

Saira Shams¹, Shabir Ahmed², Sultan Ayaz³, E. F. Abd_Allah⁴, Sumaira Shams⁵, Sadaf Niaz⁵, Irum Gul⁶, Naila Gul⁶, Riaz Ullah⁷

¹Department of Chemistry, Kohat University of Science and Technology Kohat; ²Department of Chemistry, Islamia College University, Peshawar KPK Pakistan; ³College of Veterinary Sciences and Animal Husbandry, Abdul Wali Khan University, Mardan KPK Pakistan; ⁴Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Riyadh 11451, Saudi Arabia; ⁵Department of Zoology, Abdul Wali Khan University Mardan KPK Pakistan; ⁶Department of Zoology, Kohat University of Science and Technology, Kohat; ⁷Department of Chemistry, Government College Ara Khel FR Kohat, KPK, Pakistan.

E-mail: afridiriaz@yahoo.com

Abstract

Background: Malaria is a crucial disease that causes huge mortality and morbidity, along with economic losses in term of purchasing medicines in underdeveloped countries such as Pakistan.

Materials and Methods: A total of 150 human blood samples were collected from the Districts of Karak and Kohat Khyber Pakhtunkhwa, in Pakistan, and were examined through microscopy and then all positive samples were analyzed by SDS-PAGE method for the detection of antibodies against *plasmodium*.

Result: The overall prevalence of malaria was found to be 18% (27/150). The prevalence of *plasmodium species* was “21.3% (16/75) and 14.6% (11/75) in the Districts of Karak and Kohat respectively. The prevalence of *plasmodium species* i.e. *P. vivax* and *P. falciparum*, in the Districts of Karak and Kohat, was found to be 14.6% (22/150) and 3.33% (5/150) respectively. Among these, *P. vivax* 10.6% (8/75) and *P. falciparum* 4% (3/75) were noted in the District of Kohat; while *P. vivax* 18.6% (14/75) and *P. falciparum* 2.6% (2/75) were found in the District of Karak Khyber Pakhtunkhwa, Pakistan respectively”. The serum protein of malaria patients were 57KDA and 78KDA bands, which were determined by Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) technique.

Conclusion: The SDS page method is the most accurate diagnostic assay and was recognized as a most reliable method in the diagnosis of antibodies produced against *Plasmodium spp.* The proteins bands of Serum protein of malaria patients may be undertaken for study to develop possible future vaccine.

Key words: Malaria, Serum protein, SDS-PAGE, microscopy and *Plasmodium species*

Introduction

Malaria is a mosquito-borne infectious disease of humans and other animals caused by protists (a type of microorganism) of the genus *Plasmodium*. It initiates with a bite from an infected female *Anopheles* mosquito, which introduces the protists through saliva into the circulatory system. There are about 422 species of *Anopheles* worldwide; however, only about 68 are vectors of the malaria parasites (Service et al., 1993).

The global mortality rate due to malaria is 40% and almost up to 700 million populations of humans get infected yearly. Africa, Middle East, the South Pacific, Eastern Europe, Central South America, Southeast Asia, India, and particularly Pakistan are endemic areas of malaria infection (Snow et al., 2003).

In humans, malaria is caused by *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi* (Mueller et al., 2007; Collins, 2012). The most commonly recognized specie is *P. falciparum* (~75%) followed by *P. vivax* (~20%) (Nadim & Behrens 2012). Typical symptoms of malaria include fever and headache, which in severe cases can progress to coma or death.

Along with microscopy various PCR assays (conventional and real-time PCR techniques) have been developed to identify and diagnose *plasmodium spp* (Farcas et al., 2004). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is the reliable technique to diagnose antibodies against malaria as it is an accurate method to detect and separate the presence of specific protein (Abs of *plasmodium*) from other different proteins.

The present study was planned to identify the different proteins present in patients of malaria infection, which will be helpful in future for the developing of vaccine against malaria causing species of *plasmodium*.

Materials and Methods

Study Areas

Selected study areas were District Karak and District Kohat Khyber Pakhtunkhwa Pakistan.

Blood Samples Collection

A total of 150 human blood samples were collected from different locations of District Karak and Kohat Khyber Pakhtunkhwa, Pakistan during the period February to July 2013. 5ml of venous blood was taken by syringe in a sterilized tube and transported to the molecular parasitology and virology laboratory, Department of Zoology, Kohat University of Science and Technology, Kohat Pakistan, for further processing.

Isolation of *Plasmodium* Species through Microscopic Technique

Plasmodium "Schizonts" (a dividing stage of plasmodium in R.B. C) (Fig 1a and Fig 1b) were isolated from the blood samples and were identified using the method described by researchers (Soulsby 1982; Urquhart et al., 1988).

SDS-PAGE Method

All positive samples were analyzed by SDS-PAGE method for the detection of antibodies against plasmodium.

Preparation of Protein Samples for Electrophoresis

Serum separated from blood samples were diluted by 2x sample buffer (with ratio 1:1). Then 100µl of serum samples was taken in separate eppendorf and 100µl loading dye was added. After mixing, the samples were heated for 3-5 minutes in boiling water bath for denaturation of proteins and after that 30µl from each of these samples were loaded in prepared gel.

Protein Marker for 10% GEL

A pre-stained protein ladder (fermentas) was used for running the gel.

SDS Gel Preparation

10% acrylamide gel was used to differentiate and analyze the proteins. Gel apparatus plates were assembled and sealed by using rubber pads. Wellformer (comb) were then used as template. 10% ammonium per sulphate and then N, N, N', N'-tetramethylethylenediamine (TEMED) was added for rapid polymerization. After mixing, the solution was loaded in between plates of gel apparatus. After pouring gel immediately, water saturated butanol was poured above the gel for smooth surface.

Stacking Gel Preparation

After gel preparation, the butanol was removed from surface with pipette, 4% stacking gel was poured on the resolving gel then comb was inserted. After solidification of gel, comb was removed carefully

Assembling, Loading and Running Gels

The upper and lower buffer compartments were filled with running buffer (electrode buffer). 30 µl sample of denatured protein was loaded in each well. Gels were usually run at a voltage 4.54V/cm and dye was run to the bottom of the gel by keeping apparatus away from overheating.

Staining Protein and De-Staining Gels

0.1% Coomassie Blue dye in 10% glacial acetic acid and 50% methanol was used to stain the protein bands. Acetic acid and methanol was used to de-stain the gel, by following 2 steps: i. 50% methanol and 10% acetic acid for 1-2 hours, ii. Then 7% methanol and 10% acetic acid were used to finish. The image of gel was photographed and saved for permanent record (Fig 2 and Fig 3).

Calculation of Molecular Weights of *Plasmodium* Proteins

Each protein fraction was determined by plotting a stander curve of relative mobility of stander bands of protein verses log₁₀ MW of ladder

To find the accurate molecular weights of protein bands, the log values were transformed into matching antilog figures.

Formula to calculate Relative mobility:

$$\text{"Relative mobility (R f value)"} = \frac{\text{Distance covered by a protein band}}{\text{Total distance of the dye front"}}$$

Statistical Analysis of the Data

"Data was statistically analyzed using the computer software, SPSS 16 and Duncan test, Turkey's and chi square test and ANOVA. P<0.05 was considered significant".



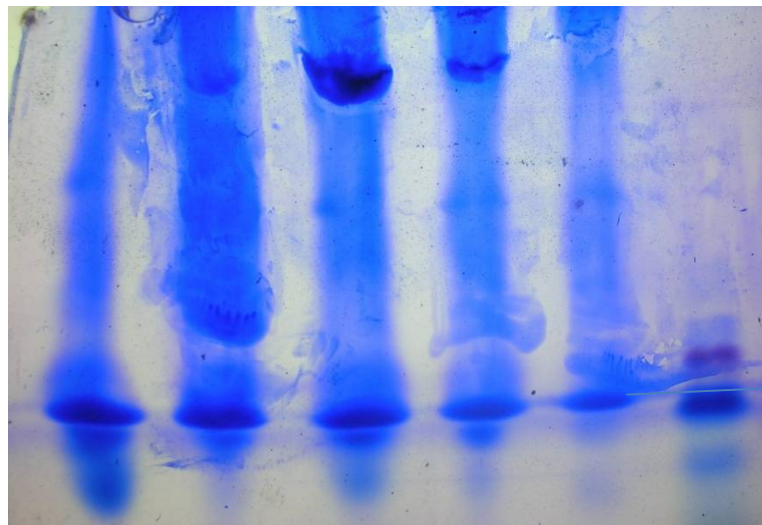
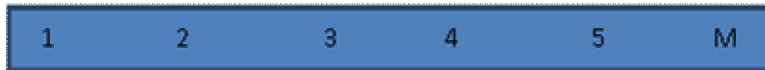
Schizont



Schizont

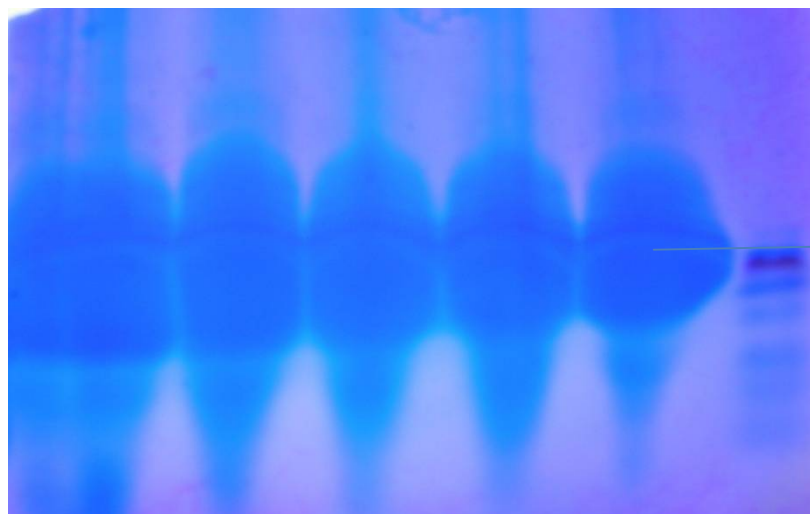
Figure 1a: "Mature Schizont of *P. vivax*"

Figure 1b: "Mature Schizont of *P. falciparum*"



57

Figure 2: "*P. vivax* SDS PAGE electrophoresis of Serum protein extracts of Malaria patients Lane M is the molecular size (KDa) marker while lane 1, 2, 3, 4, and 5 indicates protein bands of 57Kda)"



78

Figure 3: "*P. falciparum*; SDS PAGE electrophoresis of Serum protein extracts of Malaria patients Lane M is the molecular size (KDa) marker while lane 1, 2, 3, 4, and 5 indicates protein bands of 78 Kda)"

Results and Discussion

In human population, the overall prevalence of malaria among two Districts, Karak and Kohat Khyber Pakhtunkhwa, Pakistan was “18% (27/150)”, in Karak “21.3% (16/75)” and in Kohat “14.6% (11/75)” prevalence rates were determined by microscopy during the study (Table 1).

Table 1: “Prevalence of Malaria in Districts Karak and Kohat Khyber Pakhtunkhwa, Pakistan”

Districts	+ive Samples/total	Prevalence (%)
Kohat	11/75	14.6
Karak	16/75	21.3
Grand total	27/150	18

The *plasmodium* species prevalence i.e. *P. vivax* and *P. falciparum* in the population of human is in the Districts of Karak and Kohat, through microscopic technique was found to be “14.6% (22/150) and 3.33% (5/150) respectively” (Fig 4).

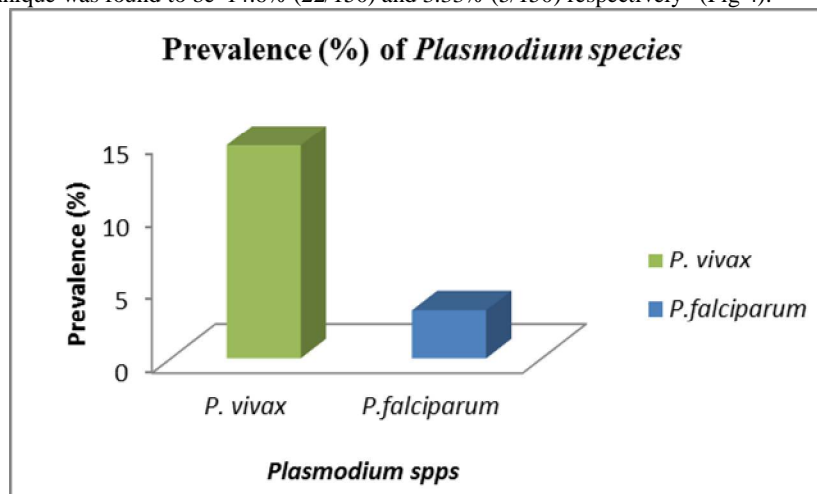


Figure 4: “Species wise Prevalence (%) of Malaria in the Districts of Karak and Kohat”

Area Wise Prevalence of *P. Vivax* and *P. Falciparum*

The total prevalence of malaria in the human population among two Districts of Khyber Pakhtunkhwa, Pakistan was noted to be “18% (27/150)”. Among these, “*P. vivax* 10.6% (8/75) and *P. falciparum* 4% (3/75)” were found in the District of Kohat, while “*P. vivax* 18.6% (14/75), *P. falciparum* 2.6% (2/75)” was observed (Fig 5).

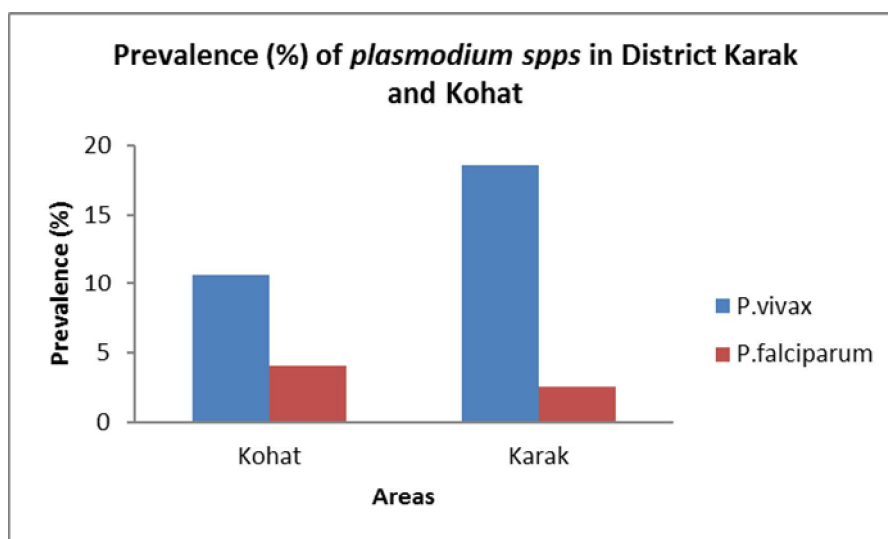


Figure 5: “Prevalence (%) of *Plasmodium* species in District Karak and Kohat Khyber Pakhtunkhwa, Pakistan”

Malaria is a protozoan parasitic infection and throughout the world it causes high morbidity and mortality among human population (CDC. 2005). The present study was carried out during the months of February to July 2013 in the Districts of Karak and Kohat Khyber Pakhtunkhwa, Pakistan. The prevalence of malaria was found 18%, which is higher than the prevalence rate of (7%) previous survey conducted by (Awan & Jan 2008). The reason for the higher overall prevalence rate in the present study may be due to difference in time duration of sample collection, different year of study and different environmental factors.

During the present study, the high rate prevalence of *P. vivax* as followed by *P. falciparum* was found. Many reports are available by researchers in different areas of District Bannu Khyber Pakhtunkhwa, Pakistan (Awan & Jan 2008).

Proteins are major part of any living cell which performs many functions. Comparison and analysis of proteins for “epidemiological/ diagnostic or taxonomic” purposes, living cells are categorized accordingly as per protein profiles (Lugtenberg & Alphen 1983). The serum protein molecular size of malarial patients was determined by SDS-PAGE method, as described by researchers with comparing to known molecular protein marker at one of the lanes of gel. However, results were cautiously interpreted because some amino acid substitutions can affect the migration rate of protein (Laemmli 1970). The results of present study (10% Acrylamide SDS-PAGE of serum protein of malaria bands) were 57 and 78 Kd respectively. Similar results were also reported by different researchers (Stranden et al., 1990; Waitayakul et al., 2006) which favors the findings of present study.

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