Prevalence and associated risk factors of malaria: a population-based study in northwestern Pakistan

By

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FACULTY OF BIOLOGICAL SCIENCES

QUAID-I-AZAM UNIVERSITY

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In

Parasitology

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"In the Name of ALLAH, the most Gracious, the most Merciful"

CERTIFICATE

This dissertation "**Prevalence and associated risk factors of malaria: a population-based study in northwestern Pakistan**" submitted by **Shafia Rehman**, is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan as satisfying the thesis requirement for the degree of Master of Philosophy in Parasitology.

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Declaration

I hereby declare that the material and information presented in this thesis in my original work. I have not previously presented any part of this work "**Prevalence and associated risk factors of malaria: a population-based study in northwestern Pakistan.**"

Shafia Rehman

Dedication

MY BELOVED PARENTS WHO HAVE BEEN PILLARS OF

SUPPORT, GUIDANCE AND LOVE IN MY LIFE

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List of Abbreviations

Abbreviated form	Full form
µl	Micro liter
Covid	Corona virus disease
D.I.Khan	Dera Ismail Khan
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleoside triphosphate
EDTA	Etheylenediaminetetraacetic acid
GYPA	Glycophorin A
GYPB	Glycophorin B
GYPC	Glycophorin C
GYPE	Glycophorin E
ml	Mili liter
mM	Mili mole
P	*Plasmodium*
PCR	Polymerase chain reaction
pH	Potential hydrogen
RBCs	Red blood cells
rpm	Revolution per minute
SPSS	Statistical package for social science
WBCs	White blood cells
WHO	World health organization

Acknowledgment

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SHAFIA REHMAN

ABSTRACT

Introduction: Malaria is the one of major health problem, predominant in the world and leading cause of illness and death in Pakistan and other developing countries. Major receptors for *Plasmodium* are glycophorin on red blood cells, which are gate for parasite entry. The present study was designed to investigate the prevalence of malaria prevalence in district Bannu, a highly endemic district of Khyber Pakhtunkhwa, Pakistan and association of glycophorin gene with malarial severity. **Material and Methods:** Blood sample were from 400 patients visiting diagnostic laboratories of DHQ hospital, KGN hospital, W&C hospital of district Bannu, examined via microscopy. A well-structured questionnaire was used to collect patient information to assess risk factors associated with malaria. DNA extraction of 50 positive blood samples was performed. GYPB gene exon 4 was amplified by PCR. **Results:** The study detected over all 113 (28.25%) confirmed positive patients, and prevalence of malaria was high in male 73(18.3%), and among age group 11-20 years, rural communities 73(18.5%), and in primary level of education 54(13.5%). Malaria infection was significantly higher at Miryan 16(4.0%) as compared to other areas of Bannu district. According to blood groups, prevalence was high among AB-ive, B+ive and O+ive (n=22, 5.5%) blood groups. Clinical factors such as fever, malaria symptoms, repeated malaria infection, home treatment with antimalarial drugs were significantly (P=<0.0001) associated with malaria. The prevalence of malaria was significantly (P=<0.0001) associated with preventive measures such as mosquito repellent, indoor residual spraying, mosquito nets used, water preservation at home, wearing sleeveless shirts. Other environmental factors such as presence of plants at home, sewage canal close to home, standing water bodies in surrounding area, environmental cleanness, holes in the walls were also found significant (P=<0.0001) risk factors for malarial prevalence. The PCR product of 245bp of exon 4 of GYPB gene was obtained. **Conclusion:** The finding suggested high malarial prevalence in Bannu district and environment and household conditions, lack of preventive measures found as major risks factors for high prevalence of malaria. Due to paucity of funds GYP gene exon 4 sequencing was not done. Further studies are required to sequence this gene to find the association with malaria severity and resistance.

Introduction

The causative agent of malaria is a small protozoon belonging to the group of *Plasmodium* species, and it consists of several subspecies. Some of the *Plasmodium* species cause disease in human. The genus *Plasmodium* is an amoeboid intracellular parasite which accumulates malaria pigment (an insoluble metabolite of hemoglobin). Of the 172 of *Plasmodium* species, five species can infect humans. These are *P.malariae*, *P. falciparum*, *P. vivax*, *P.ovale*, and *P.knowlesi* (White *et al*., 2014; Walker *et al*., 2017).

Early association of the malaria disease was associated with marshy areas. A French army surgeon, Charles Louis Alphonse Laveran, in end of 19th century, observed parasite in the blood of malaria patient. And in Hyderabad India, a British medical officer, Dr Ronald Ross, began to thought that this dangerous malaria disease is spread through mosquito, and he done the surgery of mosquito in 1897 while examining the human blood in the stomach of mosquito (Tuteja, 2007). And thus he found malaria parasite (Snow *et al*., 1999). It is the one of major health problem, predominant parasitic infection in the world and major cause of sickness and mortality in developing countries including Pakistan (Kim *et al*., 2006). Each year 300 million people are affected by malaria, as a result approximately 15, 00,000 people are dying. Majority of the people know that malaria is a disease, but most have no idea that death can also be occurred (Patra *et al.,* 2019).

1.1 Taxonomic Classification of *Plasmodium* species

Phylum Apicomplexa

Class Aconoidasida

Order Haemosporida

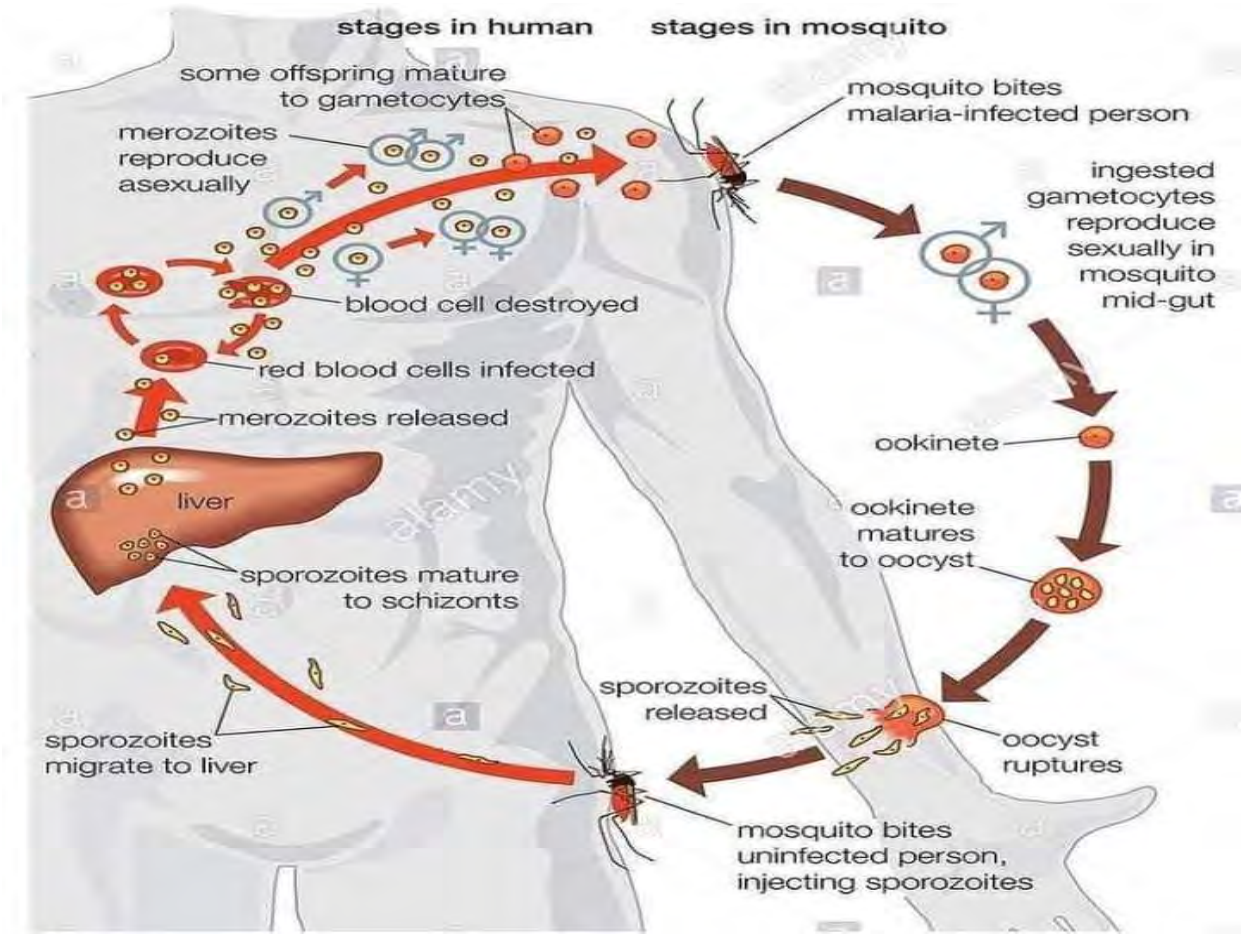
Family Plasmodiidae

Genus *Plasmodium*

(Puente *et al*., 2021)

1.2 Transmission and Life Cycle of Malaria Parasite

The life cycle (Fig. 1.1) of *Plasmodium* completes in three phases; human phase, erythrocytic phase, sexual reproduction and has two hosts: female *Anopheles* mosquito (definite host) and human (intermediate host). *Plasmodium* is present in mosquito *Anopheles* the saliva. When a mosquito bites a person, then *Plasmodium* is transmitted into the human body with saliva in sporozoites form (Zambare, 2019). The sporozoites migrate to liver through blood and multiply in liver for 5-15 days. Now sporozoites divided into huge number of merozoites. From the liver cells merozoites out, feed on red blood cell (RBC) and increasing there. Merozoites are released into the blood with lyses of RBCs, and attack new RBCs and asexual life cycle is repeated. Several merozoites are converted into gametocytes. When another mosquito bites malaria patient the gametocytes are taken inside by mosquito. In the mid gut of mosquito these gametocytes converted into male and female gametes. The male and female gametes are fuse together and form zygote (Kojin *et al*., 2021). The zygote moves to stomach wall of mosquito and develop into sporozoites after some time, then move towards the saliva of the mosquito. These sporozoites are injected by a mosquito into the blood when bites a healthy person. In this way spread of malaria takes place (Hollin, 2021). From the liver cells merozoites are released in the form of vesicles, travel towards the heart, and reach in the lungs, where they remain in lung capillaries, hence parasites start their developmental blood phase. Multiplication of merozoites takes place until the erythrocytes burst. Now they attack on additional RBCs (Le Roch *et al.,* 2003). Fever will be resulted when parasites attacked on blood cells and restarts a new cycle. Someblood cells that are infected will leave the asexual stage instead of replicating, and becomes mature cells called gametes. The female gametes that are fertilized converted into moving ookinetes, which incurred through the mosquito's mid-gut wall and produce oocytes on the external surface. Thousands of active sporozoites are produced inside the egg. The oocytes burst, and sporozoites are released into the body cavity, migrate to the salivary glands of mosquito. The human infection cycle starts all over again when a mosquito bites another person (Siciliano and Alano, 2015).



(Encyclopedia Britannica, 2022)

Figure 1.2: Life cycle of Malarial Parasite

1.3 Pathogenesis of Malaria

The prevalence of clinical complications in *vivax* malaria has been associated with various factors, such as: transmission intensity, the presence of other endemic or non-communicable disease, host characteristics (gender, age and genetic background) and drug resistance. The most common complications reported from vivax endemic areas are anemia and respiratory distress (Genton *et al*., 2008; Tjitra *et al*., 2008; Kochar *et al*., 2009; Lacerda *et al*., 2012). Between 1998 and 2008, 234 *vivax*-associated malaria deaths were reported in Brazil (Oliveira-Ferreira *et al*., 2010). One study has recently demonstrated that the World Health Organization's severity criteria for *P. falciparum* reliably identified *P. vivax*-infected patients at risk for severe disease admitted to the intensive care unit (Lanca *et al*., 2012). Anemia is the most frequent complication in Brazil, which causes increased morbidity and mortality in pregnant women and children (Haldar and Mohandas, 2009; Alexandre *et al*., 2010; Lanca *et al*., 2012). During *vivax*

infections, first erythrocytes are destroyed, but later on anemia can still be present even after clearance of parasite. This observation can be described by the fact that *P. vivax* infects reticulocytes, and the infection prevents the reestablishment of the normal erythrocyte population (Collins *et al*., 2003). Respiratory distress is another common complication in *vivax* malaria, reported distress (Anstey *et al*., 2007; Price *et al*., 2007; Tan *et al*., 2008).

1.4 Epidemiology of Malaria

The transmission of malaria can also occur by organ transplantation, blood transfusion, sharing of contaminated needles and syringes. And also can be transmitted from mother to child during delivery or before birth. The two species of *Plasmodium*, *P. vivax* and *P. falciparum* have greatest public health threat. These both parasite species are reported in Pakistan. In which the most dominant specie is *Plasmodium vivax* (>80). During infection the first symptoms of headache, chills and fever appear in 10-15 days after the mosquito bite, and these symptoms are difficult to recognize as malaria because may be mild. The untreated left malaria progress, cause severe illness and can lead to death with in a period of 24 hours (World Health Organization, 2022).

1.5 Symptoms of Malaria

Symptoms of malaria include, high grade fever, chills, shaking, headache, myalgia, fatigue, vomiting, sometimes nausea, sweating, anemia, splenomegaly, dehydration and jaundice, heamoglobinuria, retinal damage, hyperbilirubinemia, thrombocytopenia, splenomegaly and diarrhea (Ndako JA *et al.,* 2020; Beare *et al*., 2006; Nadjm and Behrens, 2012).

1.6 Receptors for Malaria Parasite on RBCs

Glycophorin rich in sialic acid are red blood cells protein that carry blood group antigen and act also as receptors for different intracellular pathogens. The invasive merozoite stage of *Plasmodium* parasite uses numerous glycophorin to penetrate the red blood cell, during intra-erythrocytic cycle. There are five types of glycophorin in which the three are GYPA, GYPB, and GYPE that can also carry MNS blood group system which is encoded by a locus on chromosome no 4. Glycophorin A and glycophorin B recognized by erythrocyte-binding antigen 175 (EBA-175) and erythrocyte binding ligand 1 (EBL1) of *Plasmodium falciparum*, and MN and Ss blood

groups are determined by variation of amino acid in GYPA and GYPB. And the other type GYPC also acts as receptor for *Plasmodium falciparum* invasion by recognizing EBA-140 on the surface of merozoites. It is not homologous to GYPA and GYPB, and carries Gerbich (Ge) blood group antigens. *Plasmodium vivax* gets interconnected with human Duffy receptor that has been lost in majority of the African population, which is adaptation for resistant to *P. vivax* infection. While *P. falciparum* malaria infection which is the cause of most of the malarial infections in Africa targets glycophorin proteins (glycosylated proteins) present on the surface of human RBCs (Wright *et al*., 2014; Jaskiewicz *et al*., 2019; Malaria Genomic Epidemiology Network (2015).

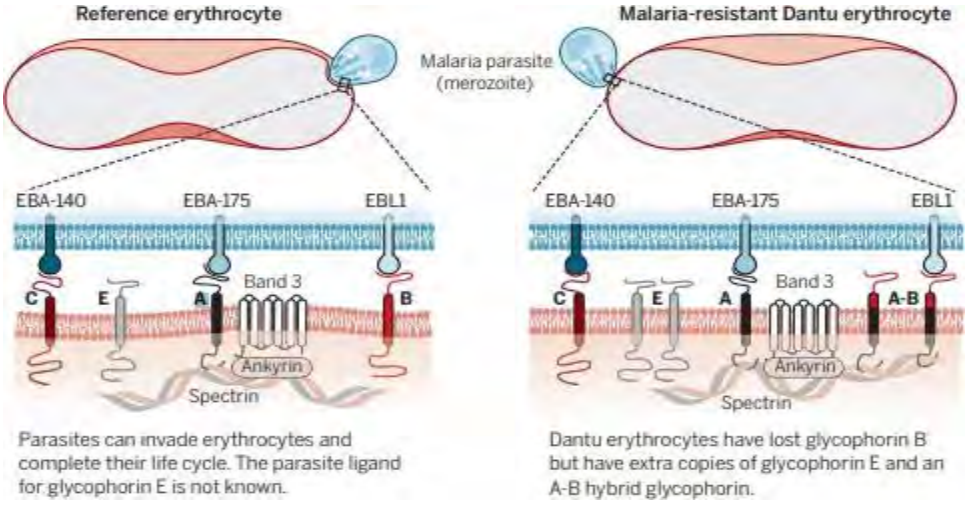


Figure 1.3: Receptors for malaria parasite on RBCs (Winzeler, E. A. (2017).

1.7 Vectors of Malaria Parasites

Vectors for malaria parasites are *Anopheles* mosquitos (arthropods) which are responsible for the transmission of *Plasmodium* species in human population (Ceccato *et al*., 2005). There are various species of *Anopheles* have been found as vectors in different part of the world. Total 460 are recognized, in which 100 species of *Anopheles* transmit human malaria. About 30-40 *Plasmodium* species commonly transmit by these mosquitoes (Naz *et al*., 2013).

1.8 Prevalence of Malaria

1.8.2 Global Prevalence

(Abah *et al.,* 2015) indicated that 63.333% of primary school students in Bayelsa State, Nigeria, had malaria infections. According to (Al Mekhlafi *et al.,* 2011), there were 78 (17.1428%) febrile patients with malaria in the highlands of Yemen from June 2008 to March 2009.The frequency of *Plasmodium* infection reported throughout from January to December 2013 in Navi Mumbai, India was 16.584% (Singh *et al.,* 2015).Almost in all parts of Afghanistan with an elevation below 1,500 meters, malaria is endemic. Here, *Plasmodium vivax* makes up 80–90% and *Plasmodium falciparum* comprises up the remaining portion of the overall malarial disease. Approximately 66% of the Islamic Republic of Iran is malaria-free, 12% has *Plasmodium vivax*, and 6% has *Plasmodium falciparum* (Naqvi *et al.,* 2020).

1.8.1 Prevalence of Malaria in Pakistan

In Pakistan, the prevalence of malaria differs by province as well as by city, depending on the climatic changes. During 2017, the most instances of malaria were reported in Khyber Pakhtunkhwa (30%), Sindh (26.5%), the Federally Administered Tribal Area (FATA) (21.9%), Baluchistan (20.5%), and Punjab (1.1%) (WHO, 2017).In the year 2018, out of 6.5 million suspected and tested malarial cases, the Directorate of Malaria Prevention and control has recorded 374,513 positive cases of plasmodium parasite in Pakistan, providing a frequency of 5.7617% for the entire year. The ratio of the disease provided on the basis of different localities was as follows: Sindh (1.9859%), Khyber Pakhtunkhwa (1.7845%), Tribal districts (1.0131%), Punjab (0.0289%) and for AJK (0.0030%) (Directorate of Malaria control, 2019).

The incidence of malaria in Khyber Pakhtunkhwa, Pakistan, to be 13.81% by applying a random effect model in which he aggregated results from 18 researches between 2003 and 2007 that were then looked up in databases (Najeeb *et al.,* 2018). Another survey published in Khyber Pakhtunkhwa found that the overall incidence of malaria was 13.8430% with 4,297 confirmed samples from a random selection of 31,041 patients during the period of August and October 2018, using rapid diagnostic test as the screening test, in the 3 highly prevalent districts of Bannu, Lakki Marwat, and D.I. Khan. Malaria prevalence recorded was (5.5894%) in Bannu, (4.2428%) in Lakki Marwat and (4.0108%) in D.I. Khan (Qureshi *et al*., 2020).

According to a survey done in the Malakand Division, 26.7% of people tested positive for malaria. *P. vivax* made up 98.6% of the positive cases, and *P. falciparum* made up 1.3% (Khan *et al*., 2019). According to a study, the overall positivity rate Punjab was 5.7% across all selected cities, while Southern Punjab had a higher disease frequency (6.1%) than Northern Punjab (5.3%). The infectivity rate in the Northern Punjabi cities where people were recruited was 6.8 and 3.1% in Jhelum and Chakwal, respectively. Contrarily, among the Southern Punjab cities Rajanpur had the highest slide positivity rate (7.1%) and Dera Ghazi Khan had the lowest SPR (5.4%). The API in each recruited city was 0.1 per 1000 people (Qureshi *et al*., 2019).

Numerous microscopic studies have been conducted from different regions in Pakistan, such as (Mahmood *et al.,* 2005) studied 348 feverish patients in Karachi and reported a 35% frequency rate of malaria. According to (Yasinzai *et al*., 2008), the parasite frequency ranged from 19.8 to 58.1% among different age groups. (Leghari *et al*., 2014) studied the five-year surveillance of Malaria in Bahawalpur from 2007 to 2011.

Numerous studies have been published from the province of Balochistan. 18.45% of the general population in Quetta had malaria in 2012 (Tareen *et al.,* 2012). According to the studies conducted by Yasinzai and Kakar Suleman Khel (2013) a frequency incidence of malaria fever in Panjgur, Balochistan was 38.5%. In Killa Saifullah region prevalence rate of 20% was published (Umer and Yasinzai, 2017).The average incidence of positivity rate, as reported by another study (Sumbal *et al*., 2018), was 19.56%, with *P. vivax* being greater (84.52%) than *P. falciparum* (6.01%) and mixed infection of both *P. vivax* and *P. falciparum* reported was (12.29%). In District Khuzdar's total prevalence was 27.9% in 2019. *Plasmodium* slide positivity rate was 18.8% in the Pishin district (Farooq *et al*., 2020).

1.9 Current investigations

Previously many studies have been conducted where the members of Phylum Apicomplexa were categorized mainly on the basis of morphology, clinical data, disease causing ability of the pathogen, locality and the intermediate host or vector involved in the spreads of the parasites. Clinical sign and symptoms associated with malaria is fever which is often accompanied with chills, vomiting, anorexia, and perspiration. The symptoms of malaria are not specific and match

with other diseases too. So the diagnoses of malaria through clinical basis is not good (Tajitra *et al,* 1999).

Microscopy is considered as the gold standard diagnostic method. Although microscopy have least sensitivity and specificity as compared to other diagnostic procedures but is still used as the most common technique throughout the world for the parasite identification. For detecting and identifying the malaria parasite the expert technician prepare Geimsa stained blood slide for different stages of the parasite (Maqsood, 2021). In case of mixed infection, it then become challenging to recognize the plasmodium species entirely on the basis of the morphological parameters (Gillani *et al,* 2020). Several test kits are also available in the market for detecting antigens produced due to the parasite of malaria; these immunologic (immunechrometographic ICT) tests are most commonly performed by using a cassette format or dipstick and give results in 5-15 minutes. In rapid diagnostic test immunochrometographic methods are used in which the detection of antigen derived from malaria parasite in lysed blood is carried out. Dipstick or test strip is used in this method having monoclonal antibodies which is used against the target parasite antigen (Mathison and Pritt, 2017). Serological assays also play an important role in the accurate identification but the major limitation to this assay is that they cannot distinguish between the recent and past infection. So, the current study used microscopic method for malaria diagnosis.

The present study was aimed to find the distribution of malarial and role of glycophorin gene in malarial infection susceptibility/resistance among inhabitants of district Bannu. The objectives of the present study were:

- To find prevalence of malaria in district Bannu of Khyber Pakhtunkhwa.
- To determine the association of demographic, clinical and environmental risk factors with malaria.
- To determine the polymorphism in exon 4 of glycophorin gene in malaria positive patients.

METHODS AND MATERIALS

2.1 Study Area

The study was carried out in district Bannu of Khyber Pakhtunkhwa, Pakistan. Its population is 552 persons/Km^2. Bannu district is listed among malaria effected areas in Pakistan (Carlton *et al*., 2008). The rivers Kurram and Gambila cross district Bannu which provide water for irrigation as well as serve as breeding sites for malaria parasites vectors i.e. mosquitos. The main mosquitos' vectors reported are *Anopheles culicifacies* and *Anopheles stephens* (Bouma *et al.,* 1996; Ministry of Health, 2010).The annual parasite incidence is above the national average (0.8), as it is 1.6-3.5 /1000 population (Bouma *et al*., 1996). Mean daily temperature range between 10.8 °C to 32.9 °C. Precipitation occurs in 2 phases i.e. in March and summer (July and August). Mostly, malaria transmission is on peak after monsoon (Ministry of Health, 2010). Bannu district has great economic importance as it is a central market of the southern region, in addition to serving as a safe shortcut to markets in Central Asia (Khatoon *et al*., 2010).

2.2 Ethical Consideration

The current study was approved by ethical committee of District Head Quarter Hospital Bannu.

2.3 Research Design

The study consist of two main parts; one is finding out the prevalence of malaria in district Bannu and the second one is the evaluation of mutation in exon 4 of GYPB gene of malaria positive patients. The overall data was collected from 400 patients through questionnaire based survey along with blood samples. The entire collected blood sample were taken on microscopic slides, and examined under microscope. Out of these 400 patients 1ml of blood was collected from only 50 random positive patients in EDTA tube for genomic DNA extraction.

2.3.1 Study Population

Blood samples and data were collected from different areas of district Bannu. People of different age groups (1- 40 years), who visited the local hospitals of district Bannu were included in this study.

In the study all those patients were included who showed malarial symptoms are willing to participate in the study filled the questionnaire and gave 1 ml of blood samples. Patients having symptoms of Covid-19, dengue, typhoid, and enteric fever, mouth sores, or any other illness that affect the studied parameters, were excluded. Patients withliver and renal disease, hypertension, diabetes, platelet problems, pregnancy were excluded too from the study.

2.3.2 Questionnaire survey

Questionnaire was designed containing 27 variables. Responses of the patients were recorded and compiled in excel sheet for statistical analysis. The 27 variables were area gender, blood group, age (in years), locality, education, environment and clinical factors.

2.4 Microscopy

Both thick and thin blood smears were formed for microscopic examination (Fig.2.1). For thirty seconds the thin film was fixed with 100% methanol after drying and were stained with freshly prepared 10 % Geimsa stain, made by combining 1 ml stock solution with 9 ml buffer (pH: 7.2). The slides were washed with tap water and hung to dry in avertical position. Blood films were examined through (100X) oil immersion lens. For quality control each fresh batch of functional Geimsa stain comprised a positive smear was used. To determine parasite densities the parasite thick film was used. All slides were double-checked, and those slides were considered to be negative having no parasite in the (100X) oil immersion lens.

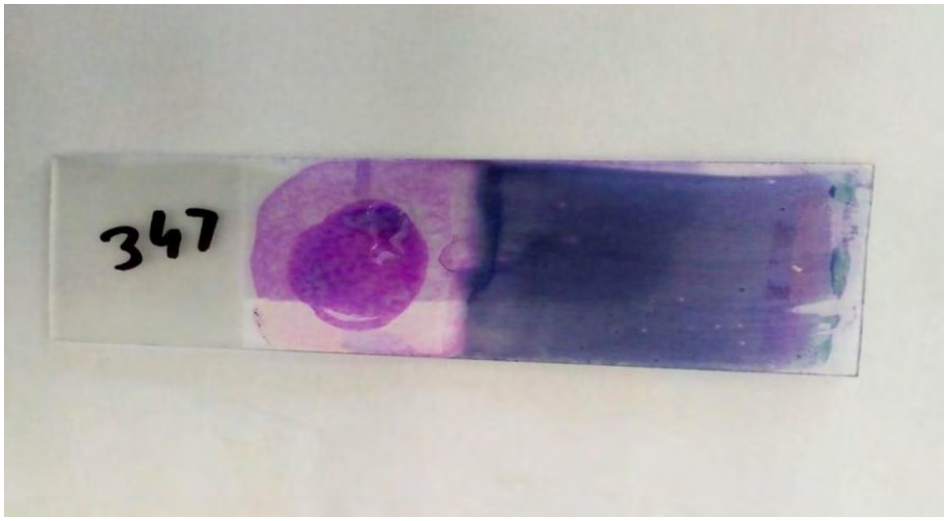


Figure 2.1 Slide prepared for microscopy

2.5 Molecular Analysis

2.5.1 Blood Collection

Blood sampling was carried out from malarial labs of different hospitals in district Bannu, i.e. Khalifa Gul Nawaz Hospital (KGN), District Head Quarter Hospital (DHQ), and Women and Children Hospital (W&CH) malarial labs. At all these labs, from total 50 malaria positive patients, 1ml blood was collected in labeled EDTA tubes. A unique number was also assigned to each tube to keep them different from each other. The blood was well shaken in EDTA tube to prevent the blood from clotting for microscopy and then was stored at -20^{o}C in freezer.

2.5.2 Extraction of Genomic DNA

Genomic DNA was extracted by Phenol-Chloroform method of DNA extraction. The protocol of phenol-chloroform DNA extraction method. Briefly, blood samples were thawed at room temperature for the lysis of RBCs. Then 750µl blood was shifted to ependorf tubes (SPL Life Sciences) from EDTA tubes. For removal of lysed RBCs, washing buffer was prepared in 50ml of falcon tube. For buffer preparation 500µl Tris, and 2µl EDTA was added in falcon tube, and fill the falcon tube up to 50ml with distal water. Now the eppendorf tubes were filled up to 1.5ml with washing buffer. 35ml of Tris-EDTA (TE) buffer (10mM Tris HCL, 2mM EDTA, pH 8.0) was added to 5ml of blood in each falcon tube. After adding washing buffer, samples were centrifuged (BECKMAN COULTER MICROFUGE) for 10 minutes, at 13000 rpm. The supernatant was discarded up to 750µl through glass pipette without disturbing the white blood cells pellet. Then the tube was again filled with washing buffer up to 1.5ml and pellet was tapped to dissolve completely in buffer. Samples were centrifuged again for 10 minutes at 13000 rpm and supernatant discarded up to 1ml without disturbing the WBCs pellet. Washing steps were repeated 3-4 times to make WBCs pellet free from hemoglobin. For digestion of pellet of WBCs protein we added 7.5µl of proteinase kinase K (PK) (20mg/ml), 20µl sodium dodecyle sulfate (SDS) and 500--l Tris-NaCl-EDTA (TNE) buffer (10mM Tris HCL, 400mM NaCl, 2mM EDTA) in each eppendorf tube and the samples were kept in incubator at 37^{o}C overnight.

On the next 2nd day 150µl of 6M NaCl was added to each sample for precipitation of proteins. After adding given forceful shake. Then, were kept in ice for 10-15 minutes. 750ml PCI (5:4:1) (25ml phenol: 20ml chloroform: 5ml iso-amyl alcohol) mixture or solution was prepared and

1ml of PCI solution was added in each tube and shake them properly, and then centrifuged then for 20 minutes at 3000rpm. Due to centrifugation three layers were formed in ependorf tube. The DNA was dissolved and present in the upper transparent layer. In the bottom two layers proteins, lipids, and salts, other cellular debris were present. The upper DNA layer was separated by pipit in another eppendorf tube by using 1000µl micropipette without disturbing the bottom two layers. In the next step chilled isopropanol was added volume equal to upper picked layer of the sample in the falcon tube, mixed it properly and DNA thread was precipitated. Then centrifuged at 13000 rpm for 10 minutes to form DNA pellet. After centrifugation supernatant was discarded carefully to leaving the DNA pellet adhered to the wall of falcon tube. Then DNA pellet was washed with 150µl chilled 70% ethanol. After adding 70% ethanol the samples were centrifuged at 13000rpm for 10 minutes. After centrifugation supernatant was discarded carefully to leaving the DNA pellet adhered to the wall of eppendorf tube. DNA pellet was dried, keeping eppendorf tube inverted for 2-3 hours. Then 40µl TE buffer was added, vortex and then placed in incubator overnight at 37^{o}C to dissolve the DNA pellet.

On 3rd day of DNA extraction in the start eppendorf tubes were sealed with paraffin strips. Then heat was given at 70^{o}C for 1 hour, to inactivate nucleases and prevent the DNA from denaturation. The tubes were placed at room temperature for 5 minutes. Then short spin was given by centrifuge to mix the buffer and DNA properly. In next step DNA samples were transferred to autoclaved screw cap tubes. The DNA containing screw cap tubes were stored at -20^{o}C in labeled storage box.

2.5.3 Agarose Gel Electrophoresis

Gel electrophoresis (1%) as done for the confirmation of extracted DNA samples. In this process 1% agarose is used. Agarose gel electrophoresis was done in the following way:

1% gel was prepared, by dissolving 0.5g of agarose powder in 50ml 1X-TBE buffer (Tris-Boric-EDTA buffer) in conical flask. This mixture was placed in microwave oven, to make the solution clear. Opening of flask was covered with aluminum foil. The mixture was allowed cool on room temperature for a few minutes. Then 5µl Ethidium Bromide (EtBr) was in the gel mixture and properly shaken. Combs were placed in casting tray, and the gel mixture was poured in the casting tray, in such way that there is no bubble formed the casting tray. Wait for solidified for

30-40 minutes at room temperature. Then the gel was removed from casting tray and placed in 1X-TBE buffer filled gel tank (Cleaver Scientific Limited, CS-3000V). Prepared the loading samples with mixing of 3µl 6X Bromophenol, and 3µl extracted DNA sample. This 6µl mixture of DNA sample and 6X Bromophenol blue (loading dye) was poured in holes of gel, which was placed in the 1X-TBE buffer filled gel tank. Then Gel electrophoresis apparatus was run at 120 volt for 20 minutes. After this process the gel was observed under UV through Gel Documentation (Cleaver Scientific Limited).

2.5.4 Primer Designing

Primers were designed by using Primer-3 software (https://primer3.ut.ee/) for the amplification of the specified region of GYPB gene exon-4. All the conditions were adjusted at optimum level. Reference sequence was obtained from the Ensemble website (https://www.ensembl.org/index.html) which was used to design the required primers. Blat like alignment tool on UCSC genome browser (https://genome.ucsc.edu/) was used to confirm the specificity of the selected primers. In-silico PCR tool on UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgPcr) was used to verify the amplicon size of the primers. Primer sequence, product size and melting temperature for each primer are given in the table 2.1.

Table 2.1: Primers for Selected Exon

Gene	Exon	Primer	Sequence (5' —3')	Primer Length	Product Size	Tm
GYPB	4	Forward	CAAGGTGATGTTATGCTGATGT	22	245bp	60 °C
		Reverse	CCTCCTAGAGCTGTTCACAC	20		

2.5.5 Polymerase Chain Reaction

Initial concentration of the ordered primers was 100 picomole /µl. 300µl PCR water was added for dilution to make final concentration of primer 300µl. The selected exon-4 of Glycophorin-B gene was amplified through polymerase chain reaction in all blood samples of malaria positive patients. PCR was performed in PCR tubes (Axygen, USA) having 200µl capacity. The following table 2.2 shows the chemical (Thermo-Scientific PCR Kit) and their volumes the PCR mixture used in PCR. The condition for PCR thermocycler was given in Table 2.3.

Table 2.2 Chemicals used in PCR mixture

Sr. No.	Chemical	Concentration	Volume (µl) for Single PCR Reaction
1	DNA	>100 ng/µl	3 µl
2	Forward Primer	10 0pmol/ µl	0.5 µl
3	Reverse Primer	10 0pmol/ µl	0.5 µl
4	DNTPs	2.5 mM	2 µl
5	10X Buffer (with Mg^{2+})	10X	2.5 µl
6	Taq Polymerase	5 U/µl	0.5 µl
7	PCR Water		16 µl
	Total Volume		**25 µl**

Table 2.3 Conditions for PCR Cycles

Step	Temperature ^{o}C	Time	Cycle
Initial Denaturation	95	5min	1X
Denaturation	94	45sec	
Annealing	50-60	45sec	40X
Extension	72	45sec	
Final extension	72	10min	1X

2.6 Statistical Analysis

The data collected from the participants was arranged in MS excel (Microsoft Corporation, 2010) and then for further analysis the data was exported to SPSS version 20. The questioners were checked regularly for missing values, logical errors etc. Chi-square test was performed to find out the relationship of prevalence of malaria and other risk factors of malaria. The level of significance was established at $P \leq 0.05$.

RESULTS

The present research was designed to determine the prevalence of malaria associated with demographic and environmental and clinical risk factors in district Bannu. The molecular analysis of exon 4 of GYPB gene of malarial positive patients was also done. The patients were divided into 5 age groups i.e. 1-10, 11-20, 21-30, 31-40, and >40. To achieve this goal, thin and thick smears of blood were prepared from all the suspected patients of malaria belonging to different localities and union councils, who visited malarial labs at Women and Children Hospital and District Head Quarter Hospital at Bannu district. And then Giemsa's staining procedure was done and followed by microscopy to observe results. In this study 113 (28.25%) patients were malaria positive and 287 (71.75%) were malaria negative.

3.1 Prevalence

3.1.1 Association of malaria among individuals of different areas of district Bannu

Table 3.1 shows association of malaria among individuals belonged to different areas of district Bannu. The results indicates total 113 positive case in which the highest rate is found in Miryan 16(4.0%), and the least malarial infection was in Bannu Township 1(0.3%) (Fig.3.1). The significant difference (χ^2=81.180, P=<0.0001) was observed in malarial occurrence from different areas.

3.1.2 Association of malaria among subjects by gender

High prevalence rate of malaria infection was seen in males 73(18.3%) as compared to females 40(10.0%), and there was no significant difference (χ^2=0.24, P=0.7) (Table 3.2, Fig. 3.2).

Table: 3.1 Association of malaria among individuals from different areas of district Bannu.

Variable	Positive n(%)	Negative n(%)	Total n(%)	Chi square	P-value
Area				81.180	<0.0001*
Baka Khel	4(1.0)	23(5.8)	27(6.8)		
Bannu City	7(1.8)	68(17.0)	75(18.8)		
Domel	3(0.8)	47(11.8)	50(12.5)		
Ghoriwala	7(1.8)	23(5.8)	30(7.5)		
Jandokhel	14(3.5)	15(3.8)	29(7.3)		
Kakki	10(2.5)	11(2.8)	21(5.3)		
Mamashkhel	8(2.0)	12(3.0)	20(5.0)		
Mandan	11(2.8)	9(2.3)	20(5.0)		
Merali	2(0.5)	3(0.8)	5(1.3)		
Mirakhel	11(2.8)	5(1.3)	16(4.0)		
Miryan	16(4.0)	17(4.3)	33(8.3)		
Nurar	6(1.5)	5(1.3)	11(2.8)		
Surani	13(3.3)	20(5.0)	33(8.3)		
Township	1(0.3)	29(7.3)	30(7.5)		

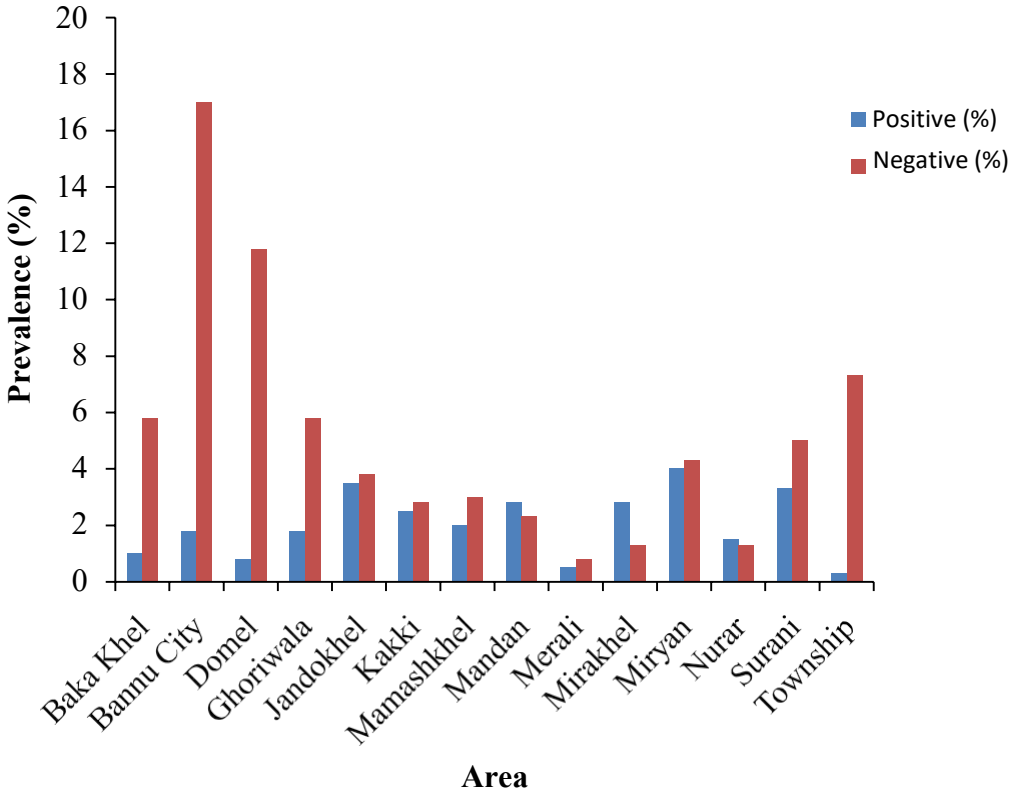


Figure 3.1: Prevalence (%) of malaria among individuals belonged to different areas of Bannu.

Table 3.2 Relationship of malaria prevalence among subjects by gender

Variable	Positive n(%)	Negative n(%)	Total n(%)	Chi square	P-value
Gender					
Female	40(10.0)	107(26.8)	147(36.8)	0.24	0.725
Male	73(18.3)	180(45.0)	253(63.3)		

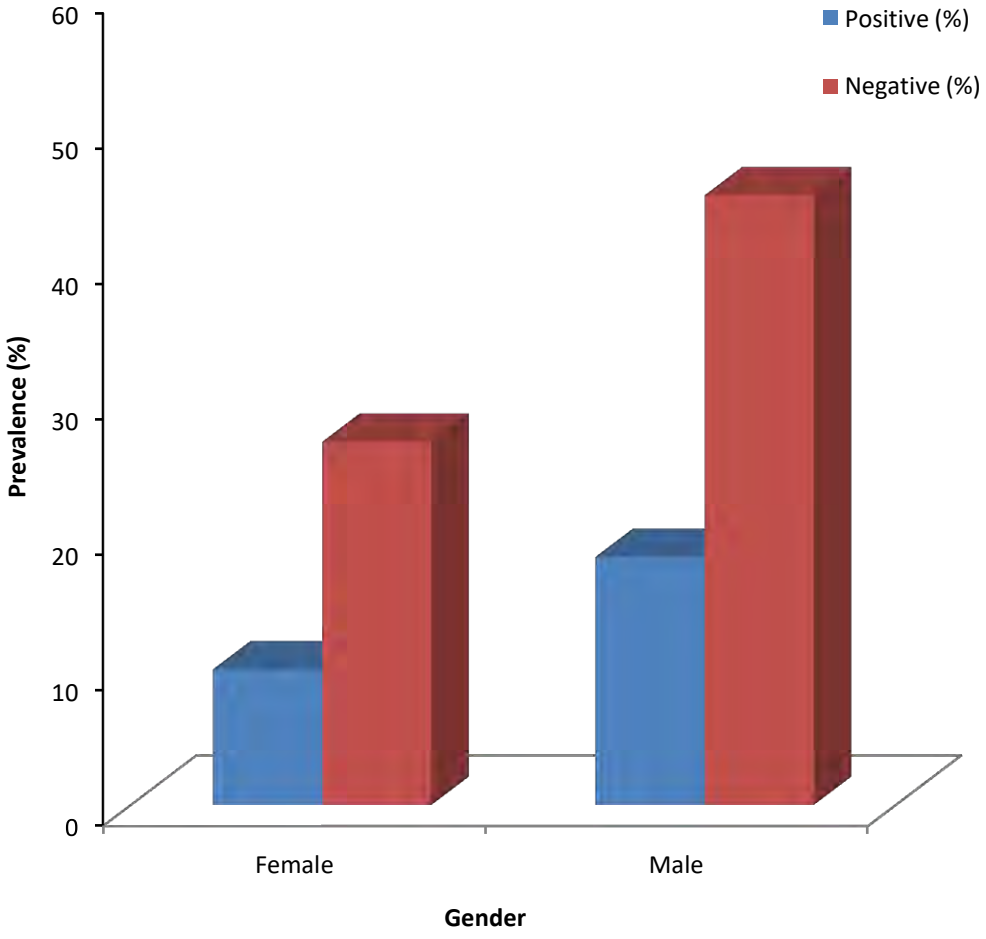


Figure 3.2: Prevalence (%) of malaria among subjects by gender

3.1.3 Relation of malaria among individuals blood groups

Prevalence of malaria was high in AB-ive, B+ive and O+ive blood groups (n=22, 5.5%), and the lowest in A-ive (n=3, 0.8%) (Table 3.3 and Fig.3.3). However, the difference was significant (χ^2= 34.337, P=<0.0001).

Table 3.3 Relation of malaria among individuals blood groups.

Variable	Positive n(%)	Negative n(%)	Total n(%)	Chi square	P-value
Blood Group					
A-ive	3(0.8)	14(3.5)	17(4.3)	34.337	<0.0001*
A+ive	11(2.8)	53(13.3)	64(16.0)		
AB-ive	22(5.5)	20(5.0)	42(10.5)		
AB+ive	14(3.5)	16(4.0)	30(7.5)		
B-ive	4(1.0)	17(4.3)	21(5.3)		
B+ive	22(5.5)	84(21.0)	106(26.5)		
O-ive	15(3.8)	14(3.5)	29(7.3)		
O+ive	22(5.5)	69(17.3)	91(22.8)		

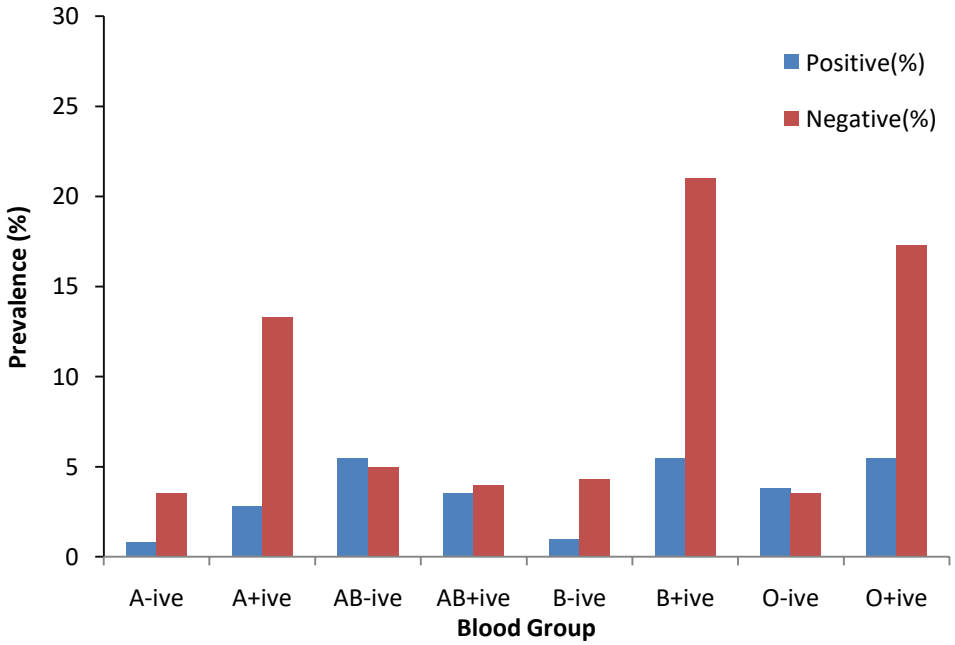


Figure 3.3: Prevalence of malaria among individuals according to their blood groups

3.1.4 Association and prevalence (%) of malaria in different age groups

Malaria infection rate was high in age group 11-20 33(8.3%), followed by 31-40 29(7.3%), 1-10 23(5.8), 21-30 19(4.8%), and was low in 40< 9(2.3%) (Table 3.4, Fig. 3.4). There was no significant difference (χ^2=7.292, P=0.121).

Table 3.4: Association of malaria among different age groups of study participants.

Variable	Positive n(%)	Negative n(%)	Total n(%)	Chi square	P-value
Age					
1-10	23(5.8)	86(21)	109(27.3)	7.292	0.121^{NS}
11-20	33(8.3)	83(20.8)	116(29.0)		
21-30	19(4.8)	41(10.3)	60(15.0)		
31-40	29(7.3)	47(11.8)	76(19.0)		
>40	9(2.3)	30(10.5)	39(9.8)		

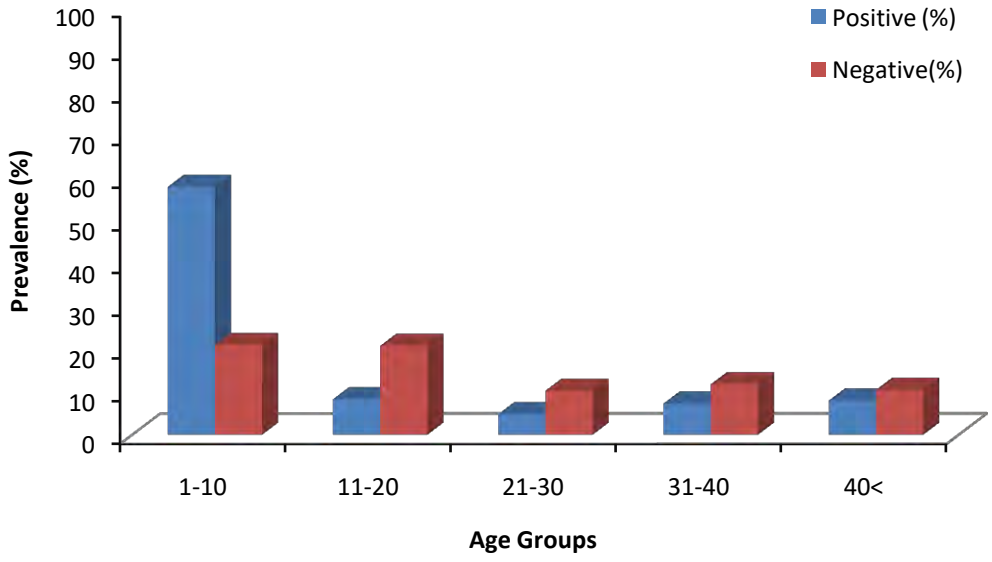


Figure 3.4: Prevalence (%) of malaria in different age groups.

3.1.5 Malaria infection prevalence according to localities

Malaria prevalence rate was high and 73(18.5%) in rural areas as compared to urban which was 39 (9.8%), and there was no significant difference among localities (χ^2=0.018, P=0.892) (Table 3.5, Fig. 3.5).

Table 3.5 Locality wise prevalence of malaria

Variable	Positive n(%)	Negative n(%)	Total n(%)	Chi square	P-value
Locality					
Rural	73(18.5)	190(47.5)	264(66.0)	0.018	0.892^{NS}
Urban	39(9.8)	97(24.3)	136(34.0)		

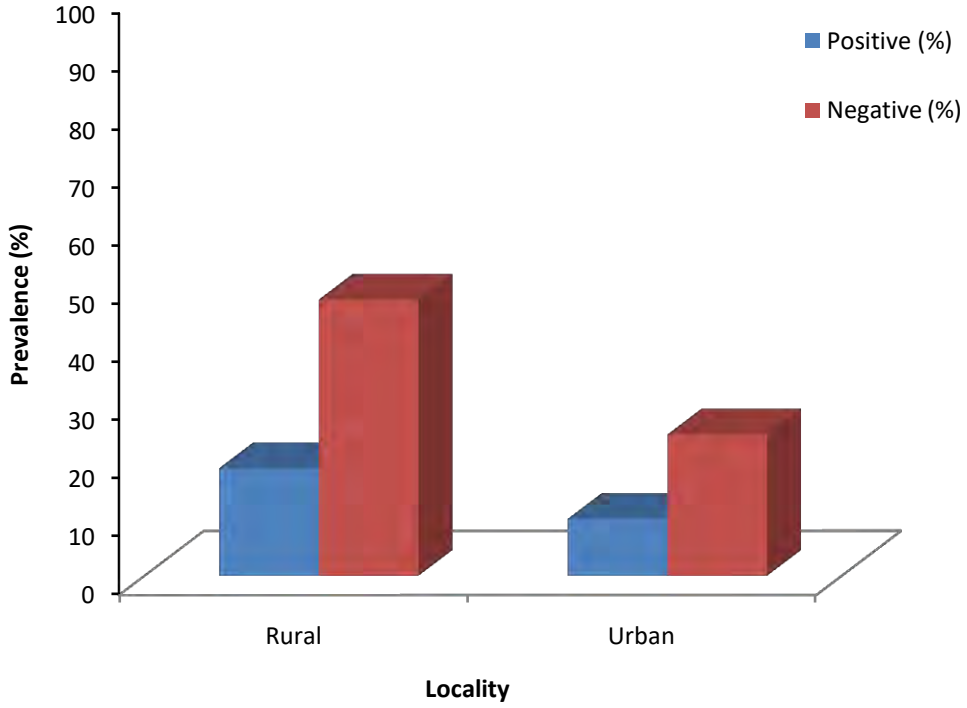


Figure 3.5: Localitywise prevalence (%) of malaria.

3.1.6 Relation and prevalence of malaria among education

Literacy level and malaria infection rate was found significantly (χ^2=26.072, P= 0.0001) associated (Table 3.6). And the prevalence was high in patients having literacy level of primary 54(13.55%), followed by secondary education 38(9.5%), and illiterate 21(5.3%) (fig.3.6).

Table 3.6Prevalence of malaria among subjects by their level of education.

Variable	Positive n(%)	Negative n(%)	Total n(%)	Chi square	P-value
Education					
Illiterate	21(5.3)	65(16.3)	86(21.5)		
Primary	54(13.5)	64(16.0)	118(29.5)	26.072	<0.0001*
Secondary	38(9.5)	158(39.5)	196(49.0)		

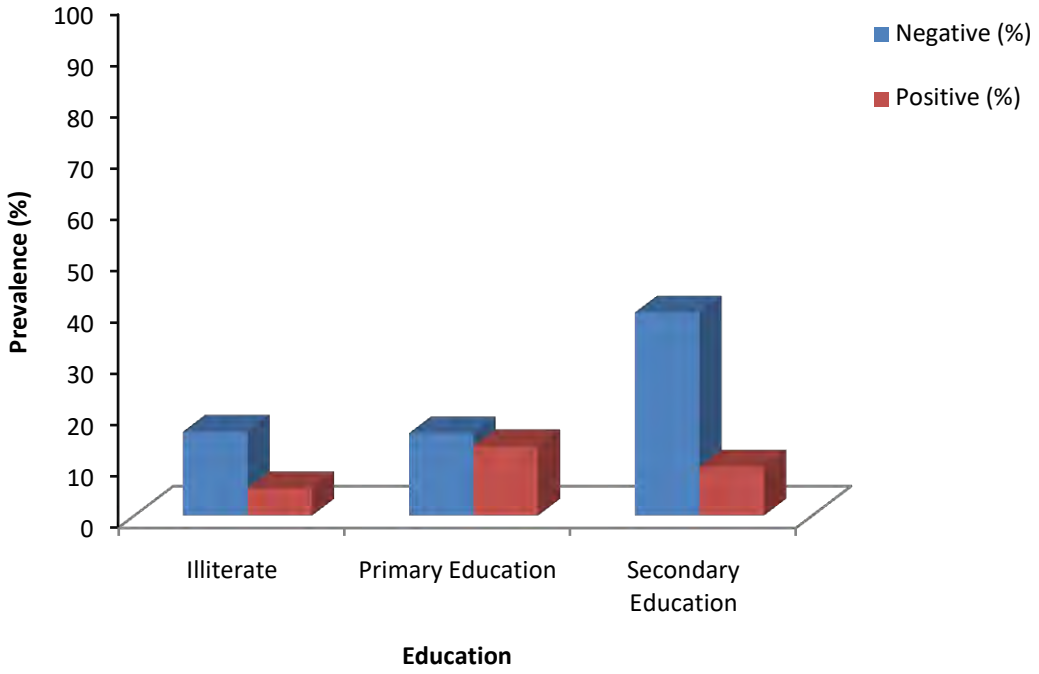


Figure 3.6: Prevalence %) of malaria among study participant by their education.

3.1.7 Association of malaria with clinical factors

Table 3.7 shows the association of clinical factors with malarial prevalence. The clinical factors such as fever was found significantly associated with malaria (χ^2=400, P=<0.0001) and all 113(28.3%) malarial positive individual have fever. Malaria symptoms were found in all malaria positive patients 113(28.3%) and were significantly associated (χ^2=400 and P=<0.0001. The prevalence of malaria in once infected by malaria individuals was 35(8.8%) and that of repeated was 78(19.5%) and was found significant (χ^2=26.686, p-value=<0.0001). The malaria in the patients having no fever in previous one year was 54(13.5%) and in those patients having fever in previous one year was 59(14.8%) and the value was found significant (χ^2=23.279, p-value=<0.0001). The prevalence of malaria in patients having treatment with antimalarial at home was also found significant (χ^2=20.393, p-value=<0.0001) and prevalence in the patients having treatment with antimalarial at home was 41(10.3%), while in those having no treatment with antimalarial at home was 72(18.0%). While the variable any knowledge about malaria was found insignificant (χ^2=0.139, p-value=0.709).

Table 3.7 Association of malaria with clinical factors.

Variables	Positive n(%)	Negative n(%)	Total (%)	Chi square	P-value
Fever					
Malaria	113(28.3)	0(0.0)	113(28.3)	400.000	<0.0001*
Other	0(0.0)	287(71.8)	287(71.8)		
Malaria Symptoms					
No	0(0.0)	287(71.8)	287(71.8)	400.000	<0.0001*
Yes	113(28.3)	0(0.0)	113(28.3)		
Infected by Malaria					
Non	0(0.0)	39(9.8)	39(9.8)		
Once	35(8.8)	42(10.5)	77(19.3)	26.686	<0.0001*
Repeated	78(19.5)	206(51.5)	284(71.0)		
History of Fever within Previous One Year					
No	54(13.5)	210(52.5)	264(66.0)	23.279	<0.0001*
Yes	59(14.8)	77(19.3)	136(34.0)		
Home Treatment with Antimalarial					
No	72(18.0)	242(60.5)	314(78.5)	20.393	<0.0001*
Yes	41(10.3)	45(11.3)	86(21.5)		
Knowledge About Malaria					
No	32(8.0)	76(19.0)	108(27)	0.139	0.709^{NS}
Yes	81(20.3)	211(52.8)	292(73.0)		

3.1.8 Association of malaria with respect to preventive measures

The prevalence of malaria was found less 50(12.5%) in the individuals using mosquito repellent as compared to those individuals using no mosquito repellent 63(15.8%) and was found significant (χ^2=57.400, P=<0.0001). Malaria prevalence relation to indoor residual spraying was also found significant (χ^2=5.665, P=0.017). Relation of mosquito nets used was found significant (χ^2=54.229, P=<0.0001), and prevalence was high in patients using no bed nets 78(19.5%), as compared those patients using mosquito bed nets 35(8.8%). While insecticide treated bed nets relation with malaria prevalence was not found significant (χ^2=1.974, p-value=0.160). Malaria association with water preservation at home was significant (χ^2=74.033, P=<0.0001). Similarly, a significantly higher infection was found (χ^2=83.029, P=<0.0001) among the individuals wearing sleeveless shirts (Table 3.8). The prevalence with preventive measures is indicated in Fig.3.7 to 3.11.

Table 3.8 Association of malaria with respect to preventive measures.

Variable	Positive n(%)	Negative n(%)	Total n(%)	Chi square	P-value
Use of Mosquito Repellent					
No	63(15.8)	51(12.8)	114(28.5)		
Yes	50(12.5)	236(59.0)	286(71.5)	57.400	<0.0001*
Indoor Residual Spraying					
No	81(20.3)	169(42.3)	250(62.5)		
Yes	32(8.0)	118(29.5)	150(37.5)	5.665	0.017*
Mosquito Nets					
No	78(19.5)	83(20.8)	161(40.3)	54.229	<0.0001*
Yes	35(8.8)	204(51.0)	239(59.8)		
Insecticide-treated Bed Nets					
No	109(27.3)	266(66.5)	375(93.8)		
Yes	4(1.0)	21(5.3)	25(6.3)	1.974	0.160^{NS}
Water storage methods					
Closed	54(13.5)	253(63.3)	307(76.8)	74.033	<0.0001*
Open	59(14.8)	34(8.5)	93(23.3)		
Clothing					
No sleeves	43(10.8)	241(60.3)	284(71.0)	83.029	<0.0001*
Sleeves	70(17.5)	46(11.5)	116(29.0)		

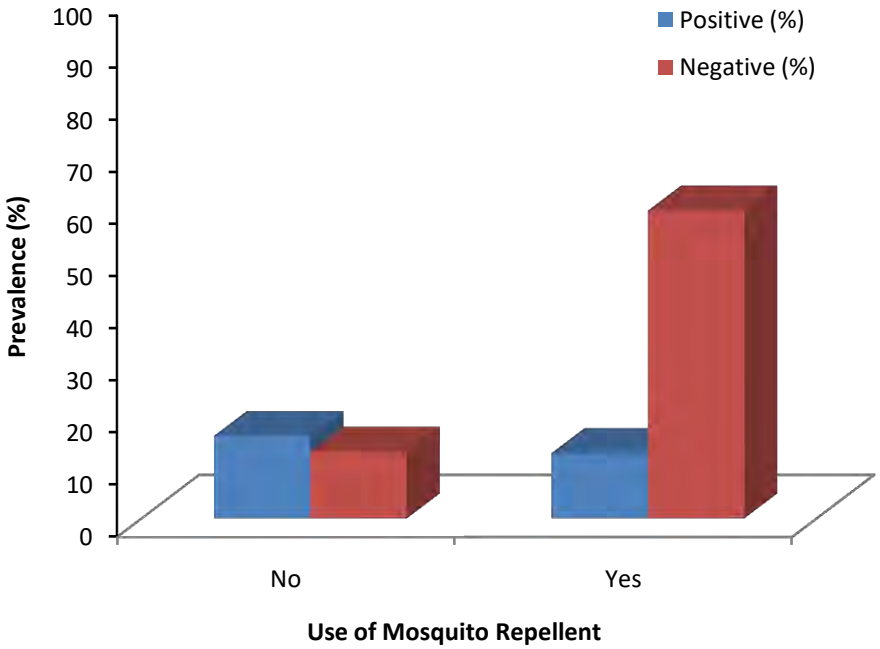


Figure 3.7: Malaria prevalence with respect to use of mosquito repellent.

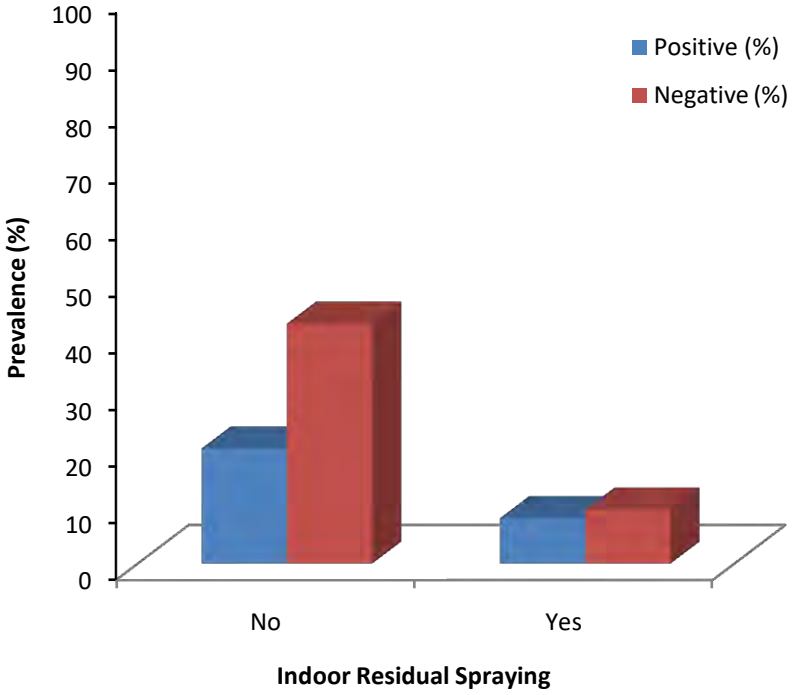


Figure 3.8: Malaria prevalence associations with indoor residual spraying.

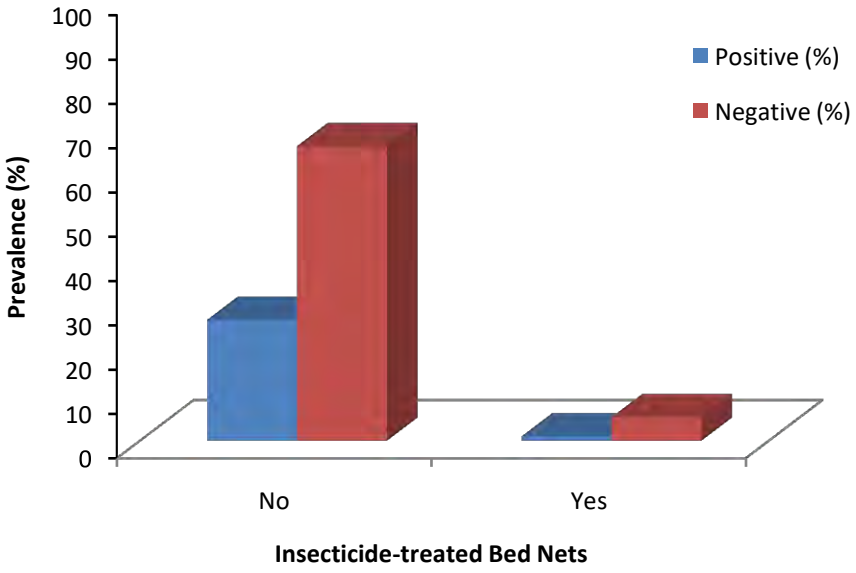


Figure 3.9: Association of malaria prevalence with insecticide-treated bed nets use.

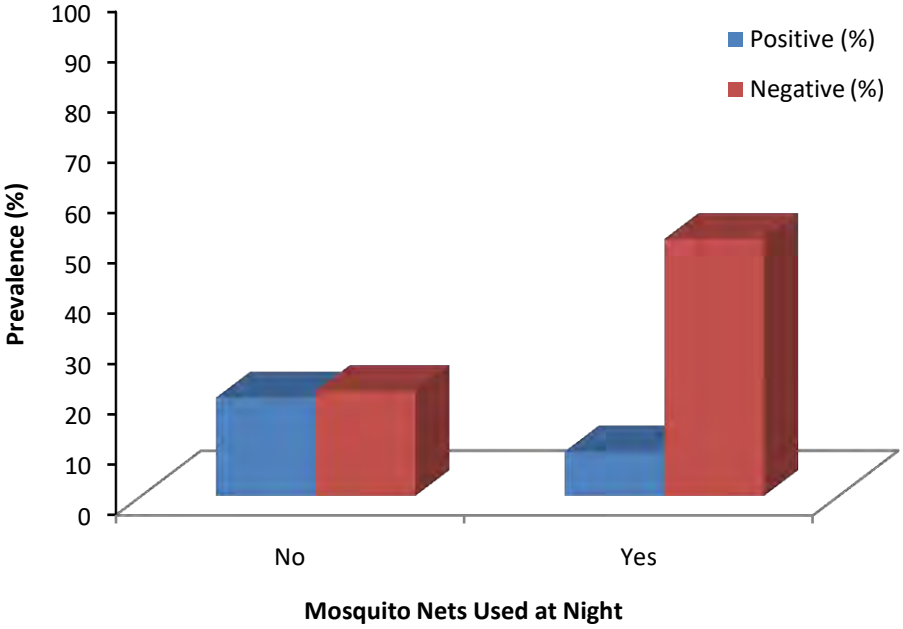


Figure 3.10: Association of malaria prevalence with mosquito nets use at night.

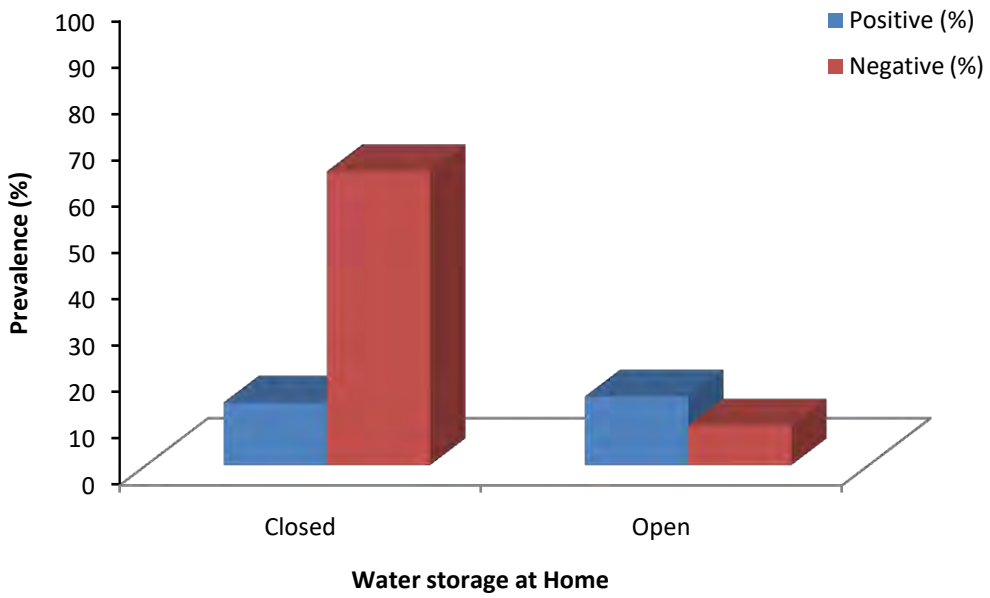


Figure 3.11: Association of malaria prevalence with water storage methods.

3.1.9 Association of malaria with environmental factors

Prevalence of malaria was found more 90(22.5%) in the patients having plants at home as compared to those having no plants 23(5.8%) and showed significant (χ^2=23.584, P=<0.0001) association. The sewage canal near the home association with malaria was high 78(19.5%) compared to patients having no sewage canal 35(8.8%) and difference was significant (χ2=23.584, P=<0.0001). The association of malaria with standing water bodies was found high 69(17.3%) as compared to others 44(11.0%). Malaria infection was high among patients with medium environmental cleanness 58(14.5%), followed by Low 41(10.3%), and high 14(3.5%), and difference was significant (χ^2=41.405, P=<0.0001). Malaria prevalence was seen much in the patients having home walls with holes 94(23.5%) as compared to those patients having no holes in the walls of home 19(4.8%) (Table 3.9).

Table 3.9.Association of malaria with environmental factors.

Variables	Positive n(%)	Negative n(%)	Total n(%)	Chi square	P-value
Presence of plants at home					
No	23(5.8)	134(33.5)	157(39.3)	23.584	<0.0001*
Yes	90(22.5)	153(38.3)	243(60.8)		
Sewage canal close to home				63.158	<0.0001*
No	35(8.8)	212(53.0)	247(61.8)		
Yes	78(19.5)	75(18.8)	153(38.3)		
Standing water bodies in surrounding area				88.977	<0.0001*
No	44(11.0)	246(61.5)	290(2.5)		
Yes	69(17.3)	41(10.3)	110(27.5)		
Environmental Cleanness				41.405	<0.0001*
High	14(3.5)	70(17.5)	84(21.0)		
Low	41(10.3)	28(7.0)	69(17.3)		
Medium	58(14.5)	189(47.3)	247(61.8)		
Holes in the Walls				132.819	<0.0001*
No	19(4.8)	227(56.8)	246(61.5)		
Yes	94(23.5)	60(15.0)	154(38.5)		

3.1.10 Relation of malaria prevalence with travelling history and any visit tohospital

Relation of malaria prevalence to travelling was found significant (χ^2=6.188, P=0.013), and the rate of infection having travelling history was more 67(16.8%) as compared to untraveled 46(11.5%). Malarial prevalence did not show significant association with visit to hospital before infection (χ^2= 0.258, p-value=0.612) (Table. 3.10).

Table 3.10.Malaria prevalence and association with travelling history and visit to hospital.

Variables	Positive n(%)	Negative n(%)	Total n(%)	Chi square	p-value
Travelling History				6.188	0.013*
No	67(16.8)	207(51.8)	274(68.5)		
Yes	46(11.5)	80(20.0)	126(31.5)		
Before Infection Visit to Hospital				0.258	0.612^{NS}
No	63(15.8)	168(42.0)	231(57.8)		
Yes	50(12.5)	119(29.8)	169(42.3)		

3.2 Molecular Analysis

3.2.1 DNA Isolation

DNA was isolated from malarial positive blood samples. The mean concentration of the isolated DNA was up to 50 ng/µl with a purity value of 1.8 for each isolated sample. The gel picture of extracted DNA is given in Fig. 3.12.

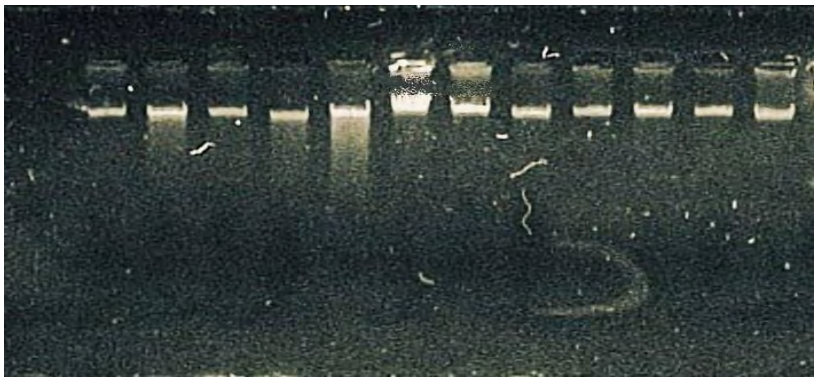


Fig. 3.12. DNA bands

3.2.2 Primer Optimization:

Gradient PCR was set for primer optimization. The annealing temperature of optimized PCR was 50°C for primer pair designed for mutation analysis of exon 4 of GYPB gene of malaria positive patients.

3.2.3 Polymerase Chain Reaction

To amplify the DNA samples of all collected patient's polymerase chain reaction (PCR) was performed. The PCR product of 245bp of exon 4 of GYPB gene was obtained (Fig. 3.13). 2% agarose gel electrophoresis was done for the confirmation of amplicon and was observed in gel documentation system (Cleaver Scientific Limited).

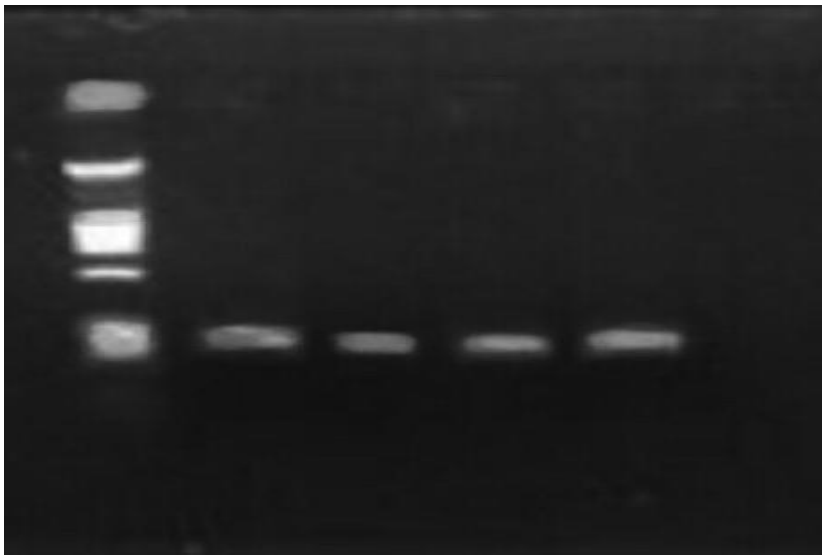


Figure: 3.13. In the first column 1kb DNA Marker was loaded and PCR products of 245 bp in subsequent columns.

3.2.4 Sequencing

The purified PCR product was obtained and stored at -20 C for further sanger sequencing. The sequencing was not done due to lack funds.

Discussion

Malaria is one of the major health problems and predominant of all parasitic infections in the world and is the major cause of sickness and mortality in developing countries including Pakistan (Shah *et al.*, 2016). Malaria has been reported in all ages of individual and nearly affects all sexes. Malaria incidence research works have been carried out before and have reported number of positive cases for the presence of *Plasmodium* parasites. Receptors for *Plasmodium* are glycophorin, present on Human and other animal erythrocytes membrane. Human glycophorin are of four types: A, B, C and D (Jaskiewicz *et al*., 2019).

The present study determined the demographic, environmental and clinical risk factors associated with malaria in district Bannu. The study includes 14 different areas of Bannu and detected overall 113/400 (28.25%) confirmed positive cases of malaria. The high prevalence rate was detected inhabitants of Miryan 16 (4.0%). The high occurrence in this area might be due to the presence of agricultural land, streams, rivers, and water channels which provide breeding grounds for mosquitoes. A study (Khan *et al*., 2012) reported high prevalence rate of 4.26% in Bada Mir Abas, Mandew, and Pir Khel Kakki, which may be due to their stagnant marshy waters, poor sanitary conditions, and high population. Another study reported increased prevalence of malaria infection of 8% in Bada Mir Abas, Mandew, and Pir Khel Kakki which might be due to huge number of rice growing fields (Awan and Jan, 2008).

Gender wise prevalence showed that males were infected more than female with 18.3 % prevalence consistent with previous findings (Khan *et al*., 2006). Another study recorded more prevalence in male population as compared to female that was 68.90% and 31.09% respectively (Ibrahim *et al*., 2014). The predominance of malaria in male in our society could be due to more outgoing behavior as compared to females (Khan *et al*., 2006) and females are usually covered.

In the present study the prevalence of malaria was high in AB-ive, B+ive and O+ive blood groups. *Plasmodium vivax* was the only species reported in the study participants. Previous study reported *P. falciparum* prevalence in blood group A was 0.7% and 1.1% in blood group B, 5.6% in AB blood group and 8 % in O blood group, while *P. vivax* prevalence was 5.1% in O blood group (Alemu and Mama, 2015). Similar findings are reported in subsequent studies, with blood group O having a

higher malarial prevalence than blood group A in malaria-endemic tropical regions (Epidi *et al*., 2008; Sirina *et al.*, 2013; Tadesse *et al.*, 2013; Mandefro *et al.*, 3014; Oladeinde *et al.*, 2014). While studies in malaria-free cold regions revealed that malaria prevalence was higher in blood group A than in blood group O (Uneke *et al.,* 2006; Mourant *et al.,* 1976). Results from the study (Alemu and Mama, 2015), thus support the idea that *P. falciparum* has evolved to shape the distribution of the ABO phenotype.

The current study observed different prevalence rates of malaria in different age groups; the prevalence rate of malaria parasite in the age group of 11-20 years was 8.3% higher from other studied age groups. Similarly, a study recorded the highest 19.3% prevalence rate of malaria in age group 5-15 years as compared to other groups (Umaru *et al*., 2015), consistent with other studies (Noland *et al*., 2014; Mawili-Mboumba *et al*., 2013). The primary cause of the change in the prevalence of malaria in this age group may be because younger children are given more attention than older ones, such as by benefiting from control measures like sleeping under mosquito bed nets. Another fact is that older kids typically spend time away from home for boarding school and other reasons, they might not receive the same level of direct parental care and supervision (Umaru *et al.,* 2015).

Locality wise results showed that malaria prevalence was high 18.5% in rural areas as compared to urban areas, consistent with previous study in rural areas of district Bannu (Khan *et al*., 2013). The reason may be due to the fact that rural areas have agricultural land, which provides more breeding places for mosquitoes. The results also recorded a high prevalence in patients having low literacy rate, consistent with previous study that primary level education of patients or guardian is a risk factor for malaria infection (Dike *et al*., 2006).

According to Konradsen's (2003) study, housing conditions such as poorly built houses with mud walls and thatched roofs are also helpful in the higher risk of malaria. The present study also found that poor mud houses with holes in the walls are high risk factors for malaria. Contrarily, according to Konradsen *et al.,* (2003), homes made of cement and bricks can reduce the risk of malaria. This means that homes with thatched roofs, mud walls and walls with holes act as habitats for mosquitoes and raise the risk of malaria.

The current study also found that the risk of malaria was higher in patients with a nearby sewage canal (78%), while it was lower in patients without a nearby sewage canal. Malaria was found to have a high association with water bodies in the studied area, such as lakes, ponds, or standing water. The

importance of larval habitats close to houses was further highlighted by a study that claimed people with open sewage systems at home have a higher risk of developing malaria than people with closed sewage systems. The present study recorded malaria prevalence was found to be higher 22.5% in patients with plants in their homes as compared to those without plants, which highlights the how plants serve as habitat for mosquitoes and raise the risk of infection (Hasyim *et al.,* 2019).

In case of prevention the prevalence of malaria was found less in the individuals using mosquito repellent, having indoor spray, using mosquito bed nets and using insecticide treated bed nets. Poor knowledge regarding malaria control, utilization of preventative measures and malaria treatment has been observed as a risk factor for high occurrence of malaria (Khan *et al*., 2021).

The present study attempted to amplify genetic association of erythrocyte membrane protein glycophorin (GYP) GYP-B gene exon 4 with occurrence of malaria. Fifty malarial positive samples were amplified for GYPB gene exon 4. Many of the glycophorin variants that are common in regions where malaria is endemic lead structural change in receptors (Race and Sanger 1975; Mourant *et al.,* 1976). Huang *et al.* (2000) recognized that Africans population tends to have more variations. A study reported mutations in and around exon 5 of GYPB leads absence of S antigen at the surface of RBC. Another mutation at nucleotide position 251 (C/G) in GYPB*s allele was also recorded (Storry *et al*., 2003). Leffler *et al*. (2017) identified a specific variant that encodes hybrid glycophorin proteins and showed that this variant is associated against severe malaria protection. Ko *et al*. (2011) identified a common polymorphism in GYPB at exon 4 that determines the Ss blood. Cserti *et al*. (2007) recorded mutations in the glycophorin gene, which may aid the host's immune system in controlling the malaria infection. Therefore, further research on sequencing of glycophorin gene in Pakistani population is required.

Conclusion

It is concluded from the present study that children and teenagers were at high risk of malaria as compared to adults. Poor hygienic conditions of environment and household, water bodies and vegetation, lack of preventive measures remain major risks factors for high prevalence of malaria. All malarial cases were caused by *Plasmodium vivax* and there was no single case of other species or mixed. It is concluded that the possible reason for negativity of *Plasmodium falciperum* could be polymorphism in GYP receptors. According to blood groups high prevalence of infection was found in AB-ive, B+ive and O+ive blood groups.

Future Recommendation

- Further studies should be carried out to sequence glycophorin gene in Pakistani population.
- Awareness seminars should be arranged to spread knowledge about malaria among people.
- Control measures (spraying, providing of bed nets and better sewage system) should be taken by government to lower the risk of malaria.

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Annexure

Composition of solution used in DNA extraction

Solution	**Composition**
TE Buffer	2mM EDTA, 10mM Tris HCl (pH=8.0)
Proteinase Kinase	10 mg/ml
TNE Buffer	10mM Tris HCl, 400 mMNaCl, 2mM EDTA
SDS	20%
NaCl	6M
PCI	P(50): C(48): I(2)
Isopropanol (chilled)	100%
Ethanol	70%

Composition of agarose gel and other required chemicals

Solution	**Composition**
1% Agarose Gel (50ml)	1X TBE (50ml) Agarose (0.5g) Ethidium Bromide (2µl)
2% Agarose gel (50ml)	10X TBE (5ml) Agarose (1.0g) Ethidium Bromide (5µl) Distal Water (45ml)
Gel preparation buffer (10X TBE)	Boric Acid (27.5g) EDTA (3.65g) Tris (54g) Deionized water (45)
Gel Running Buffer (1X TBE)	10X TBE (1 part) Distal water (9 part)
Ethidium Bromide (50ml)	Autoclaved filter water (50ml) Ethidium Bromide (0.5)
Loading Dye (25ml)	Autoclaved filter water (25ml) Bromo-phenole blue (0.0875g) Sucrose (10g)

FASTA Sequence of Selected Exon:

CTAGCCTCTGAAATATCTCACAGAAATAATACTGTGAGTTAAAGAAATTAAAACAATGTGGCAAAGCACAG
AAATGATACATGTGACCATGAAATAGTGGGCCAGATAAAGGGGACCTAATAGTGCGGTGGTGCGGAGGGTC
TGTGGGCAAAATGAGTTCAGCTCAGACCTGGGCTCAGCTCTATCCCAGCTGCTGACCCAGGGTGAGTTGCCC
TGCAGGGTTTCTATCCCATTAATTTTAAAATGGGGCCAATAACACAGTACTTATCTCACAGCATTTCTCTAAA
GGCTAAATAAGAAGAAGTGTCTAAAAGTTATTAGCTCAGAGCCTCACACATTCTCAGTGACTGATAAACAA
TAAGCAAAGCTGGGTGCTGAGATAAGAGTAATCTGGTGGCAGTCTCTCTTGTTAGTTTTCAGGGGAGAAGA
AGAAATTCTGGAGCTGCTGCTGGGAGGGATGTGGGAGAATTTGTCTTTCATGATACGCTGTATGTCCACGCA
GTCACCTCATTCTTGTTCCCTTTCTCAACTTCTCTTATATGCAG**ATAAGCACAAACGGGACACATGTCCAG**
CTCATACTGCTAATGAAGTTTCAGAAATTTCTGTTACAACTGTTTCCCCTCCAGAAAAGAAAAACGTTA
TGTTCTTAGTTTTAAATAGTTTCTCTGGAGTCATTGTTGTGATTGAACTCTATTTACACGAGCTGTAAC
TCATGACAGTTCTCAAACTTTCGTGACAGAAAACCCAACTCTTTTACTCCAAAGCCCATATAGCACCC
ACAACTATTAACTGTGACCAAGAAAGAGAAGGCAAGCCCCAATTAACCTTTGTACGTAAAGCCTAAAG
AATGAAAAAATATACCTGAATCCTCAATCATCAAACAACATAGCATATACTAAGTAATTTGTAATAATT
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GACAAAAGTAGGATGTGGACTGAGAGGGAAGGTTAGCACAGGTGGAACAGTAAGGCAACCATACTATCAA
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GTGGAGTAATGGCTCCATATGCCTCCTTTCTTCTCATTATTTTTTACATGTTTTAAAAATGCATTGCTTCTTGT
GTAAGTCAATAAGTGATTCTTCCAATACTTTCTCATTCCTTTCCCCTCAGTTATGAGACAATTTGCTTATTTCT
CATCCATGAATACGTGTTGGGTCATTAAAAGTAGATACTGAAATTACTAATGGTAAGACTGACACATTACCT
CATAAATGTTACTAGCTAGATGTTGAAAGTTGACCAACAACTCTCAAAATATGATTAAGAAAAGGAAACCC
GCAGAACAGTTTGATTCCAAAATGATTTTTTTCTTTGCACATGTCTTTCTTATTTGGACTTACATTGAAATTTT
GCTTTATAG**GAGAAACGGGACAACTTGTCCATCGTTTCACTGTACCAGGTATGTTAATATTTGACAAAG**
AATAAAAGTCATTCCATTTTAAACTATCCATTGCTTGTTTCAAATGCCTAAGAAAATGTGTCTATCTTA
AAACAGAAGAGCATATGTTGTTAACTTTATTCACACGAAATTGTAAAGACAAAGAAAATATTCTCTTTT
TAAAATTAAAATAGGCATTTCTTATTTTTAAAAACATTTTGGGGGCCAGGGGCCGTGGCTCATGCCTATAAT
CCCTATAATTTTGGGAGGCCAAGCCTGGCTAATCACTTGAGCCCAGGAATTTGAGAACAGCCTGGGCAATAT
GGCAAAATCTATCTCTACAAAAAATACAAAAATTAGCTGGGATGGGGCATGCACCTGTAGTCTCAACTGCTT
GGGAGGCTGGCTGAGGTGGGAGGATCGGATCCATTGCCTGAGCCTGGGAGTTTGAGGCTGCAGTGAGCTAT
GACTGTGCCACTGTACTCTAGCCTTGGTAAGACCCTGTCTCAAAAACAAATACATAAGTAAATAAAAATAA
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TGAGTATCACTGTGTGTGCTCATTTGCTATTCTGCATTCTAACCCTCCCTTCACACTTGGCCGTGTCCATGGCT
CACAGGGTAAAAAGCACATCATAGAACTTCATCACTATCGCATACATTCAAGCTAAGTGGTCAAGAAGGCT
GGGCAACACCAGCAAGAGGAAATGCTACTTTTACTTTTTGTGAATAATTTAAATATTAATTAGGCAAATAAA
TGAGCCATTTTACCTGTATGTCTAGCCTTCCATTCTATTTACTTCATCTGGAAGTACTACAAATATGCTATAA