Isb-3  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Bra-   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Ind-11 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Thai-4 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#China  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Chess  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Ajk-2  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Rwp-2  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Br781T HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#NK-2   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Ecu-41 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Sri-83 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#CHi-1  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Sri-10 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Thai   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Sal1-  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Br1B   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Br56   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#China  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Thil   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Iran-6 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#India2 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Bra-08 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#India1 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#kor23  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#chin   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Thai12 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Iran   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Ajk-3  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Sal1   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Sal1-  HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Kor-   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Ajk5   HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Sr-421 HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
#Papau- HGEKAAEKVGE STKAHSDEAQ QENKNAKDAS EEAENRAVDA LEEAYAVEAH LARTKNAAES AKSATDMSEL EKAKEEAIDA ANIAHQKWLK ATQAATIAKE KKEAAKVAAE KAQTAANVVKD    [670]
```

Semi-Conserved

```
#Br69    AK-------D EAIKETGKPK SQ-------- --NTTKAVTM ATEEEKKTKD EAQTASEKAG KTAEEAQKEV GKETADDDKE VSQLEEEIKE LERILKIVKD LASEASSASD NAKKAKLKTQ  [ 360]
#Br1T    AK-------E VAIKETGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTVKE LASNAEDASK NAKKEMAKAQ  [ 360]
#Br69    AK-------E ETIKETGTSE SE-------- --IVTKAVAT ATAEEEKTQK EAQTASEKAD KAVEETQKEV DKGIEDESKE TSDLE----- --DILKSVKE LASSAEDASK NAKKEMTKAQ  [ 360]
#India   AK-------E ETIKETGTSE SE-------- --IVTKAVAT ATAEEEKTQK EAQTASEKAD KAVEETQKEV DKGIEDESKE TSDLE----- --DILKSVKE LASSAEDASK NAKKEMAKAQ  [ 360]
#EcuT    AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKGAQEEV EKEIKDESKE KLELE----- --GILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Rwp-4   AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKGAQEEV EKEIKDESKE KLELE----- --GILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Ajk-1   AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKGAQEEV EKEIKDESKE KLELE----- --GILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Isl-2   AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAQTAVKMQI SSKRSSREEV EKEIKDESKE KLELE----- --GILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Rwp-2   AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKGAQEEV EKEIKDESKE KLELE----- --GILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#China2  AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKGAQEEV EKEIKDESKE KLELE----- --GILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#China8  AK-------E EAIKETDKPK LQ-------- --NATKAVTM TTEEEEKTKK EAKTASEKAG NAAEEAQKEE KKGIADEDKE ISDLEIEIKE LEGILNKVKD LASNAEVASK KAKKANLKTQ  [ 360]
#Bangl   AK-------D EAIKETGTSK SE-------- --NATKAVAM ATEEEEKTKN QANIASENAD NAAKEAQKEV EKEITDEDKE ITELG----- --DILKKVEV LASDASSASE EAKKANLKIQ  [ 360]
#SL57    AK-------E ETIKETGTSE SE-------- --IVTKAVAT ATAEEEKTQK EAQTASEKAD KAVEETQKEV DKGIEDESKE TSDLE----- --DILKSVKE LASSAEDASK NAKKEMAKAQ  [ 360]
#SL6     AK-------E ETIKETGTSE SE-------- --IVTKAVAT ATAEEEKTQK EAQTASEKAD KAVEETQKEV DKGIEDESKE TSDLE----- --DILKSVKE LASSAEDASK NAKKEMAKAQ  [ 360]
#Rwp1    AK-------E EAIKETDKPK LQ-------- --NETKAVAM VKKEEEETKK EAKTASEKAG NAAEKAQKEV KKGTADEGKR ISDLENEISE LEDILKEVGA LASQASSASE KAKTAKLKTQ  [ 360]
#Bra-N   AK-------D EAIKETGTSK SE-------- --NATKAVAM ATEEEEKTKN QANIASENAD NAAKEAQKEV EKEITDEDKE ITELG----- --DILKKVEV LASDASSASE EAKKANLKIQ  [ 360]
#Viet-T  AK-------E EAIKETDKPK LQ-------- --NETKAVAM VKKEEEETKK EAKTASEKAG NAAEKAQKEV KKGTADEDKK ISDLENEISE LEDILKEVGA LASQASSASE KAKTAKLKTQ  [ 360]
#Ind3    AK-------E EAIKETDKPK LQ-------- --NETKAVAM VKKEEEETKK EAKTASEKAG NAAEKTQKEV KKGTADEDKK ISDLENEISE LEDILKEVGA LASQASSASE KAKTAKLKTQ  [ 360]
#Iran-   AE-------D EAIKETGTSK SE-------- --NATKAVAM ATEEEEKTKN QAIIASENAD NAAKEAQKEV EKEITDEDKE ITELG----- --DILKKVEV LASDASSASE EAKKANLKIQ  [ 360]
#China-  AK-------E EAIKETDKPK LQ-------- --NETKAVAM VKKEEEETKK EAKTASEKAG NAAQKAQKEV KKGTADEDKK ISDLENEISE LEDILKEVGA LASQASSASE KAKTAKLKTQ  [ 360]
#Chess   AK-------E VALKETDTSK SQ-------- --YATKAVDM ATREEGKTKK EAQTASEKAD EAAKEAQKEV EKEIKDEDKD ISVLQNEITE LEGILETVKK LTSKASSALE EAKKAKLKTQ  [ 360]
#Kor-T   AK-------E VALKETGTSK SQ-------- --YATKAVDM ATKEEGKTKN EAQTASENAD KAAKAAQEEV EKEIKDENKE ILELDNEITK LGGILSTVKP LVSEALSASE KAKKANLKTQ  [ 360]
#Thai-T  AK-------E VALKETDTSK SQ-------- --YATKAVDM ATREEGKTKK EAQTASEKAD EAAKEAQKEV EKEIKDEDKD ISVLQNEITE LEGILETVKK LTSKASSALE EAKKAKLKTQ  [ 360]
#Iran    AK-------D EAIKETGKPK SQ-------- --NTTKAVTM ATEEEKKTKD EAQTASEKAG KTAEEAQKEV GKETADDDKE VSQLEEEIKE LERILKIVKD LASEASSASD NAKKAKLKTQ  [ 360]
#China   AK-------D EAIKETGKPK SQ-------- --NTTKAVTM ATEEEKKTKD EAQTASEKAG KTAEEAQKEV GKETADDDKE VSQLEEEIKE LERILKIVKD LASEASSASD NAKKAKLKTQ  [ 360]
#Ind-    AK-------E EAIKETDKPK LQ-------- --NATKAVTM TTEEGEKTKK EAKTASEKAG NAAEEAQKEE KKGIADEDKE ISDLEIEIKE LEGILNKVKD LASNAEVASK KAKKANLKTQ  [ 360]
#Ecua    AK-------D EAIKETGKPK SQ-------- --NTTKAVTM ATEEEKKTKD EAQTASEKAG KTAEEAQKEV GKETADDDKE VSQLEEEIKE LERILKIVKD LASEASSASD NAKKAKLKTQ  [ 360]
#Thai    AK-------D EAIKETGKPK SQ-------- --NTTKAVTM ATEEEKKTKD EAQTAGEKAG KTAEEAQKEV GKETADDDKE VSHLEEEIKE LERILKIVKD LASEASSASD NAKKAKLKTQ  [ 360]
#Thai    AK-------D EAIKETGKPK SQ-------- --NTTKAVTM ATEEEKKTKD EAQTASEKAG KTAEEAQKEV GKETADDDKE VSQLEEEIKE LERILKIVKD LASEASSASD NAKKAKLKTQ  [ 360]
#Sri     AK-------D EAIKETGKPK SQ-------- --NTTKAVTM ATEEEKKTKD EAQTASEKAG KTAEEAQKEV GKETADDDKE VSQLEEEIKE LERILKIVKD LASEASSASD NAKKAKLKTQ  [ 360]
#India   AK-------E VAIKETGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTVKE LASNAEDASK NAKKEMVKAQ  [ 360]
#China   AK-------E VAIKETGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTVKE LASNAEDASK NAKKEMVKAQ  [ 360]
#Br56    AK-------E VAIKETGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENKE ISQLENEIKE LEGILNKVKD LASNAEVASK KAKKANLKAQ  [ 360]
#Kor-11  AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTVKE LASNAEDASK KAQKAKLKAL  [ 360]
#Sal1-T  AK-------E VAIKETGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTVKE LASNAEDASK NAKKEMVKAQ  [ 360]
#Pak-12  AK-------E VAIKEAGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENEE ISQLEYEITK LGDILNTLKT LASNAEDASR KAQKAKLKAQ  [ 360]
#Papua-4 AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EGKTASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTVKE LASNAEDASK KAQKAKLKAQ  [ 360]
#Braz-40 AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTVKE LASNAEDASK NAKKEMVKAQ  [ 360]
#India-  AK-------E VAIKETGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTVKE LASNAEDASK NAKKEMVKAQ  [ 360]
#Kor     AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKGAQEEV EKEIKDESKE KLELEG---- ---ILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Iran    AK-------E VAIKETGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAG NAAEEAQKEE KKGIADEDKE ISDLEIEIKE LEGILNKVKD LASNAEVASK KAKKANLKTQ  [ 360]
#Saril   AK-------E VAIKETGTSK SE-------- --NVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#China-  AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKGAQEEV EKEIKDESKE KLELEG---- ---ILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Africa  AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASGNAD KAAKGAQEVV EKEIKDESKE KLELEG---- ---ILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Sri-    AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAAKGAQEEV EKEFKDESKE KLELEG---- ---ILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Thai-44 AK-------E VAIKETDNLN SQ-------- --NATKAVIT ATEQEEETKK EAKTASENAD KAVKGAQEEV EKEIKDESKE KLELEG---- ---ILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Sri-4   AK-------E VAIKETDKLN SQ-------- --NATKAVTT ATEQEEETKK EAKTASENAD KAVKGAQEEV EKEIKDESKE KLELEG---- ---ILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
#Kor-4   AK-------E VAIKETGTSK SENVTKAIDM AENVTKAIDM AKKEEEETKN QASIASENAD KAAKAAQEEV KKEIKDENKE ISQLENEITK LGDILNTLKT LASNAEDASK KAQKAKLKAQ  [ 360]
```

DISCUSSION

The occurrence and incidence of malaria can be reduced as a result of active and passive case detection (Zoghi *et al*., 2012). Definite and exact estimation of *Plasmodium* infection can be helpful in arising malaria control interventions and surveillance in the Twin cities and AJK. So, in the present study to attain and keep up the malaria elimination operation in the Twin cities and AJK. In AJK the occurrence of malarial parasites was determined for the first time, by using microscopy and nested-PCR methods.

All the samples were collected from subjects presenting with symptoms in all seasons at each locality. Abrupt rains and immediate weather fluctuations can alter the peaks of *P. falciparum* and *P. vivax.* Regional variations in peak times also occurred. These effects must be taken into account during the evaluation of minor variances in the specified incidence of malaria and of the ratio in cases of *P. falciparum* and *P. vivax* between areas. In spite of these limitations, the records existing here add to complete and recent description of malaria in the Twin cities and AJK.

In the present study the prevalence of *Plasmodium* in Rawalpindi is (n=372) 74.4%, in Islamabad is (n=384) 76.8% and in AJK is (n=311) 62.2%. In twin cities the *P. vivax* and *P. falciparum* are predominant species as it was confirmed by other studies (Khattak *et al*., 2013). Both these species are also responsible for malaria in AJK. The prevalence of *Plasmodium* in the twin cities is highest while in the AJK it is lowest.

In this study, 49.4% were *P.vivax*, 26.6% were *P. falciparum*, 45.6% were *P.vivax*, 28.4% were *P. falciparum*, 41% were *P.vivax*, and 20.6% were *P. falciparum* in Islamabad, Rawalpindi and AJK respectively. Yasinzai and Kakarsulemankhel in 2008 reported the incidence of malaria parasites and found high rates of *P. vivax* and low rates of *P. falciparum* in the Multan district, while studying malarial parasites in Kashmiri refugees settled in Muzaffarabad found higher incidence of *P. vivax* than of *P. falciparum*. Mixed infections (*P. vivax* and *P. falciparum)* detected in the current study were 1% in Kashmir, 3.8% in Islamabad and 1.6% in Rawalpindi. Similar results were of mixed infections were also observed in Multan district.

Infection of *P. malariae* and *P. ovale* were not noticed in any one sample and these results support the study conducted in Multan and Abbottabad (Yasinzai & Kakarsulemankhel, 2008). High transmissibility of *P. vivax* cause an increase in *vivax* malaria. It is correlated to its representative biological features. These features include quick gametocyte production, short sporogony and presence of the dormant hypnozoite stage (Abdullah *et al.,* 2013) .

The transmission outline of malarial parasite varies according to different endemic areas. The temperature of the twin cities is suitable for mosquito's growth. AJK shows minimum growth due to unfavorable temperature. Environmental fluctuations results in change of appropriate vector habitats. The extensive agricultural practices, comprehensive irrigation network, and prolonged monsoon rain act together to support a favorable environment for the broadcast and development of these vectors. The malarial infection in twin cities had a distinguishing summer monsoon seasonal peak. The transmission also occurs throughout the year. Inter annual climatic changes and variability have been noted as a vital factor of epidemics and climate predictions can be used as early warning of changes of risk in epidemic prone parts of the country.

Microscopy has historically been the backbone of the diagnosis of malaria. A clinical diagnosis of malaria currently depends on the visualization of parasites by light microscopy of giemsa-stained thick and thin blood smears. This procedure is simple and cheap, but it requires well-trained personnel and is a labour intensive procedure. Many studies have demonstrated greater sensitivity and specificity of PCR compared to thick blood films. Undetectable low levels of *P. vivax* and *P. falciparum* parasitemia can be detected by PCR (Kazmi & Pandit, 2001; Manning *et al*., 2011; Rowland & Nosten, 2001). The present study between microscopy and nested PCR showed that the results obtained by PCR were not equivalent to those obtained by microscopy, in that all microscopy-positive samples were not positive by PCR. In addition, the PCR test was able to detect mixed infections that were missed by microscopy. This may be due to the tendency of one specie to be dominant over other species. It is important to diagnose malaria accurately and to treat it correctly. Assessment of the eminence of microscopy in the twin cities and AJK might aid in recognizing areas for better identification and treatment.

Due to misdiagnosis malaria is not considered as a serious disease which cause main reason for transmission. Misdiagnosis of mixed species infections result in inappropriate or incomplete treatment, especially in the Twin cities and AJK. In the current survey, we have emphasized that microscopic examination of blood smears depend on experienced and skillful examiners. Therefore, it is important to standardize and improve diagnostic techniques. So that species-specific parasitemia can be determined in as more specialized method. It will make easy to calculate the ratio of mixed *Plasmodium* species infection and the observing of patients who are receiving antimalarial cure.

When parasitemia is very low than (during the detection of mixed species infections) the information's achieved by microscopy is limited. There is a lack of ability to dedicate the compulsory time to the inspection of blood smears. A missed diagnosis of *P. vivax* co-existing with *P. falciparum* is problematic since these species might cause relapses, thereby morbidity (Tangpukdee *et al*., 2009). Because of negative microscopic diagnosis untreated patients may be carriers of malarial parasites in these particular areas.

By enlightening the behavioral patterns, my study recommended that almost 75% of malaria patients in AJK and 25% in Pakistan did not strive for any treatment and therapy. Mostly self-treatment is observed. A large proportion of people consulted unqualified dispensers and doctors of the village. In AJK, drug sellers have been linked to the choice of treatment and therapy. Various studies also clarified this in Pakistan.

Low socioeconomic status and lack of education and awareness was closely related to insufficient knowledge of the local population on malaria transmission, prevention and treatment. Several risk factors for malaria have been proposed, including a continuous influx of refugees, unfortunate hygienic condition, poor drainage system, lack of education and use of presumptive treatments.

In the current studies of Twin cities number of male exposure to malaria are increasing because they are working outside. They are not covered well. It increases bites of infected Anopheles. Females are well covered or they may have reduced access to health precautions amenities. Females may not strive for medical care as regularly as males. Khatoon *et al* (2013) also supports these results. In the case of AJK, females are more

affected than males and these results do not support results of Khatoon *et al* (2013) because females struggle more for surviving as compared to women of twin cities.

In the current survey, one reason of malaria prevalence is antimalarial drug resistance. In Pakistan, chloroquine resistance for the first time was reported in the Punjab province in 1984 (Khatoon *et al*., 2009). Mefloquine resistance has also been recorded between 1996 and 1999 (Organization *et al*., 2009). Soon after, in 2000 to 2006 disappointment rates have been found for the combined therapy of sulphadoxine-pyrimethamine and chloroquine. Finally, the chloroquine primaquine combination has been in use since 2007. A recent study of 2013 observed a considerable failure rate in patients receiving this regimen (Raza *et al*., 2013). In the present study, patients with suspected *P. falciparum* of 78% received chloroquine even though it is ineffective for this specie. About 17% malarial patients were not treated with any antimalarial drug.

Insecticide treated bed nets propose with an attractive solution. Users attain some fortification. In the current study, it is observed that ITN proved 60% protection against *P. falciparum* and 40% against *P. vivax* and the same results were proved by Kakar (2010). In recent years a major ratio of the refugees are motivated to buy nets through social advertising schemes. Nets are effective, but peoples are generally unwilling for nets paying. Mostly people are poor and they cannot buy bed nets, especially in the villages of AJK.

Mutation is the main cause of novel alleles. Genetic diversity in the normal population is generated by the introduction of novel alleles. Furthermore chemotherapy performs vital roles in selection. It has an impact on the frequency of novel alleles in parasite residents. The investigation of parasitic response to humanoid interventions like vaccines or drugs. Fixation of advantageous alleles will favor directional selection in the population, and thus reduction in genetic diversity.

In spite of *Plasmodium* importance, there is a lack of records of two malarial parasite species on their genetic diversity circulating in AJK. There is no study analysis of the diversity of *P. vivax* in AJK. There is a need for comprehensive analyzing of the diversity of *P. vivax* in AJK.

In the case of *P. vivax* molecular markers are used for epidemiological studies such as *msp*3 gene. In the current study, we use *Pvmsp*3α and *Pvmsp*3β polymorphism of *P.*

vivax for identification of new variants and to perceive the epidemiology in the Twin cities and AJK. In spite of the malaria control program, it still remains a great challenge. Keeping in view the results of the present investigation, Directorate of Malaria Control Program, should effectively arrange a malaria control program in the Twin cities and AJK**.** A combined struggle in this respect is to be organized by Health Department, Irrigation Department and Local Government eliminate the optimistic epidemiological factors, which support the malaria spread, so as to make sure the public health of the populations of mentioned areas.

The polymorphic markers of malarial parasites, particularly encoding surface antigens that play an important role in the drug and vaccine development. For the analysis of these polymorphic markers PCR/RFLP is the most common and useful technique (Rice *et al*., 2013). In addition, these markers also play a basic role in differentiating repetition, re-infections and relapse (Rungsihirunrat *et al*., 2011). *Pvcsp*, *Pvmsp*1, *Pvmsp*3α and *Pvmsp*3β and other several polymorphic markers are extensively used, using the PCR⁄RFLP techniques for the genetic studies of recurring *P. vivax* malaria and parasitic genotyping (Kim *et al*., 2006).

The *Pvmsp*3α and *Pvmsp*3β gene has been widely used as a molecular marker for epidemiologic and genetic studies of *P. vivax* malaria in the epidemic and pan-endemic countries. In assessing molecular diversity and dynamics of the malarial parasite in Papua New Guinea, India, Pakistan and Thailand various researchers have identified more than 20 alleles of the *msp*-3α gene in *P. vivax* based on PCR-RFLP analysis using two restriction enzymes Hha1 and Alu1. In the current study, we identified at least 14 alleles based on Alu I digestion, which is in consistent with the findings of Bruce (1977) in the Papua New Guinea and Lubna (2013) in Pakistan (Kim *et al*., 2006 ; Raza *et al*., 2013 ; Rungsihirunrat *et al*., 2011). This study aims that the population of *P.vivax* in Pakistan and Kashmir are equally diverse despite of low to intermediate prevalence of the disease. The high genetic diversity of the *P. vivax* parasite can be explained by different factors, including inherent biological characteristics of the *P. vivax* malaria, such as deterioration and early gametocytemia, the meiotic recombination and cross-fertilization of divergent parasite genotypes within vectors species. Second, the migration of parasite-carriers is also the

leading cause of the genetic diversity of the parasite. Third is the drug resistance that affects the period of infection and develop the diversity of the gene pool in mosquitoes. The findings of researchers from Pakistan discussed that the association between the genetic makeup and transmission intensity of the malarial parasites seems to be nonlinear. The findings of our current work further sustenance the same argument. Sequence analysis of *Pvmsp*-3α from different isolates identified the regions of deletions of different variants, but the main are B and C. Both B and C variants shows deletions on the central alanine rich domain of N-terminus. In other malarial region both variant types were found in isolates. Survival of a parasite does not depend on these deletions. Reduce fitness is due to the presence of these two variants in parasite genotypes are less than 30% (Gonzalez-Ceron *et al*., 2013). In the result of the comparison between type C and type B deletion of 26 amino acids upstream are found. Most important portion is this and >6% of parasitic genotype fit for this type. To cope the malaria infection, this most polymorphic region must be selected by the host immune systems (Rungsihirunrat *et al*., 2011).

The detail phylogenetic comparison of geographically separated clones were not possible because *Pvmsp*-3α gene sequences are available in limited number. Therefore limited number of countries with multiple sequences of *Pvmsp*3α were selected. Pakistani isolates are more indefinitely related to the clones from Brazil and China, recommend that certain degrees of geographic isolation exist. This primary analysis will aid as the basis for future detailed studies of population structure of *P. vivax* in different geographic regions. The current malaria parasite diversity has been molded by numerous discerning forces such as malaria control programs, drug resistance and travelers etc. Equally the malaria control and eradication development progresses, it is anticipated that the level of malaria endemicity will shrink, which should directly affect the effective population size (Zakeri *et al*., 2011).

Various discerning forces have been used to affect the current diversity of malaria parasite gene pool, for example traveler, drug resistance and malaria eradications Programs. Equally the malaria eradication development progresses and control, it is predictable that malaria endemicity level will therapist. It would straightly affect the population effective size.

In numerous states of GMS (Greater Mekong Subregion) of the Southeast Asia, malarial parasite is forced to the regions of international border and more or less survives in isolated pockets, flow of more confining gene between populations of parasites (Manning *et al*., 2011). In the present study, we inspected the genetic diversity and *Pvmsp*3β gene evolution in parasites from Islamabad and Rawalpindi of Pakistan and Azad Jammu and Kashmir. We have more inveterate that *Pvmsp*3β sequencing deals significantly higher power for defining the genetic diversity of parasite linked with the PCR-RFLP simple method (Ghanchi *et al*., 2010).

A coiled-coil tertiary structure of protein is vital for its attachment on the merozoite surface and this structure was suggested by the structural and expressional feature found in the sequence of *msp*3β (Rungsihirunrat *et al*., 2011).

Pvmsp-3ß is anticipated to be associated with further MS molecules, may be via protein-protein interactions by its coiled-coil structure in the same manner as *msp*3 of *P. falciparum*. These super coiled-coil configurations were finely conserved in total *P. vivax* isolates from Rawalpindi, Islamabad and Azad Jammu Kashmir. The central coiled-coil domains of *Pvmsp*3β were same as that of *msp*3α and more diverse than the C and N-terminal sites and that are comparatively conserved. These findings of our current study are reliable with those of earlier investigations, so that in the *Pvmsp*-3ß overall tertiary assembly is conserved in isolates of *P. vivax* regardless of the sequence difference between these isolates. Nucleotide and protein sequence examines were accomplished to inspect the genetic differentiation and diversity on *Pvmsp*3ß between isolates from Pakistan, Kashmir and the whole world.

The normal numeral of pair-wise nucleotide differences (K) of *Pvmsp*-3ß aimed at the N-terminal domain was built on the incidence in the sequence of minor indels and the degenerate repeated occurrence of one or the other indels flank. There is a possible method anticipated for producing multiplicity of *Pvmsp*-3ß named as DNA polymerase slippage (Ezebialu *et al*., 2012). In the same way, isolates of *P. vivax* in Korea with minor flanking and indels degenerate repeats recognized in sequences of *Pvmsp*-3ß. Which proposed that, for *Pvmsp*-3ß diversity glided-strand mispairing contributed during DNA replication (Rawasia *et al*., 2012).

In the big -scale insertion, eleven amino acid-length of frequent elements were recognized. They were also conserved in the N-terminal domain end and *Pvmsp*-3ß end of insert A of Asian *P. vivax* isolates as well as *Pvmsp*-3α and is sustained by balancing selection (Contreras *et al*., 2002). Intragenic recombination events are known to generate *Plasmodium* antigenic diversity. In the same way, allelic diversity of *Pvmsp*-3ß is also caused by another factor known as recombination. Levels of endemicity and force of transmission are the factors, which affects genetic diversity of malarial parasite. The force of transmission and endemicity levels are closely linked to the genomic variations of malarial parasite (Kolaczinski *et al*., 2005).

The *P. vivax* populace in hyperendemic regions shows high diversity and multi-infections e.g. Papua New Guine. However, in hypo-endemic areas, complicated genomic configurations have been recognized in population of *P. vivax* e.g. China, Thailand and Salvador. Several current studies have recommended that in the present year, high genetic diversity in the *P. vivax* populace is found. As low transmission intensity and endemicity in Pakistan and neighboring countries, it leftovers vague that in present year in what way genomic variation of *P. vivax* in Pakistan is quickly distributed and sustained. Allelic variation increasing in Pakistan might be higher by an increase entry refugees and international travelers. From other endemic areas, *P. vivax* population starts increasing (Kazmi & Pandit, 2001; Manning *et al*., 2011).

Furthermore, in the case of Pakistan, *P. vivax* population has developed underneath the pressure of evolution and it possibly associated with the immune response of the host. More analysis of genomic variations in the Kashmir, population of *P. vivax* reveals that they have a close similarity to the Indian isolates. The main reason of the changing diversity pattern is due to continue the influx of foreigners and tourist (Hewitt *et al*., 1994; Rowland & Nosten, 2001).

Major task for malaria vaccine development is greater genetic diversity of vaccine candidates. Then operative immunity in contradiction of these vaccine entrants is frequently strained surpassing. Antibodies for 1 strain cannot persist operative in contrast to other (Rwland & Nosten, 2001; Rice *et al*., 2013).

In the distribution region, it needs to be well-thought genetic diversity of vaccine candidates. Highly polymorphic and conserved domains are found in numerous merozoite surface proteins. They represent the effects of functional constraints and varying selection. This profound investigation shows that *Pvmsp*3α and *Pvmsp*3β proteins resembles the domain configuration through N terminal sites exposed going on merozoite surface. They also subject in the direction of resilient balancing selection via host immunity. This has possible suggestions designed for permitting *Pvmsp*3α and *Pvmsp*3β vaccine possibilities. The antibodies contrary to these dominions are frequently having allele specificity.

The high genetic diversity is found in the N-terminal region, which argues for attention against the development of vaccines. The favorable region for vaccine development is conserved C terminal region. Asia is an endemic area and it has advanced antibodies against *Pvmsp*3α. Immunogenicity of both proteins is indicated by *Pvmsp*3β. On those antibodies considerably more people are identifying portion of the C-terminal of the protein *Pvmsp*3β. Subsequently, in a parasite strain, about 12 paralogs are found in the *Pvmsp*3 gene. Possibly their roles are redundant. For conserved members, an assessment of vaccine would be needed.

REFERENCES

Abdullah, N. R., Barber, B. E., William, T., Norahmad, N. A., Satsu, U. R. & Muniandy, P. K. (2013). *Plasmodium vivax* Population Structure and Transmission Dynamics in Sabah Malaysia. *Public Library of Science*, 8(12): 1-10.

Alemu, A., Fuehrer, H. P., Getnet, G., Kassu, A., Getie, S. & Noedl, H. (2014). Comparison of Giemsa microscopy with nested PCR for the diagnosis of malaria in North Gondar, North West Ethiopia. *Malaria Journal*, 13(1): 174.

Anstey, N. M., Russell, B., Yeo, T. W. & Price, R. N. (2009). The pathophysiology of *vivax* malaria. *Trends in Parasitology*, 25(5): 220-227.

Aponte, J. J., Menendez, C., Schellenberg, D., Kahigwa, E., Mshinda, H., Vountasou, P. & Alonso, P. L. (2007). Age interactions in the development of naturally acquired immunity to *Plasmodium falciparum* and its clinical presentation. *Public Libarary of Science medicine*, 4(7): 242.

Bailey, J. W., Williams, J., Bain, B. J., Parker-Williams, J. & Chiodini, P. L. (2013). Guideline: The Laboratory diagnosis of malaria. *British Journal of Haematology*, 163(5): 573-580.

Bannister, L. H. & Sherman, I. W. (2009). *Plasmodium*. *Encyclopedia of Life Sciences Journal* 1(1): 10-12.

Batchelor, J. D., Malpede, B. M., Omattage, N. S., DeKoster, G. T., Henzler-Wildman, K.A. & Tolia, N. H**.** (2014). Red blood cell invasion by *Plasmodium vivax*: structural basis for DBP engagement of DARC. *Public Library of Science Pathogens*, 10(1): 1-14.

Batista-dos-Santos, S., Raiol, M., Santos, S., Cunha, M. G. & Ribeiro-dos-Santos, A. (2012). Real-time PCR diagnosis of *Plasmodium vivax* among blood donors. *Malaria Journal*, 11(1): 345.

Binello, N., Brunetti, E., Cattaneo, F., Lissandrin, R. & Malfitano, A. (2014). Oligohydramnios in a pregnant Pakistani woman with *Plasmodium vivax* malaria. *Malaria Journal*, 13(1): 1-4.

Bozdech, Z., Mok, S., Hu, G., Imwong, M., Jaidee, A. & Russell, B. (2008). The transcriptome of *Plasmodium vivax* reveals divergence and diversity of transcriptional regulation in malaria parasites. *Proceedings of the National Academy of Sciences*, 105(42): 16290-16295.

Bruce, M. C., Galinski, M. R., Barnwell, J. W., Snounou, G. & Day, K. P. (1999). Polymorphism at the merozoite surface protein-3alpha locus of *Plasmodium vivax*: global and local diversity. *The American Journal of Tropical Medicine and Hygiene*, 61(4): 518-25.

Carneiro, I., Roca-Feltrer, A., Griffin, J. T., Smith, L., Tanner, M., Schellenberg, J. A. & Schellenberg, D. (2010). Age-patterns of malaria vary with severity, transmission intensity and seasonality in sub-Saharan Africa: a systematic review and pooled analysis. *Public Library of Science*, 5(2): 1-10.

Chuquiyauri, R., Penataro, P., Brouwer, K. C., Fasabi, M., Calderon, M., Torres, S. & Vinetz, J. M. (2013). Microgeographical differences of *Plasmodium vivax* relapse and reinfection in the Peruvian Amazon. *The American Journal of Tropical Medicine and Hygiene*, 13: 60.

Cornejo, O. E. & Escalante, A. A. (2006). The origin and age of *Plasmodium vivax*. *Trends in Parasitology*, 22(12): 558-563.

Contreras, C. E., Cortese, J. F., Caraballo, A. & Plowe, C. V. (2002). Genetics of drug resistant *Plasmodium falciparum* malaria in the Venezuelan state of Bolivar. *The American journal of tropical medicine and hygiene*, 67(4): 400-405.

Cox, F. (2010). History of the discovery of the malaria parasites and their vectors. *Parasites and Vectors*, 3(1): 5.

Cui, L., Escalante, A. A., Imwong, M. & Snounou, G. (2003). The genetic diversity of *Plasmodium vivax* populations. *Trends in Parasitology*, 19(5): 220-226.

Dondorp, A. M., Lee, S. J., Faiz, M. A., Mishra, S., Price, R. & Tjitra, E. (2008). The relationship between age and the manifestations of and mortality associated with severe malaria. *Clinical Infectious Diseases*, 47(2): 151-157.

Dzeing-Ella, A., Obiang, P. C. N., Tchoua, R., Planche, T., Mboza, B. & Mbounja, M. (2005). Severe *falciparum* malaria in Gabonese children: clinical and laboratory features. *Malaria Journal*, 4(1): 1.

Ekpuka, B., Okogun, G., Obodo, B., Itua, E., Olagboye, J. & Obhakhan, J. (2014). The Prevalence of Malaria Parasitic Infections in Cord Blood: Association with Some Socio Demographic Profile. *International Journal of Basic, Applied and Innovative Research*, 2(2): 40-45.

Escalante, A. A., Cornejo, O. E., Freeland, D. E., Poe, A. C., Durrego, E., Collins, W. E. & Lal, A. A. (2005). A monkey's tale: the origin of *Plasmodium vivax* as a human malaria parasite. *Proceedings of the National Academy of Sciences of the United States of America.* , 102(6): 1980-1985.

Ezebialu, I. U., Eke, A. C., Ezeagwuna, D. A., Nwachukwu, C. E., Ifediata, F. & Ezebialu, C. U. (2012). Prevalence, pattern, and determinants of placental malaria in a population of southeastern Nigerian parturients. *International Journal of Infectious Diseases*, 16(12): 860-865.

Falk, N., Maire, N., Sama, W., Owusu-Agyei, S., Smith, T., Beck, H. P. & Felger, I. (2006). Comparison of PCR-RFLP and genes can based genotyping for analyzing infection dynamics of *Plasmodium Falciparum*. *The American Journal of Tropical Medicine and Hygiene*, 74(6): 944-950.

Fakruddin, M. & Chowdhury, A. (2012). Pyrosequencing: an alternative to traditional Sanger sequencing. *American Journal of Biochemistry and Biotechnology*, 8(1): 14-20.

Frenkel, S. & Western, J. (1988). Pretext or prophylaxis racial segregation and malarial mosquitos in a British tropical colony: Sierra Leone. *Annals of the Association of American Geographers*, 78(2): 211-228.

Garnham, P. (1988). History of discoveries of malaria parasites and of their life cycles. *Journal of History and Philosophy of the Life Science,* 10: 93-110.

Ghanchi, N. K., Martensson, A., Ursing, J., Jafri, S., Bereczky, S., Hussain, R. & Beg, M. A. (2010). Genetic diversity among *Plasmodium falciparum* field isolates in

Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2. *Malaria journal*, 9(1): 1.

Gomez, D., Chaparro, J., Rubiano, C., Rojas, M. O. & Wasserman, M. (2002). Genetic diversity of *Plasmodium falciparum* field samples from an isolated Colombian village. *The American Journal of Tropical Medicine and Hygiene*, 67(6): 611-616.

Gonzalez-Ceron, L., Mu, J., Santillan, F., Joy, D., Sandoval, M. A., Camas, G. & Su, X. (2013). Molecular and epidemiological characterization of *Plasmodium vivax* recurrent infections in southern Mexico. *Parasites and Vectors*, 6: 109.

Haque, U., Ahmed, S. M., Hossain, S., Huda, M., Hossain, A., Alam, M. S. & Haque, R. (2009). Malaria prevalence in endemic districts of Bangladesh. *Public Libarary of Sciences*, 4(8): 6737.

Hewitt, S., Kamal, M., Muhammad, N. & Rowland, M. (1994). An entomological investigation of the likely impact of cattle ownership on malaria in an Afghan refugee camp in the North West Frontier Province of Pakistan. *Medical and Veterinary Entomology*, 8(2): 160-164.

He, L., Nan, T., Cui, Y., Guo, S., Zhang, W., Zhang, R. & Cui, L. (2014). Development of a colloidal gold-based lateral flow dipstick immunoassay for rapid qualitative and semi-quantitative analysis of artesunate and dihydroartemisinin. *Malaria Journal*, 13(1): 127.

Hsu, E. (2006). Reflections on the "discovery "of the antimalarial Qinghao. *British Journal of Clinical Pharmacology*, 61(6): 666-670.

Kakar, Q., Khan, M. & Bile, K. (2010). Malaria control in Pakistan: new tools at hand but challenging epidemiological realities. *East Mediterr Health Journal*, 16(1): 54-60.

Kazmi, J. H. & Pandit, K. (2001). Disease and dislocation: the impact of refugee movements on the geography of malaria in NWFP, Pakistan. *Social science and medicine*, 52(7): 1043-1055.

Kebede, S., Aseffa, A., Medhin, G., Berhe, N. & Velavan, T. P. (2014). Re-evaluation of microscopy confirmed *Plasmodium falciparum* and *Plasmodium vivax* malaria by nested PCR detection in southern Ethiopia. *Malaria Journal*, 13(1): 48-48.

Khairnar, K., Martin, D., Lau, R., Ralevski, F., Pillai, D. R. (2009). Multiplex real-time quantitative PCR, microscopy and rapid diagnostic immuno-chromatographic tests for the detection of *Plasmodium* spp: performance, limit of detection analysis and quality assurance. *Malaria J*ournal, 8: 284.

Khan, S. N., Khan, A., Khan, S., Ayaz, S., Attaullah, S., Khan, J. & Shah, A. H. (2014). PCR/RFLP-Based Analysis of Genetically Distinct *Plasmodium vivax* Population of Pvmsp-3alpha and Pvmsp-3beta genes in Pakistan. *Malaria Journal*, 13(1): 355.

Khatoon, L., Baliraine, F. N., Bonizzoni, M., Malik, S. A. & Yan, G. (2009). Prevalence of antimalarial drug resistance mutations in *Plasmodium vivax* and *P. falciparum* from a malaria endemic area of Pakistan. *The American Journal of Tropical Medicine and Hygiene*, 81(3): 525-528.

Khatoon, L., Baliraine, F. N., Bonizzoni, M., Malik, S. A. & Yan, G. (2010). Genetic structure of *Plasmodium vivax* and *Plasmodium falciparum* in the Bannu district of Pakistan. *Malaria Journal*, 9(1): 112.

Khatoon, L., Jan, M. I., Khan, I. U., Ullah, F. & Malik, S. A. (2013). Genetic diversity in human malarial parasites of Khyber Agency Pakistan. *Acta Parasitologica*, 58(4): 564-569.

Khattak, A. A., Venkatesan, M., Nadeem, M. F., Satti, H. S., Yaqoob, S., Khatoon, M. S. & Plowe, C. (2013). Prevalence and distribution of human *Plasmodium* infection in Pakistan. *Malaria Journal*, 12(1): 297.

Kim, J. R., Imwong, M., Nandy, A., Chotivanich, K., Nontprasert, A. & Tonomsing, N. (2006). Genetic diversity of *Plasmodium vivax* in Kolkata, India. *Malaria Journal*, 5(1): 71.

Kim, J. Y., Ji, S. Y., Goo, Y. K., Na, B. K., Pyo, H. J., Lee, H. N. (2013). Comparison of rapid diagnostic tests for the detection of *Plasmodium vivax* malaria in South Korea. *Public Library of Sciences*, 8(5): 1-6.

Kim, S. H., Hwang, S. Y., Shin, J. H., Moon, C. S., Kim, D. W. & Kho, W. G. (2009). Molecular genetic characterization of the merozoite surface protein 1 Gene of *Plasmodium vivax* from reemerging Korean isolates. *Clinical and Vaccine Immunology*, 16(5): 733-738.

Kochar, D. K., Das, A., Kochar, S. K., Saxena, V., Sirohi, P., Garg, S. & Gupta, V. (2009). Severe *Plasmodium vivax* malaria: a report on serial cases from Bikaner in northwestern India. *The American Journal of Tropical Medicine and Hygiene*, 80(2): 194-198.

Kolaczinski, J., Graham, K., Fahim, A., Brooker, S. & Rowland, M. (2005). Malaria control in Afghanistan: progress and challenges. *The Lacent Journal*, 365(9469): 1506-1512.

Kumar, P. (2009)**.** Kumar and Clark's Clinical Medicine. *International Edition*, page 286.

Larru, B., Molyneux, E., Ter-Kuile, F. O., Taylor, T., Molyneux, M. & Terlouw, D. J. (2009). Malaria in infants below six months of age: retrospective surveillance of hospital admission records in Blantyre, Malawi. *Malaria Journal*, 8: 310.

Laveran A. (1881). Un nouveau parasite trouvé dans le sang de malades atteints de fièvre palustre. Origine parasitaire des accidents de l'impaludisme. *Bulletins et memoires de la Societe medicale des hopitaux de Paris*, 17:158-164.

Leclerc, M., Durand, P., Gauthier, C., Patot, S., Billotte, N., Menegon, M., Renaud, F. (2004). Meager genetic variability of the human malaria agent *Plasmodium vivax*.*Proceedings of the National Academy of Science of the United States of America*, 101(40): 14455-14460.

Lim, C. S., Tazi, L. & Ayala, F. J. (2005). *Plasmodium vivax*: recent world expansion and genetic identity to *Plasmodium simium*. *Proceedings of the National Academy of Sciences United State of America*, 102(43): 15523-15528.

Liu, W., Li, Y., Learn, G. H., Rudicell, R. S., Robertson, J. D., Keele, B. F. (2010). Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. *Nature*, 467(7314): 420-425.

Mangold, K. A., Manson, R. U., Koay, E. S., Stephens, L., Regner, M., Thomson, R. B. & Kaul, K. L. (2005). Real-time PCR for detection and identification of *Plasmodium* spp. *Journal of Clinical Microbiology*, 43(5): 2435-2440.

Manning, L., Laman, M., Law, I., Bona, C., Aipit, S., Teine, D. & Warrell, J. (2011). Features and prognosis of severe malaria caused by *Plasmodium falciparum*, *Plasmodium vivax* and *mixed Plasmodium* species in Papua New Guinean children. *Public Library of Science*, 6(12): 29203.

Manson P. (1898) Surgeon-Major Ronald Ross's recent investigations on the mosquito-malaria theory. *British Medical Journal*, 1: 1575-77.

Marchiafava, E. and Celli, A. (1885) Nouve richerche sulla infezione malarica. *Archives of Science Medical College*. 9: 311-340.

Medana, I. M. & Turner, G. D. (2006). Human cerebral malaria and the blood-brain barrier. *International Journal for Parasitology*, 36(5): 555-568.

Mitsui, H., Arisue, N., Sakihama, N., Inagaki, Y., Horii, T., Hasegawa, M., Hashimoto, T. (2010). Phylogeny of Asian primate malaria parasites inferred from apicoplast genome encoded genes with special emphasis on the positions of *Plasmodium vivax and P. fragile*. *Gene*, 450(1): 32-38.

Mu, J., Joy, D. A., Duan, J., Huang, Y., Carlton, J., Walker, J. (2005). Host switch leads to emergence of *Plasmodium vivax* malaria in humans. *Molecular Biology and Evolution*, 22(8): 1686-1693.

Okiro, E. A., Al-Taiar, A., Reyburn, H., Idro, R., Berkley, J. A. & Snow, R. W. (2009). Age patterns of severe paediatric malaria and their relationship to *Plasmodium falciparum* transmission intensity. *Malaria Journal*, 8(1): 4.

Organization, W. H., for Research, S. P., Diseases, T. in T., Diseases, W. H. O. D. of C. of N. T., Epidemic, W. H. O. & Alert, P. (2009). *Dengue: guidelines for diagnosis, treatment, prevention and control*. World Health Organization.

Panchal, H. K. & Desai, P. B. (2012). ICT-A rapid, innovative but simple technique for malaria diagnosis. *International Journal of Pharmacy and Life Sciences*, 3(4): 1581-1584.

Perkins, D. J., Were, T., Davenport, G. C., Kempaiah, P., Hittner, J. B. & Ong'echa, J. M. (2011). Severe malarial anemia: innate immunity and pathogenesis. *International Journal of Biological Sciences*, 7(9): 1427.

Phimpraphi, W., Paul, R., Witoonpanich, B., Turbpaiboon, C., Peerapittayamongkol, C., Louicharoen, C., (2008). Heritability of *P. falciparum* and *P. vivax* malaria in a Karen population in Thailand. *Public Library of Science*, 3(12): 3887.

Poespoprodjo, J. R., Fobia, W., Kenangalem, E., Lampah, D. A., Hasanuddin, A., Warikar, N. & Price, R. N. (2009). *Vivax* malaria: a major cause of morbidity in early infancy. *Clinical Infectious Diseases*, 48(12): 1704-1712.

Rasheed, A., Saeed, S. & Khan, S. A. (2009). Clinical and laboratory findings in acute malaria caused by various plasmodium species. *Journal of Pakistan Medical Association*, 59 (4): 220-223.

Rawasia, W. F., Sridaran, S., Patel, J. C., Abdallah, J., Ghanchi, N. K., Barnwell, J. W. & Escalante, A. A. (2012). Genetic backgrounds of the *Plasmodium falciparum* chloroquine resistant transporter (*pfcrt*) alleles in Pakistan. *Infection, Genetics and Evolution*, 12(2): 278-281.

Raza, A. & Beg, M. A. (2013). Population genetics and drug resistance markers: an essential for malaria surveillance in Pakistan. *Journal of the College of Physicians and Surgeons-Pakistan*, 23(12): 878-884.

Raza, A., Ghanchi, N. K., Khan, M. S. & Beg, M. A. (2013). Prevalence of drug resistance associated mutations in *Plasmodium vivax* against sulphadoxine-pyrimethamine in southern Pakistan. *Malaria Journal*, 12(1): 261.

Raza, A., Ghanchi, N. K., Thaver, A. M., Jafri, S. & Beg, M. A. (2013). Genetic diversity of *Plasmodium vivax* clinical isolates from southern Pakistan using pvcsp and pvmsp1 genetic markers. *Malaria Journal*, 12: 16.

Rich, S. M., Leendertz, F. H., Xu, G., LeBreton, M., Djoko, C. F. & Aminake, M. N. (2009). The origin of malignant malaria. *Proceedings of the National Academy of Sciences,* 106(35): 14902-14907.

Rice, B. L., Acosta, M. M., Pacheco, M. A. & Escalante, A. A. (2013). Merozoite surface protein-3 alpha as a genetic marker for epidemiologic studies in *Plasmodium vivax*: a cautionary note. *Malaria Journal*, 12: 288.

Rollinson, D., Hay, S. I., Price, R. & Baird, J. K. (2013). *The Epidemiology of Plasmodium vivax: History, Hiatus and Hubris*, 1st edition.

Ross R. (1923). Memoirs with a Full Account of the Great Malaria Problem and its Solution London: *John Murray*.

Rowland, M. & Nosten, F. (2001). Malaria epidemiology and control in refugee camps and complex emergencies. *Annals of tropical medicine and parasitology*, 95(8): 741-754.

Rungsihirunrat, K., Chaijaroenkul, W., Siripoon, N., Seugorn, A. & Na-Bangchang, K. (2011). Genotyping of polymorphic marker (*MSP3 alpha and MSP3 beta*) genes of *Plasmodium vivax* field isolates from malaria endemic of Thailand. *Tropical Medicine & International Health*, 16(7): 794-801.

Safi, N., Adimi, F., Soebiyanto, R. & Kiang, R. (2010). Toward malaria risk prediction in Afghanistan using remote sensing. *International Archives of the Photogrammetry, Remote Sensing and Spatial Information Science*, 9: 125.

Sahar, S., Akhtar, T., Bilal, H. & Rana, M. (2010). Prevalence of *Plasmodium falciparum,* Malarial Parasite in Muzaffargarh District, Punjab, Pakistan: a Two year Study. *Pakistan Journal of Medical Science*, 64: 64-66.

Salem, M. S. O. A., Ndiaye, M., OuldAbdallahi, M., Lekweiry, K. M., Bogreau, H., Konate, L., (2014). Polymorphism of the merozoite surface protein-1 block 2

region in *Plasmodium falciparum* isolates from Mauritania. *Malaria Journal*, 13(1): 26.

Sanger, F., Nicklen S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceeding of National Academy of Sciences*, 74: 5463-5467.

Sattabongkot, J., Tsuboi, T., Zollner, G. E., Sirichaisinthop, J. & Cui, L. (2004). *Plasmodium vivax* transmission: chances for control *Trends in Parasitology*, 20(4): 192-198.

Seman, N. A., Isa, N. A. M., Li, L. C., Mohamed, Z., Ngah, U. K. & Zamli, K. Z. (2008). Classification Of Malaria Parasite Species Based On Thin Blood Smears Using Multilayer Perceptron Network. *International Journal of the Computer, the Internet and Management*, 16(1): 46-52.

Schellenberg, D., Menendez, C., Kahigwa, E., Font, F., Galindo, C. & Acosta, C. (1999). African children with malaria in an area of intense *Plasmodium falciparum* transmission: features on admission to the hospital and risk factors for death. *The American Journal of Tropical Medicine and Hygiene*, 61(3): 431-438.

Stephens, J. W. W. (1922). A new malaria parasite of man. *Annals of Tropical Medicine and Parasitological Journal*, 16: 383-388.

Tanabe, K., Escalante, A., Sakihama, N., Honda, M., Arisue, N., Horii, T. (2007). Recent independent evolution of msp1 *polymorphism* in *Plasmodium vivax* and related simian malaria parasites. *Molecular and Biochemical Parasitology*, 156(1): 74-79.

Tangpukdee, N., Duangdee, C., Wilairatana, P. & Krudsood, S. (2009). Malaria diagnosis: a brief review. *The Korean Journal of Parasitology*, 47(2): 93-102.

Taviad, P. P., Javdekar, T., Selot, B. A. & Chaudhari, V. P. (2011). Specificity and sensitivity for malaria detection by Rapid Detection Test and Microscopy method. *National Journal of Community Medicine*, 2(3): 440-442.

Tek, F. B., Dempster, A. G. & Kale, I. (2009). Computer vision for microscopy diagnosis of malaria. *Malaria Journal*, 8(1): 153.

Trampuz, A., Jereb, M., Muzlovic, I. & Prabhu, R. M. (2003). Clinical review: Severe malaria. Critical care London, 7(4): 315-323.

Tuteja, R. (2007). Malaria an overview. *Federation of European Biochemical Societies Journal*, 274(18): 4670-4679.

Wickramasinghe, S. & Abdalla, S. (2000). Blood and bone marrow changes in malaria. *Best Practice & Research Clinical Haematology*, 13(2): 277-299.

Yasinzai, M. I. & Kakar, J. K. (2012). Prevalence of human malaria infection in bordering areas of Baluchistan with Sindh province: district Jaffarabad. *Journal of Postgraduate Medical Institute (Peshawar-Pakistan)*, 26(3): 277-282

Yasinzai, M. I. & Kakarsulemankhel, J. K. (2008). Incidence of human malaria infection in desert area of Pakistan: District Kharan. *Journal of Agriculture and Social Sciences*, 4: 39-41.

Yang, Z., Miao, J., Huang, Y., Li, X., Putaporntip, C., Jongwutiwes, S., & Cui, L. (2006). Genetic structures of geographically distinct *Plasmodium vivax* populations assessed by PCR/RFLP analysis of the merozoite surface protein 3beta gene. *Acta Tropica*, 100(3): 05-212.

Zakeri, S., Kakar, Q., Ghasemi, F., Raeisi, A., Butt, W. & Safi, N. (2010). Detection of mixed *Plasmodium falciparum & P. vivax* infections by nested-PCR in Pakistan, Iran & Afghanistan. *Indian Journal of Medical Research*, 2(1): 31-35.

Zakeri, S., Afsharpad, M., Ghasemi, F., Raeisi, A., Kakar, Q., Atta, H. & Djadid, N. D. (2011). *Plasmodium vivax*: Prevalence of mutations associated with sulfadoxine-pyrimethamine resistance in *Plasmodium vivax* clinical isolates from Pakistan. *Experimental parasitology*, 127(1): 167-172.

Zoghi, S., Mehrizi, A. A., Raeisi, A., Haghdoost, A. A., Turki, H., Safari, R. (2012). Survey for asymptomatic malaria cases in low transmission settings of Iran under elimination program. *Malaria Journal*, 11(1): 126.

Zucker, J. R. (1996). Changing patterns of autochthonous malaria transmission in the United States: a review of recent outbreaks. *Emerging Infectious Disease Journal*, 2(1): 37-43.