Prevalence and Distribution of Human *Plasmodium* Infection in Bannu, Khyber Pakhtunkhawa Pakistan

By

Kalsoom

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

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Declaration

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

Kalsoom

IN THE NAME OF ALLAH

THE MOST BENEFICENT

THE MOST MERCIFUL

Dedication

With profound love & deep respect this dissertation is dedicated to my

Parents

and

honorable supervisor

Dr Naveeda Akhtar Zureshi.

List of Abbreviations

Abbreviations	Full Name
API	Annual Parasite Incidence
BER	Blood Examination Rate
SPR	Slide Positivity Rate
IDW	Inverse Distance weighting Interpolation Method
EDTA	Ethylenediaminetetra-acetic acid
PCR	Polymerase Chain Reaction
Ml	Mili liter
GIS	Geographical Information System
UV	Ultra violet
RDT	Rapid Diagnostic Test

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Kalsoom

ABSTRACT

Malaria is a vector borne disease of old world that is regaining epidemic situation in many countries of the world including Pakistan. *P. vivax* and *P. falciparum* major are the most frequent causative agents of malaria in Pakistan. The objective of the current study was to investigate the prevalence of malaria in District Bannu. In the previous two years (2016-2017), a total of 193419 of blood films were examined microscopically in different Health Care Center. An estimated of 1258516 population living in Bannu is at risk of contracting malaria at making it the leading public health problem. The temporal analysis of malaria data could be important to evaluate the performance of malaria prevention programs. The present study based upon the recorded data of 193419 malarial patients registered in 2016-2017. Chi square and Inverse Distance weighting model (IDW) were used to analyze the data. In 2018 a total of 500 blood samples were collected from malaria suspected patients. Microscopy and nested-PCR were used for detection of species of *Plasmodium* causing human malaria. The following patient data were retrieved from laboratory registration logbook for analysis; sex, age, residence, blood film (BF) microscopy result, type of malaria parasite identified, year and month when the patients visited Health Care Centers. Among the total 28700 microscopically confirmed cases 50.8% were males and 49.1% were females. The overall prevalence of malaria was 16.43% annual parasite incidence in 2016 was 1.32% and in 2017 was 0.9%. The two species of malaria parasite identified were *P. falciparum* 3.8% and *P. vivax* 95.05%. Mix infection 1.10% was also found in the study area. Relatively higher proportions of cases were documented in the months of August, September and October respectively. Similarly, patients in the age group above >14 were more likely to be infected than individuals 5-14 and <5, as 61.64%, 27.7% and 22.05 respectively. The PCR result showed that out of 500 samples 281 were positive through microscopy and 315 samples were positive through PCR. Negative samples confirmed through microscopy were 219 samples and through PCR 185 samples. The current study showed that *P. vivax* is the most prevalent species in District Bannu.

INTRODUCTION

The malaria is a vector borne protozoan parasitic disease of the genus *Plasmodium* belonging to *phylum Apicomplexan* (Gamit and Trivedi, 2014). Malaria parasites are transmitted through the bites of adult female mosquito. Five known species cause human malaria viz., *P. falciparum, P. vivax, P. ovale, P. knowlesi* and *P. malariae* (Majid *et al.*, 2016). Human malaria parasites are transmitted by *Anopheles* mosquitoes. There are 465 formally recognized species, more than 50 members of species complexes are still unnamed (Sinka *et al*., 2012). Almost 70 species of *Anopheles* mosquitoes have ability to transmit human malaria parasite, 4 being the major vectors viz., (*A. gambia*, *A. funestus*, *A. arabiensis* and *A. melas*). In the highly dense and forested areas *A. gambia* is responsible for the transmission of *Plasmodium* species. *A. funestus* has a bumpy distribution while *A. Miles* is a saltwater species (Nmadu *et al.*, 2015). Malaria parasite was discovered for the first time by a French army officer, Charles Louis Alphonse Laveran in 1880. For his discovery, Laveran was awarded the nobel prize in 1907. In 1897 Ronald Ross discovered the species of mosquito which act as a vector for transmission of avian malaria and recorded as carrier for human malaria by Italian scientists, between 1898 and 1900 (Cox, 2010).

1.1. Global Distribution of Malaria

The malaria disease is not only confined to the tropical region (Yasinzai and Kakarsulemankhel, 2007) but is a major threat to economic development and human health worldwide (Khatoon *et al.*, 2010). According to the report of the World Health Organization 106 countries are at high risk of malaria infection around the globe (WHO, 2011). Malaria is one of the deadliest killers of human populations (Yasinzai and Kakarsulemankhel, 2008) leading to 1.5 to 2.7 million cases of mortality annually (Trampaz *et al*., 2003). Malaria is endemic in Africa, Southeast Asia, Oceania, Haiti, parts of the Amazon basin of South America, and the Dominican Republic (Bardach, 2015). According to an estimate of the World Health Organization in 2006 about 247 million people suffer from malaria globally on an annual basis and resulting in one million deaths per year, primarily children under the age of 5 years living in sub Saharan Africa (Messina *et al*., 2011). In 2013, the major outbreak was reported,

globally and approximately 584,000 deaths were reported with 72.2% cases from South America, e.g., Brazil, Venezuela and Colombia and a minor proportion, 2% from Latin America and 10% from Central America (Vasquez-Jimenez *et al*., 2016). Among all *Plasmodium* species *P. falciparum* is the most pathogenic (Price *et al.,* 2007) and most prevalent in Africa, but in most areas where malaria is endemic multiple sympatric species and co-infection is common in human hosts or in mosquito's population (Gnémé *et al.,* 2013).

The *Plasmodium falciparum* and *P. malariae* is most frequently found in Africa. The co-infecting species in human host can change transmission potential and modify host dynamics (McKenzie *et al.,* 2007). *Plasmodium falciparum* causing over 2 million cases per year and inflicting virtually all 300,000 annual deaths recorded mainly in children of Sub-Saharan Africa (Loy *et al.,* 2017). *Plasmodium vivax* is less common in Africa but is the most prevalent species in Asia, Oceania, central and south America, where it affect 6 million people which represent half of all malaria cases outside the Africa. *Plasmodium vivax* has low virulence than *P. falciparum* has but been neglected largely in control (Khatoon *et al*., 2010). Recent reports from Indonesia, Thailand, India and Papua New Guinea showed that 21-27 % patients with malaria have *P. vivax* (Price *et al.,* 2009). In the highly endemic area of Nigeria, 80 % peoples were affected by *P. falciparum* with a death rate of 30 % of children, 11 % of maternal death and 50 of outpatient consultations (Okeke *et al*., 2010).

The prevalent disease throughout the Sudan is malaria, were 7.5-10.0 million cases occur every year leading to l35, 000 mortality. The prevalence of *Plasmodium* parasites was also studied in Indonesian population and among 350,000 positive cases with 51% patient infected with *P. falciparum* (WHO, 2011). In 2009, about 2.85 billion people were at high risk of *P. vivax* infection and 91% of these occurred in Central and South East Asia (Naing *et al.,* 2014). *Plasmodium vivax* is the predominant and accounts for the 1/3 of all malarial cases in India. In 2014, approximately 5.14 million confirmed cases of *P. falciparum* were reported globally, 18% of which occurred in India mostly in children under the age of 1-14 year. Asia become second to Africa in term of malaria infection, where malaria is endemic in 19

countries of Asia affect 2.35 billion peoples (WHO, 2013), in 2010 about 34.8 million cases were reported from Asia with 456,00 death causalities (WHO, 2011).

Pakistan is the endemic country for both *P. falciparum* and *P. vivax* (Khattak *et al.*, 2013) with 1.5 million cases reported annually (Majid *et al.*, 2016). In Pakistan 7% of its populations are at high risk of malaria infection. According to an estimate 1.6 million positive cases are reported annually in Pakistan (Tareem *et al*., 2012). Round about 60% of cases 64% diseases are caused by *P. vivax* and 35% by *P. falciparum* (Khattak *et al.*, 2013).

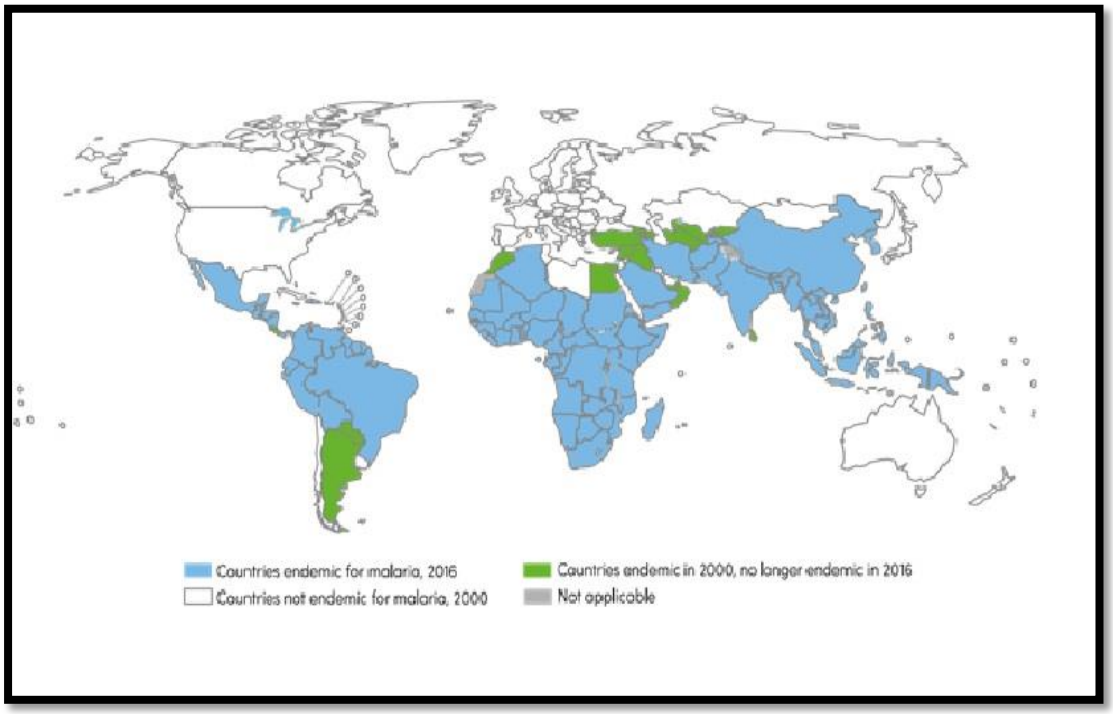

Figure 1.1: Global map of malaria distribution 2016 (Adopted from WHO, 2016).

1.2. Prevalence of Malaria in Pakistan

Pakistan is in the list of moderately malaria endemic countries (Murtaza *et al*., 2009). As a tropical and agriculture country, most of the population is poor and living in rural areas, increasing the incidence of malaria in those regions. Several factors are involved in providing a suitable environment for the transmission of malaria (Khan *et al*., 2013). These factors include poverty, environmental deterioration and especially resistance to chloroquine (Rowland *et al*., 1997). Malaria transmission is also associated with seasonal variation as most of the malaria infections occur in post

monsoon season, i.e. from September to November (Khattak *et al.,* 2013). Several other factors, including, urbanization, an extensive irrigation network, continuously migrations of Afghan refugees, increase in population and sudden environmental changes due to excessive rainfall, floods and water development projects acts together, making the environment suitable for *Plasmodium* species in many regions of Pakistan (Tareem *et al*., 2012).

According to malaria control program in Pakistan 500,000 positive cases were reported in 2011, with of which 50,000 death causalities (Khattak *et al.,* 2013). In Pakistan the transmission period for *P. vivax* malaria is June to September and April to June. For *P. falciparum* malaria this period is August to December. Malaria infection caused due to *P. falciparum* and *P. vivax* is the major health problem in Pakistan. In Pakistan malaria occurs in the province of Balochistan, Khyber Pakhtunkhwa (KP) and Sindh (Khattak *et al.,* 2013).

The incidence of malaria is high in Balochistan and Federally Administered Tribal Area (FATA) (Rahman *et al*., 1999). In Quetta region (Balochistan), a total of 1662 positive slides was confirmed for malaria of which 505 were positive for *P. falciparum* with cerebral malaria (Durrani *et al.,* 1997). About 41% of the cases were observed in District Zhob. According to an estimate in 2012 about 1896 positive cases were reported from District Charsada (KP) 1616 cases with *P. vivax* and 280 with *P. falciparum* (Ali *et al.,* 2013). According to the World Health Organization the *Plasmodium* frequency increased from 34% to 54% between 1987- 1990 and from 45% to 68% between 1995 to 2006 in the city of Quetta (*Balochistan)* and in the Jhangara city of Sindh (Khattak *et al*., 2012). In 2010, a total of 83857 malaria cases was reported in Pakistan, of which 240,591 cases were infected by *P. falciparum* (Khattak *et al*., 2013).

The mixed infection is also common in Pakistan. Association of *P. falciparum* with *P. vivax* was reported from Balochistan and KP with 10% and 8% of the cases, respectively (Khattak *et al.,* 2013). The higher prevalence of *P. falciparum* in Pakistan may be due to chloroquine resistance (Nizamani *et al.,* 2006). Resistance in *P. falciparum* against chloroquine was reported for the first time from Pakistan in 1984 (Ghanchi *et al*., 2016) and confirmed as widespread (Nizamani *et al.,* 2006).

According to a recent study conducted by Rawasia *et al.* (2012) about 90% of the *P. falciparum* samples were collected from the provinces of Sindh and Balochistan with pfcrt 76 T allele, which is responsible for chloroquine resistance. In Pakistan the high prevalence of *P. falciparum* may also be related to the heavy influx and presence of refugees from Afghanistan. A survey conducted by Kakkar *et al.* (2010) from 2005-2009 showed variation in malaria endemicity.

The various disasters happened in the past are the reason of increasing malaria burden such as recently occurred seasonal floods with some 10 million people affected in 60 Districts of Pakistan (Khattak *et al*., 2013).

The aim of the current study was to evaluate the prevalence of malaria in District Bannu. Bannu has a plane area located in the Southern part of KP Pakistan and has a high rate of transmission of malaria. Due to the presence of marshy and stagnant water, poor sanitary condition in villages of study area results an increase in the vector populations. Rice fields and dates dropping to the stagnant water also provide a suitable environment for mosquito breeding in these areas (Khan *et al.,* 2013).

1.3. Clinical Signs and Symptoms of *Plasmodium* Infection

The clinical symptoms of malaria are different for all five species of *Plasmodium* (Azikiwe *et al.,* 2012). Initial manifestations of malaria common to all species of *Plasmodium* are nonspecific and similar to flu like syndrome (Bartoloni and Zammarchi, 2012). Early nonspecific symptoms include malaise, anorexia, dizziness, with a desire to stretch limbs and yawn, headache, backache in the lumbar and sacroiliac region, myalgias, nausea, vomiting, diarrhea, chillness and fever (Ali *et al*., 2013). At first time the temperature is irregular then leading to high temperature with shivering and mild chills (Bartoloni and Zammarchi, 2012). *Plasmodium falciparum* causes cerebral malaria, anemia, pulmonary edema and kidney problem, in some cases may progress leading to coma, convulsion and sometimes death. Adults have most likely had jaundice while children suffer from enlarges liver. Splenomegaly and chronic anemia are found in young children where malaria transmission is stable. The severity of *P. falciparum* malaria depends on age. Hypoglycemia and anemia are mostly found in children, whereas pulmonary, edema kidney injury and jaundice are more common in adults (Ashley *et al.,* 2014). *Plasmodium vivax* is an important cause

of malaria caused morbidity and mortality in Papua province, Indonesia, and in New Guinea causing chronic anemia, especially in young infants and children (Bartoloni and Zammarchi, 2012). The mortality rate rises when the ratio of infected blood cells exceeds 2%, although the relationship between prognosis and parasite density in case of *P. falciparum* is variable. When treated properly with antimalarial drugs, the mortality rate due to *P. falciparum* decreases up to about 0.1% (Ashley *et al.,* 2014).

1.3. Diagnosis of Malaria Infection

The effective treatment of malaria and accurate diagnosis is necessary to prevent morbidity and mortality (Willson, 2012). There are three methods used currently for diagnosis of malaria, which include microscopic examination of stained slides (Giemsa, Field, Wright, or alcidine orange stained films), detection of parasite antigens (histidine-rich protein 2, plasmodial lactate dehydrogenase and molecular biology method, (polymerase chain reaction, PCR). Microscopy is the cost effective and widely used technique for the diagnosis of malaria at the peripheral level (Azikiwe *et al*., 2012) but leads to many technical mistakes or low expertise in the peripheral health units using Giemsa stained slides. Sometime low parasite burden or infection in early stage is not detectable using microscopy, therefore PCR is a best and accurate technique used for the detection of parasite (Molla, 2016).

The microscopy (Moll, 2016), and rapid diagnostic test based on antigen detection is used which is the easiest technique and does not require special training or equipment to perform accurate result. It can be carried out in short time (Azikiwe *et al.,* 2012). The results are available within 12 to 15 minutes (Tangpukdee *et al.,* 2009) but it is unable to detect mix infection and differentiate between species of *Plasmodium*. For the detection of mix infection and low level of Parasitemia, as few as 1-5 parasites/ µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/ µl of blood by microscopy or RDT (Rodulfo *et al.*, 2007) PCR is used for the identification of all four human species of *Plasmodium,* but it also have some drawbacks, being highly cost, specific, and workload, have complex methodology and need special trained experts cannot routinely use in laboratories (Rodulfo *et al.*, 2007).

1.4. Life Cycle and Transmission of Malaria Parasite

The five species of *Plasmodium* causing malaria have very similar life cycle (Cox, 2010). Life cycle of *Plasmodium* starts when the female anopheles mosquito injected sporozoites into the blood while feeding on human. These sporozoites circulates into the blood stream for some time and then enter into the hepatocytes, where they undergo asexual reproduction (exoerythrocytic schizogony) and produce a large number of uninucleat merozoites per liver cell (Trampaz *et al*., 2003). In case of *P. vivax* and *P. ovale* some of the merozoites remain in the liver passes through dormant stages, may be for a month or year called hepnozoites. *Plasmodium falciparum* has no hepnozoites stage. The merozoites exit the liver and re-enter into the blood circulation and invade the erythrocytes, continuous the asexual multiplications. Within the erythrocytes parasite developed into uninucleate vacuolated ring shaped trophozoite (erythrocytic schizogony).

The internal replications occur and these infected RBC's are rupture and further release 8-16 daughter merozoites which attack other non-infected RBC's and a new schizogony cycle is started hence developing into gametocytes after several cycles (Garcia, 2001). The infected host exclusively infective to the female anopheles mosquito by taking gametocytes during blood meal and after ingestion these gametocytes mature and develop into male and female gametes become fused and form zygote inside the gut of mosquitoes (Shahabudin and kaslow, 1994). These ookinetes then penetrate the gut wall of the mosquito and rounds up to form oocyst, where again sporozoites produced and migrated toward silvery gland. The mosquito then transfer these sporozoites into the non infective human host during blood meal and the cycle are started again (Cox, 2010).

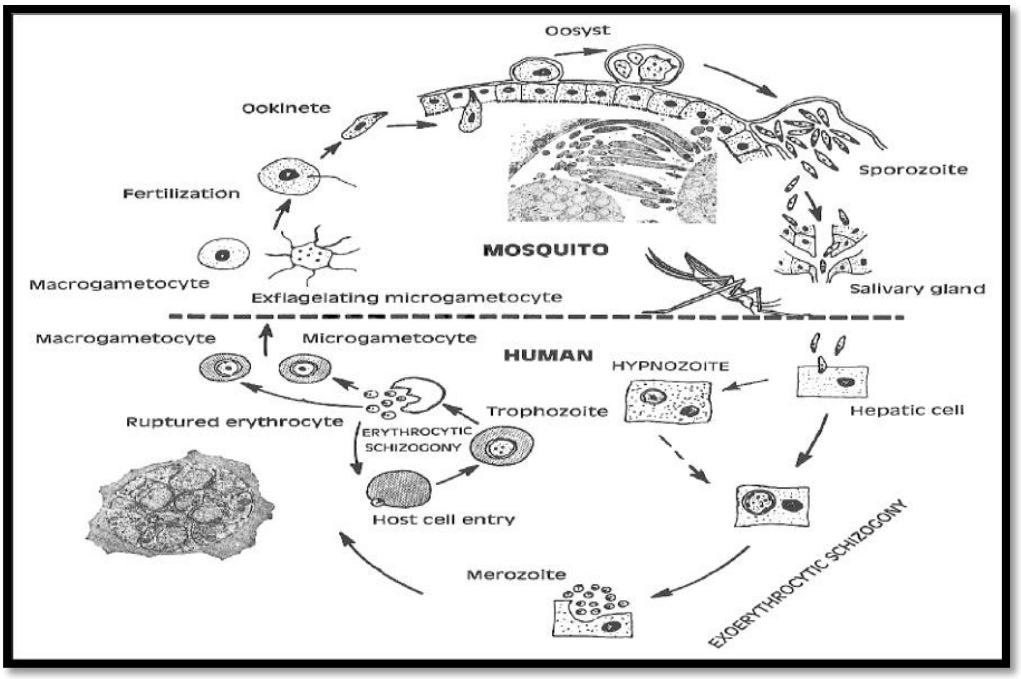

Figure 1.2: Life cycle of *Plasmodium* parasite (Fujioka and Aikawa, 2002).

1.6. Recurrent or Persistent Malaria

The *P. malariae*, remains in blood for months or years when not treated properly. Antigenic variation leads to gametocytaemia and parasitaemia. In tropical region, *P. vivax* relapses approximately every 3-4 weeks or every 6-8 weeks when treated as the drugs suppress the first relapse where as in temperate regions *P. vivax* can remain for 8-10 months between first relapse and primary infection (White, 2011). Recurrent *P. vivax* and *P. Falciparum* malaria has adverse effect on child development, growth and schooling (Williams, 2012).

1.7. Factors Affecting Malaria Transmission

Factors like the environment, urbanization, agriculture, (Messina *et al*, 2011), socioeconomic condition of individuals (Coleman *et al.*, 2006), migration (Tatem *et al.*, 2013), poor health facilities and quality of health services (Snow *et al.*, 2003), can affect the transmission of malaria (Messina *et al*, 2011). Environmental factors include land cover, rainfall; altitude and temperature have great effect on the mosquito breeding site and have been used to forecast malaria transmission risk. An area with high temperature and precipitation provides a favorable environment for mosquito breeding and also for parasite reproduction within the mosquito. Agriculture

factors, i.e. highly cultured area also provides appropriate habitat for most of the primary vectors which are non-forest and prefer sunlight while urbanized area have reduced mosquito breeding habitat although decreased hygienic condition in urban areas promote mosquito breeding in some instance (Messina *et al.*, 2011).

1.8. Effect of Temperature

The malaria transmission can be affected by temperature in several ways. Temperature plays an important role in the life cycle of the malaria vector, affect the development and survival rate of vector and malaria parasite (Zhou *et al.*, 2004). Temperature between 25°C and 30°C is the optimum temperature for the development of the parasite in the female anopheles mosquito (sporogony) and the development of parasite cease down below the 16°C (Mordecai, 2012). Temperature above 32°C leads to vector mortality due to production of weak individuals (Musa *et al.*, 2012). The decreasing patterns of temperature highly affect the number of cases due to destruction in the population of vectors and also increase in their developmental duration (Craig *et al*., 1999). Both human malaria and avian malaria vectors show high sensitivity to temperature fluctuations (Okanga *et al*., 2013). Temperature is strongly associated with altitude; the temperature drops by 0.5 at every 100 meter increase in altitude (Snow *et al*., 1999).

1.9. Rainfall and Humidity

The important factors like temperature, rainfall and relative humidity that affect the large scale distribution and transmission of malaria (Musa *et al*., 2012). Rainfall provides suitable relative humidity for mosquito survival and breeding site to lay their eggs (Ezihe *et al.*, 2017). Rainfalls make the environment ideal for mosquito breeding sites by aid in the accumulation of stagnant water (Kurup *et al*., 2017). Climatic perspectives of Pakistan are tropical with an extensive irrigation system. In monsoon season due to heavy rainfall, large quantity of water are stored in dikes which provide proper habitat for mosquito breeding. After rainy seasons huge number of malaria cases but transmission of the disease is continuing throughout the year (Zeb, 2015). In regions having large lakes due to the heavy rain or floods the chances of malaria infection is more common. Rainfall and humidity increase the breeding sites for mosquito to lay their eggs, without water surface the female anopheles

mosquito cannot lay their eggs. Humidity over 60 percent increases the longevity of adult vector (Snow *et al.,* 1999).

The heavy rainfall provide breeding site for mosquitoes as they creating temporary pool for mosquitoes. But it also has adverse effect on mosquito transmission cycles as it washes out the larvae as well as adults and destroy the mosquito breeding pools by converting them into the stream (Gemperli *et al*., 2004). Altered rainfall pattern may also cause a decrease in endemicity. A decrease was observed in malaria transmission after the drought in the 1970 in the Saher (Ermert *et al*., 2012). The behavior and survival of anopheles mosquito are affected by the interaction among rainfall and temperature evaporation runoff, which control the humidity and ambient air (Gemperli *et al*., 2004). The life span of the vector is shortened when the monthly average humidity is less than 60°C which make it unsuitable to transmit malaria (Musa *et al*., 2012).

1.10. Malaria Transmission Season in Pakistan

The malaria transmission is influenced by various factors like rainfall, humidity and also by seasonal variation in temperature, which the predominant factor in explaining the geographical distribution of the diseases. The unhygienic and low level of irrigation systems are located in some Districts in the south of Pakistan providing an ideal surrounding for mosquitoes breeding (Khan, 2014). The malarial infection due to *P. falciparum* was noticed high and touches the peak in warmer months (June-September) while relapse occurs in April to June (Molineaux, 1988). Likewise the highest prevalence was also recorded in the month of August in Multan and Balochistan (Mastung and Khuzdar) whereas the rate of infection decreased in November (Bhali, 2001). In District Buner of KP province which is situated in hilly areas showed that the highest incidence of malaria is increasing in hot and dry months, whereas the number of cases decrease in the Winter season of the year (Khatoon *et al.,* 2013).

1.11. Malaria Treatment Overview

The neglected disease in the last 20 years was malaria, which causes about 1 million deaths annually. This high mortality from malaria was due to reliance on a failing drug (Chloroquine) for treatment (Trape *et al.,* 1998). Then, due to substantial malaria control program the death rate from malaria reduced about one third, however, according to the WHO report in 2012 there were approximately 627,000 deaths from malaria (WHO, 2014). The recent success in malaria control is the contribution of a highly effective drug (Artemisinin) therapy. Hence, the emergence in Southeast Asia of *P. falciparum* parasites that are partially resistant to artemisinin is of great concern (Dondorp *et al.,* 2009; Phyo *et al.,* 2012).

Aim and Objectives

- To determine the spatio- temporal pattern of malaria incidence.
- To study the current status of malaria in District Bannu.
- Diagnosis of *Plasmodium* species by microscopy and PCR.

MATERIALS AND METHODS

2.1. Study Area

The District Bannu lies in the temperate zone, situated in the south of Khyber Pakhtunkhwa at 32° North latitude and 70° East longitude (figure 2.1). Its borders North Waziristan to the Northwest, Karak to the North East, Lakki Marwat to the South East, and South Waziristan to the southwest. The population of Bannu is about 1.168 million. The area of Bannu occupies 1,227 square kilometers with altitude of 363 meters from sea level. The climate of Bannu referred to as local steppe climate. During the year there is little rainfall. The least amount of rainfall occurs in November. The average in this month is 4mm. Most of the precipitation here falls in July, averaging 69 mm. June is the hottest month with an average temperature of 33.6°C and January is the coldest month with an average temperature of 11.7°C, with an average annual temperature of 23.6°C and average annual of rainfall is 327mm.

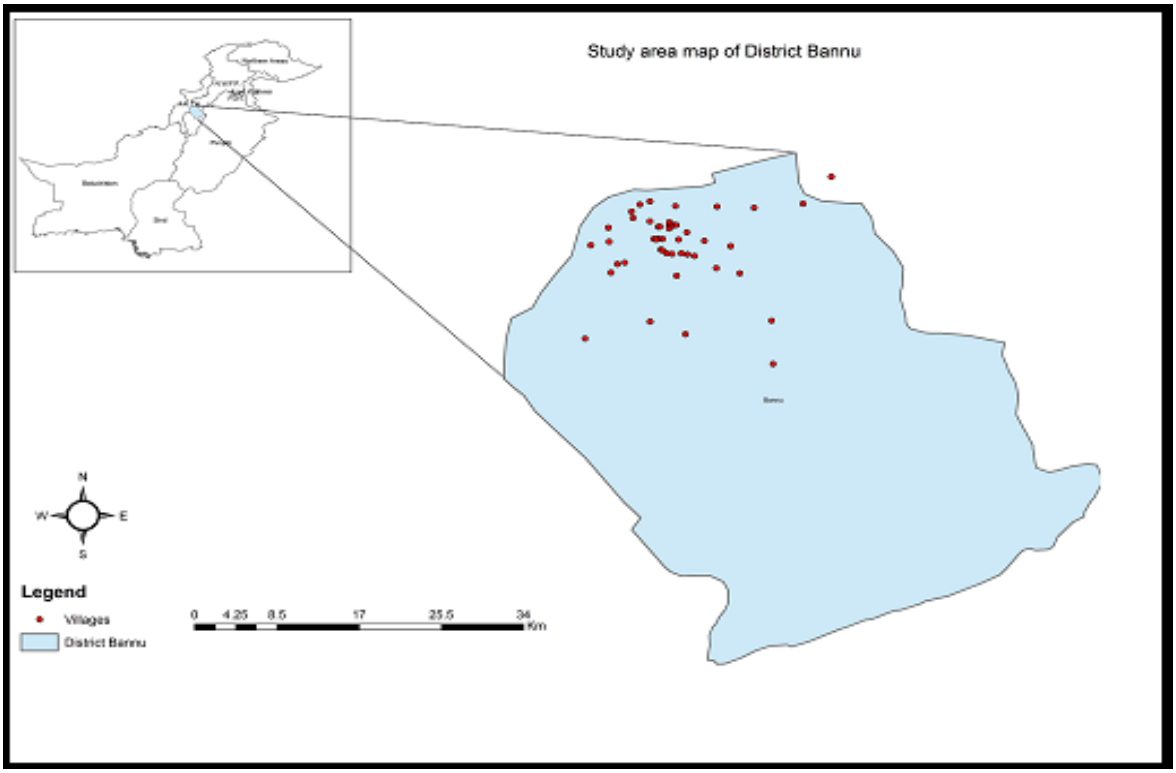

Figure 2.1: Study area map of District Bannu.

2.2. Data Collections

The data of previous two years (2016 -2017) was collected from the District Hospitals and District Malaria Control Centers of Bannu. The malaria control office collects data at the end of each month from District Health Care Centers. Laboratory technicians who examined blood films have 7–10 years of experience. To maintain the validity of this examination, well-prepared and well-stained thin and thick blood films are used as the gold standard in confirming the presence of the malaria parasite as WHO protocol. The staining techniques and blood film examination for malaria parasite detection were conducted according to a standard operating procedure stated in manual for the laboratory diagnosis of malaria in each referral hospital throughout the country. Therefore, data on sex, age, type of *Plasmodium* species and season were collected on suspected malaria cases requested from 2016-2017.

2.3. Analysis of Data

The secondary data was analyzed using various parameters; the annual parasitic incidence, blood examination rate, and slide positivity rate and temporal basis like (monthly, seasonally and annually). Village's wise species differentiation was also assessed based on *P. falciparum* and *P. vivax* ratio.

The slide positivity rate (SPR), annual parasite incidence (API) and annual blood examination rate (ABER), *P. falciparum* ratio and *P. vivax* ratio was calculated by following formulas (Laghari *et al*., 2013).

$$\mathbf{SPR} = \frac{\text{Total positive cases}}{\text{Total observed cases}} \times 100$$

$$\mathbf{API} = \frac{\text{Total positive samples in a year}}{\text{Total Population}} \times 1000$$

$$\textbf{BER} = \frac{\text{Total slide examined}}{\text{Total Population}} \times 100$$

$$\textbf{PfR} = \frac{\text{Total } P.falciparum \text{ ratio}}{\text{Total positive cases}} \times 100$$

$$\textbf{AvR} = \frac{\text{Total } P.vivax \text{ ratio}}{\text{Total positive cases}} \times 100$$

$$\textbf{Prevalence} = \frac{\text{Total positive cases}}{\text{Total suspected cases}} \times 100$$

The Chi- Square test was used to compare the significance of difference of malaria in males and females and also between the different age groups. Finally P <0.05 was considered as statistically significant level.

2.4. Ethical Considerations

The hospital-based study and blood samples were carried out after ethical approval from Quaid-i-Azam University ethical committee and Health Care Center of Bannu.

2.5. Blood Sampling

The blood samples were randomly collected from malaria suspected patients in the hospital for microscopy and molecular characterization/ confirmation of human malaria parasite, by taking blood smear on slides and in EDTA tubes.

The 500 samples were collected for thick and thin smear microscopy and molecular diagnosis through venipuncture technique. The patient was positioned at sitting on a chair and the epidermis was disinfected by using 70% ethanol soaked cotton where the vein was clear and easy for blood collection. The region above the elbow was wrapped around it. After this the needle was injected into the vein and 3-4ml blood was

taken, the needle was removed in backward direction carefully and cotton was placed on puncture side. The blood was transferred into (EDTA) tube (Perandin *et al.,* 2004). The microscopy and nested PCR for species identification were carried out to cross check the accuracy of technicians working in different Health Care Centers of Bannu.

2.5.1. Microscopy

The microscopy was used for the detection of *Plasmodium* and for the species identification. Thick smear was prepared for *Plasmodium* and thin films were used for species identifications. Because of storage of ethylenediaminetetra-acetic acid (EDTA) morphological changes of parasites occur so film were made within one hour of sample collection. Thin film was exposed to acetone for one minute, air dried, methanol fixed and then stained with Giemsa (Tek *et al*, 2009). Thick film was dried at room temperature for 15 minutes, exposed to acetone for 10 minutes and stained using Giemsa (for 15 minutes). Prepared slides were examined under (10 and 100X) oil immersion (Moody, 2002).

2.6. DNA Extraction: Standard Phenol-Chloroform Procedure

The extraction of genomic DNA the stored EDTA tubes containing blood were kept at room temperature for 1 hour. The blood (750µl) was taken into 1.5ml eppendorf tube and solution A (0.32 M Sucrose, 100mM Tris, 5mM Mgcl2) of equal amount was added to the tube containing blood. To mix the blood and solution A, the eppendorf tube was inverted 5-7 times, the tube was then kept for 5-10 minutes at room temperature. The supernatant was discarded after the centrifugation of the mixture for 1 minute at 13,000rpm. Solution A (400µl) was added to suspend the nuclear pellet followed by centrifugation at 13,000rpm for 1 minute. The supernatant was discarded and the pellet obtained was re-suspended in 400µl solution B (10mM tris, 400mM Nacl, 2M EDTA), 14µl 20% SDS, 5-8µl of proteinase K (10 mg/ml stock). To digest the pellet completely the tube was incubated overnight at 37°C. Completely digested pellet was obtained after 24 hours, for each sample fresh solution C+D was prepared by mixing 250µl of solution C (phenol) and an equal amount (250µl) of solution D (chloroform, isoamylalcohol). The solution was mixed properly until it gave a milky color, and added solution (C+D) 500µl

to the sample, mixed properly and centrifuged at 13,000rpm for 1 minute. After centrifugation three layers were formed, the upper aqueous layer was picked with the help of the 1000µl wide pipette in a new labeled eppendorf tube, and Solution D (500µl) was added to the tube and centrifuged at 1300rpm for 10 minutes. The upper aqueous layer was again transferred into a new labeled eppendorf tube and added sodium acetate 55µl (3M, pH) and chilled iso-propanol (3M, pH 6) 500µl (stored at -20˚C) to precipitate the DNA. To observe the DNA floating in the tube the mixture was mixed by inverting the tube for several times. The sample was centrifuged for 10 minutes at 13,000rpm to settle the precipitated DNA the sample and supernatant was discarded carefully without disturbing the pellet. To the tube containing settled DNA pellet chilled 200µl, 70% ethanol (stored at -20˚C) was added and centrifuged at 13,000rpm for 7 minutes. Ethanol was discarded and the pellet was obtained at the bottom of eppendorf tube, the pellet was air dried for 10-15 minutes. To dissolve the DNA pellet Tris-EDTA (TE) 150-200µl buffer was added to the tube. In order to suspend the DNA completely in Tris-EDTA buffer the sample was kept in an incubator at 37˚C for overnight (Lahiri and Nurnberger, 1991).

2.7. Gel Electrophoresis

The agarose gel for electrophoresis was prepared by dissolving 0.5g agarose in 5ml 10X TBE buffer and 45ml distilled water. Mixture was boiled for 2 minutes and 5µl 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 5µl extracted DNA mixed with 5µl loading dye and loaded into the wells. The gel was run for 40 minutes at 120V, 50W and 50mA. At last it was visualized under UV-trans-illuminator. The image was recorded.

2.8. PCR Amplification

The nested PCR reconfirmation was also carried out for species identification. In the first round the genus *Plasmodium* specific region were amplified having 1100bp, in the second round species specific gene amplification were carried out which gives 120bp**.**

The final volume of 50µl PCR recipe was used for each round of nested PCR. The gene used for PCR amplification were 18S ssrRNA genes for *P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae*, in which specific primers were used, as described by (Siwal *et al.*, 2018).

Table2.1: List of primers used for PCR amplification of genus *Plasmodium*

Genus	Primers	Sequences	Size
Plasmodium	rPLU5	5'CCTGTTGTTGCCTTAAACTTC3'	1100bp
	rPLU6	5'TTAAAATTGTTGCAGTTAAAAC3'	1100bp

Table 2.2: List of primers used for PCR amplification of *Plasmodium* species

Species	**Primers**	**Sequences**	**Size**
P. vivax	rVIV1	5'CGCTTCTAGCTTAATCCACATAACTGATA3'	120bp
	rVIV2	5'ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA3'	120bp
P. falciparum	rFAL1	5`TTAAACTGGTTTGGGAAAACCAAATATATT3`	205bp
	rFAL2	5'ACACAATGAACTCAATCATGACTACCCGTC3'	205bp
P. malaria	rMAL 1	5'TAACATAGTTGT ACG TTA AGAATAACCGC3'	144bp
	rMAL 2	5'AAAATTCCCATGCATAAAAAATTA TAC AAA3'	144bp
P. ovale	rOVA 1	5`ATC TCT TTT GCT ATT TTT TAGTAT TGG AGA3`	780bp
	rOVA 2	5`GGA AAA GGA CAC ATT AAT TGTATC CTA GTG3`	780bp

The PCR master mix was prepared containing PCR water 33.3µl, *Taq* Buffer 5µl, $MgCl_2$ 4µl, dNTPs 1µl, forward primer 2µl, reverse primer 2µl and *Taq* polymerase 0.7µl for each tube. These contents were mixed by overtaxing and short spin at 6000rpm for 30

second and 2µl DNA template was added at last. Following are the thermo cycler conditions for *Plasmodium* genus detection in first round PCR.

Table 2.3: PCR cyclic conditions for genus *Plasmodium*

Stage	**Setup**	**Temperature**	**Time**	**Cycle**
1	Initial Denaturation	95^{o}C	5 min.	1
2	Denaturation	94^{o}C	45 sec.	35
	Annealing	56^{o}C	45 sec.	
	Extension	72^{o}C	45 sec.	
3	Final Extension	72^{o}C	10 min.	1

Table 2.4: Nested PCR cycle for *Plasmodium* species identification

Stage	**Setup**	**Temperature**	**Time**	**Cycle**
1	Initial Denaturation	95^{o}C	5 min.	1
2	Denaturation	94^{o}C	45 sec.	35
	Annealing	60^{o}C	45 sec.	
	Extension	72^{o}C	45 sec.	
3	Final Extension	72^{o}C	10 min.	1

The cyclic conditions for nested PCR used for species identification were same as above except the annealing temperatures. Annealing temperature for *P. vivax* was 60^{o}C. In nested cycle second amplification product of the second round was used as a template. Annealing temperatures for *P. malariae* was 63^{o}C and *P. ovale* was 59^{o}C.

2.9. Confirmation of the Amplified PCR products

The agarose gel was prepared for the electrophoresis, by dissolving 1g agarose in 5ml 10X TBE buffer and 45ml distilled water. The mixture was heated for 2 minutes in micro oven and 5µl ethidium bromide was added then poured into gel tray. The combs were placed, till the gel was solidified and then combs were removed. Gel tray was put into the 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 120V, 50W and 50mA. At last it was visualized under UV-trans-illuminator. The image was recorded

2.10. Database Planning

The spatial distribution of malaria cases was used to generate and store the data bank in ArcGIS 10.2. The geographical coordinates of the patient's residence were determined using Google Earth Software Version (7.3.0). After Assortment, malaria cases and were analyzed through Arc GIS 10.2 and Spatial analysis.

2.11. Spatial Analysis

The spatial analyst tool was used to generate the incident rate of *Plasmodium* infection across the District Bannu including 41 villages. This parasitic incidence rate implements the assumptions of that factors which are closely related to more subject than those that are farther apart. Furthermore, spatial risk of malaria in Bannu was determined using ArcGIS 10.2, temporal and spatial analysis.

2.12. Inverse Distance Weighting Interpolation Method (IDW)

The smoothed map (neighboring type smooth, smoothing factor 0.5, angle zero) of the spatial pattern of malaria prevalence in the Bannu was created in a geographic information system (GIS) using IDW spatial interpolation (Messina *et al*., 2011) in ArcGIS 10.2. This is a deterministic estimation method where values at unsampled points are determined by a linear combination of values of known sampled points. It employs Tobler's Law (everything is related to unknown measurements as weighted averages the

known measurements as nearby points, the greatest weight to the nearest. More specifically, IDW assume that each measured point has local influence that with distance.

RESULTS

3.1. Analysis of Annual Parasitic Incidence, Blood Examination Rate and Slide Positivity Rate

The previous two years (2016-2017) a total of 193,419 blood films were examined microscopically in different Health Care Centers of Bannu. The average blood examination rate (BER) from 2016-2017 was 7.8% while average slides positivity rate (SPR) was 16.43%. There was a significant higher blood examination rate 8.05% and slide positivity rate 16.43% in 2016 compared to 2017 (average BER = 7.5% SPR=13.18%). According to the Government Health Centers the average annual parasitic incidence (API) in the last 2 years was 14.83%. The APR recorded was higher in 2016 (1.32%) than 2017 (0.9%). There was a significant decline in examined and confirmed cases of malaria in 2017 as compared to 2016. There was a successive reduction in the prevalence of malaria in 2017. Regarding the identified *Plasmodium* species, both the species of *Plasmodium* were reported in each year in the study area. Incidence rate of *P. falciparum* was 3.08% while *P. vivax* was 95.05%, which indicated *P. vivax* dominancy. The AR of *P. falciparum*, *P. vivax* and mix infection in 2016 was 2.81%, 94.67% and 2.50%, respectively, while these in AR in 2017 was 2.66%, 95.45% and 1.10% respectively. The percentage of *P. falciparum* was decreasing while *P. vivax* was increasing which that there was a trend shift from *P. falciparum* to *P. vivax* in the study area. The AR of mix infection show significant decrease 2.50% in 2016 to 1.10% in 2017.

Table 3.1: The SPR, BER, API, *P. falciparum* and *P. vivax* ratio from 2016-2017

Year	Population	Slide Examined	Positive Slides	BER	SPR	AfR	AvR	Mix Ratio	APR
2016	122,14,16	983,34	161,61	8.05	16.43	2.81	94.67	2.50	1.32
2017	125,85,16	950,85	125,39	7.5	13.18	2.66	95.45	1.10	0.9
Total	134,72,32	193,419	287,00	7.8	16.43	3.08	95.05	1.10	014.8

(BED= Blood Examination Rate**, SPR=**Slide Positivity Rate**, AvR**=Annual *vivax Rate***, AfR=** Annual *falciparum* Rate**, APR=**Annual Parasite Rate**)**

3.2. Gender Wise Incidence of Malaria from 2016-2017

The secondary data showed a significant relationship between gender and *Plasmodium* infection the data shows that males were more susceptible to malaria infection as compare to female every year. The overall gender wise average prevalence of infection was 50.8% in male and 49.1% in female showed that males are at high risk to malaria infection as compared to female. According to the previous data for the last two years in high rates of infection were in 2016 (16.43%) and prevalence rate slightly decreasing in 2017 (13.18%). In 2016, out of total 16161 microscopically confirmed malaria cases 50.7% of infection were found males and 49.2% was in females. In 2017, malaria incidence was found to be low as 50.9% males and 49.0% females as compared to 2016.

The possible reasons of low prevalence in female were due to the restricted movement from their houses and female wear shuttle cock the body is full covered which reduces the chances of mosquito biting. As males work on the fields and spend most of their time outside their houses that make them more exposed to the mosquito biting. Chi square 0.1 indicated that the data was not significant which indicates that the males and female ratio values are quite distant from each other.

Table 3.2: Gender wise incidence of malaria from 2016-2017

Year	Male	%	Female	%	Total	%
2016	8208	50.7	7953	49.2	16161	16.43
2017	6390	50.9	6149	49.0	12539	13.18

3.3. Month Wise Incidence of Malaria

The malaria is one of the vector-born infectious diseases, seasonal variation and climatic changes have a major role in malarial infection, so that's why malarial incidence is greatly fluctuated with temperature and humidity from time to time. To correlate and assess the effect of the above mentioned factors on malarial infection, month wise data was also collected and analyzed. In the two year study from 2016-2017, a total of 28,700 cases was reported.

The higher values of malaria infection were noted in August and September followed by October and July in the current study. The lowest incidence rate was founded in February, January and December. It revealed that in Winter season the vector cannot survive. The reappearance of hepnozoites stage of *P. vivax* in the Winter season revels only in relapse cases. The above discussed months showed a peak and moderate rate of infection while malaria prevalence in the remaining months of the study period was noted as reduced. Similarly slide positivity rate (SPR %) was also higher from July to September and then a slight decrease in October. Again the lowest ratio of slide positivity rate was found from January to March.

The blood examination rate (BER) was also fluctuating in different months of the study period, but on the average it was found to be higher in the month of June to September and then slightly decreased as the Winter starts, indicating the Summer season (high temperature) as a high risk of malaria infection in Bannu. The highest peak of infection mostly starts during the Summer when rainy season (June-

September) starts while the small peak would be due to increased temperature just before the Summer that increase the vector development and multiplication.

The current data showed that malaria incidence is high during the Summer when the rainy season starts (June-September) but in Winter the population of vectors decreases the vector can't survive and thus the rate of infection decrease when the Winter starts.

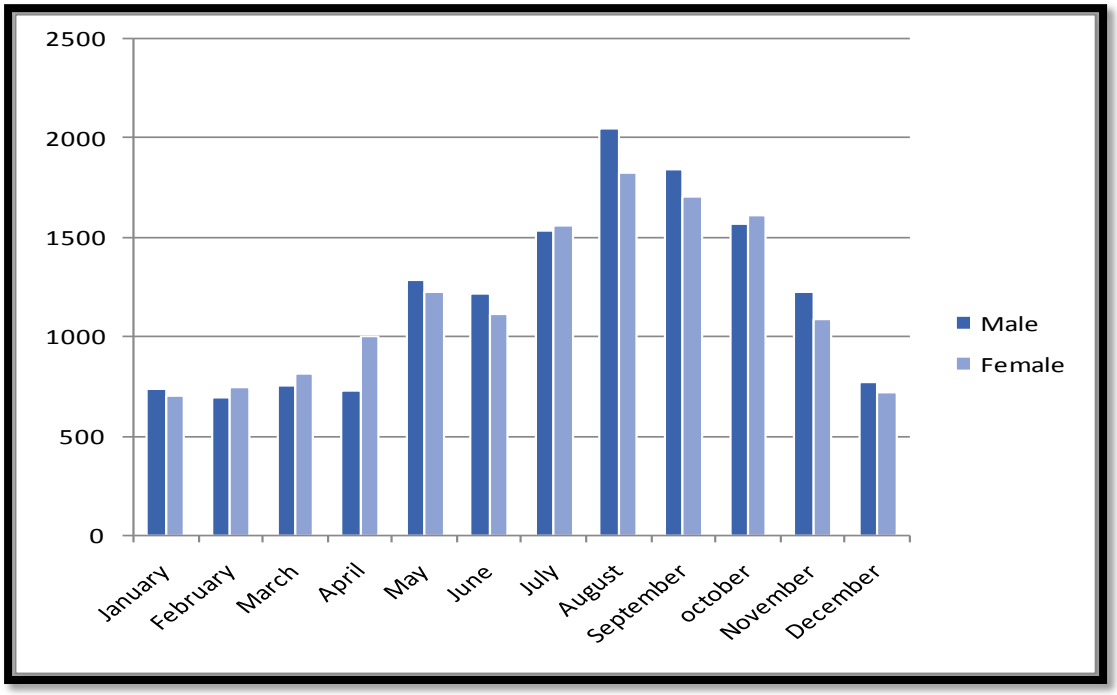

Figure 3.1: Month wise incidence of malaria from 2016-2017.

3.4. Malaria Incidence in Different Age Groups

The current study revealed that malaria was also studied in different age groups. Malaria parasite infect people of all ages, but most common in the age of >14 years in the current study. According to previous data of last 2 years total 15864 positive cases of malaria were confirmed, in the age group of >14 years. Among the infected individuals of the mentioned age group about 48.0% of the infection was found in male, while the remaining 51.9 % was noted in female.

The total of 7133 cases was declared as positive for age group 5-14 which showed a clear decrease of infection in, a comparison of the age group of >14. The ratio of infection in males were noted in this age group of 5-14 was found 54.2%

males and 45.7% in females. In age group <5 only 2206 positive cases were recorded, the ratio of infection 57.6% in males and 42.3% in females was found.

The above results showed that malaria prevalence is more common in the age group of >14 and females 51.9% were more susceptible to malaria infection than male 48.0%. The percentage of malaria infection was less common in the age group of 5-14 and the percentage of infection in males 54.2 % is higher than female 45.7%. The prevalence of malaria is less common in the age group of <5 than the age group of >14 and 5-14 and the malaria infect in male 57.6% is higher in these group than females 42.32%.

Table 3.3: Malaria incidence in different age groups

Age Group	**Male**	**%**	**Female**	**%**	**Total**	**%**
<5 years	3398	58.8	2378	41.1	5776	22.05
5-14 years	3936	45.7	3265	45.7	7133	27.7
>14 years	7620	48.03	8244	51.9	15864	61.64

3.5. Distribution of *Plasmodium* species in District Bannu

The *P. vivax* and *P. falciparum* are the most common cause of public health concern in Pakistan among the all *Plasmodium* species. In the current study, the distribution of malaria parasite species was also studied in the general population of Bannu shown in Figure 3.3. Two species of *Plasmodium* are found in the study area i.e. *P. vivax* and *P. falciparum* from 2016 to 2017. In the population mix infection was also found. The data reveal a much higher ratio of *P. vivax* than that of *P. falciparum* and mix infection. The average percentage of prevalence for *P. vivax* and *P. falciparum* and mix infection was recorded as 93.8% and 2.7 % and 1.9% respectively in the last 2 years. In 2016, total 16161 slides were examined as positive for malaria parasite in which only 455 cases 2.8% were microscopically confirmed as *P. falciparum* and 405 (2.5%) as mix infection. The total of 10323 cases in 2017 (13.7%)

positive cases were reported for malaria parasite in which 289 cases (2.8%) and 130 cases (1.2%) were confirmed for *P. vivax* and mix infection respectively and the remaining 9904 (95.9%) for *P. vivax* infection. The rate of *P. falciparum* and mix infection decreases in 2016-2017 while the rate of *P. vivax* increases from 94.6% in 2016 to 95.9 % in 2017.

Table 3.4: Distribution of positive cases of *Plasmodium* species in District Bannu

Year	***P. vivax***		***P. falciparum***		**Mix infection**		**Total**	
	Cases	**%**	**Cases**	***%***	**Cases**	**%**	**Cases**	**%**
2016	15301	(94.6%)	455	(2.8%)	405	(2.5%)	16161	(16.4%)
2017	9904	(95.9%)	289	(2.7%)	130	(1.2%)	10323	(13.7%)

3.6. Monthly Trends of Malaria from 2016-2017

The month and season have a direct role in the transmission of malaria. In our study, the prevalence of *P. vivax* throughout the year revealed that it seems stable transmission. The highest ratio for *P. vivax* was observed from August to September in which total 3787 and 3408, cases were positively confirmed with the rate of 15 % and 13%, respectively, which was followed by a July 3038 (12%) to June 2443 (9.6%). The low prevalence ratio for *P. vivax* was observed in December in total 572 positive cases were reported with a ratio of 2.6%. The prevalence for *P. falciparum* and mix infection was high in the month of November where total 123 and 122 positive cases were reported with a rate of 0.4% while the lowest prevalence ratio for *P. falciparum* was observed in April 21 (0.08%) and for mix infection was found in June (0.07%) cases.

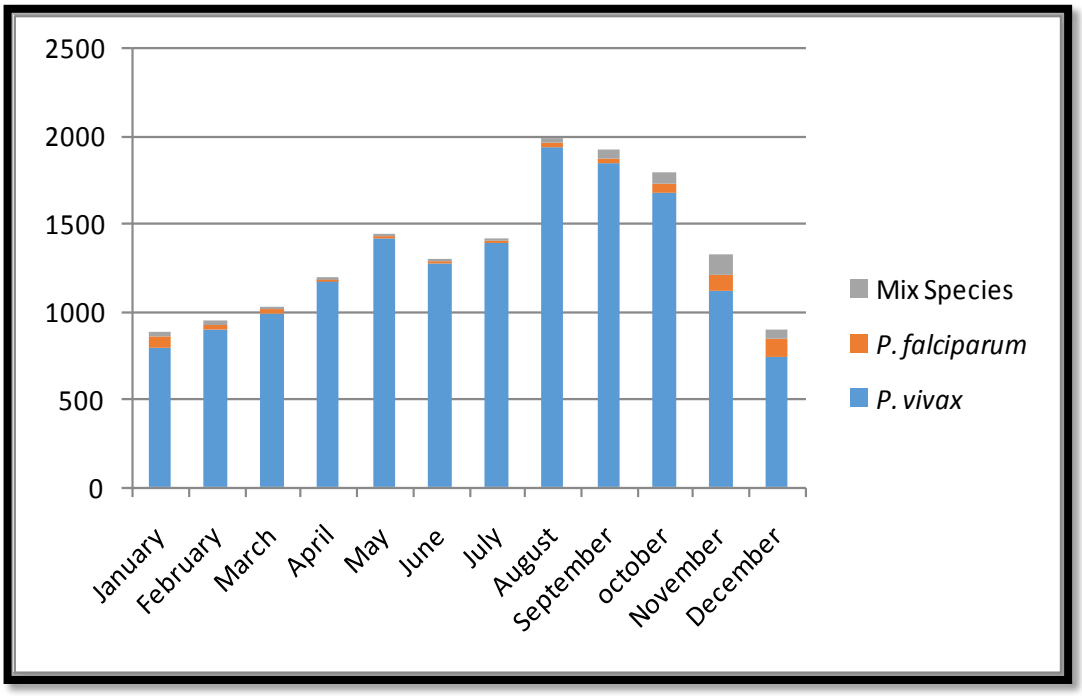

Figure 3.2: Month wise incidence of malaria from 2016-2017.

3.7. Seasonal Distribution of Malaria

The malaria incidence is greatly affected by variation in temperature and humidity. Table 3.5 shows the overall seasonal distribution of malaria in Bannu from 2016-2017. Throughout the two years *P. vivax* ratio is high and low for *P. falciparum* and mix infection. *Plasmodium vivax* incidence dominate from June to August in (Summer) followed by September to November (Autumn) while the moderate and lower rate of incidence was recorded in March to May (Spring) and December to February (Winter) respectively. Analysis of two years data revealed that malaria load in Bannu touches the peak value in Summer as indicated in Table 3.5.

The dominance of *P. vivax* was seen as 4614 cases in 2016 and its high incidence is shown to occur from June to August (Summer), whereas lower incidence was recorded from December to February (Winter) as 2437 cases. From March to May a gradual trend of increase in infection was noted. *Plasmodium falciparum* infection was higher during Autumn 170 cases, Winter 188 cases and Summer 51 cases, but very low ratio in spring only 46 cases. But the mix infection was higher during Autumn 242 cases, Winter 104 cases, and Summer 44 cases and very low during Spring 15 case. In 2017, highest incidence of *P. vivax* parasite occurs from

June to August (Summer) 145,22 cases. A moderate ratio of incidence is also seen from March to May (Spring) 2220 cases.

The slightly decreases occur in the month of September to November (Autumn) 2091 cases, despite of it some cases were reported. From December to February (Winter) 1071 cases a lower ratio of infection exists. The prevalence ratio for *P. falciparum* was observed very high in Spring 111 cases, Winter 85 cases and Autumn 90 cases but low in Summer 28 cases. For mix infection the prevalence ratio is high in Spring 58 cases, Summer 28 cases and Winter 37 cases but very low in Autumn. Chi square was applied the value 0.001 which showed that the data of 2016 was highly significant.

Table 3.5: Seasonal distribution of *Plasmodium* species

Seasonal Duration	**Positive cases in year 2016-2017**					
	2016			**2017**		
	P. vivax	***P. falciparum***	**Mix**	***P. vivax***	***P. falciparum***	**Mix**
Winter	2437	188	104	1071	85	37
Spring	3601	46	15	2220	111	58
Summer	4614	51	44	14522	49	28
Autumn	3718	170	242	2091	90	7

3.8. Spatio-temporal Distribution of Malaria in 2016

The *Plasmodiu*m distribution was done in 41 villages of Bannu. A retrospective approach was used to draw a clear picture of malaria parasite in these regions. The highest incidence in 2016 was recorded in village Kausar Fateh Khel followed by Ismail Khel and Salema Sikander Khel. These three villages have the peak ratio of malaria incidence.

The highest incidence in Kausar Fateh Khel might be due to dense population which may facilitate the anthropogenic transmission of malaria parasite. The second high incidence was found in Kot Qalandar village followed by village Surani. The reason of high malaria incidence of Kot Qalandar and Surani may be due to dense population, stagnant water in the fields and in dates, high vegetation and connection of many people with forming. The lowest incidence was recorded in Kakki and Takhti Khel villages. The possible reasons for low incidence of malaria in these villages may be due to its low population and scarcity of water.

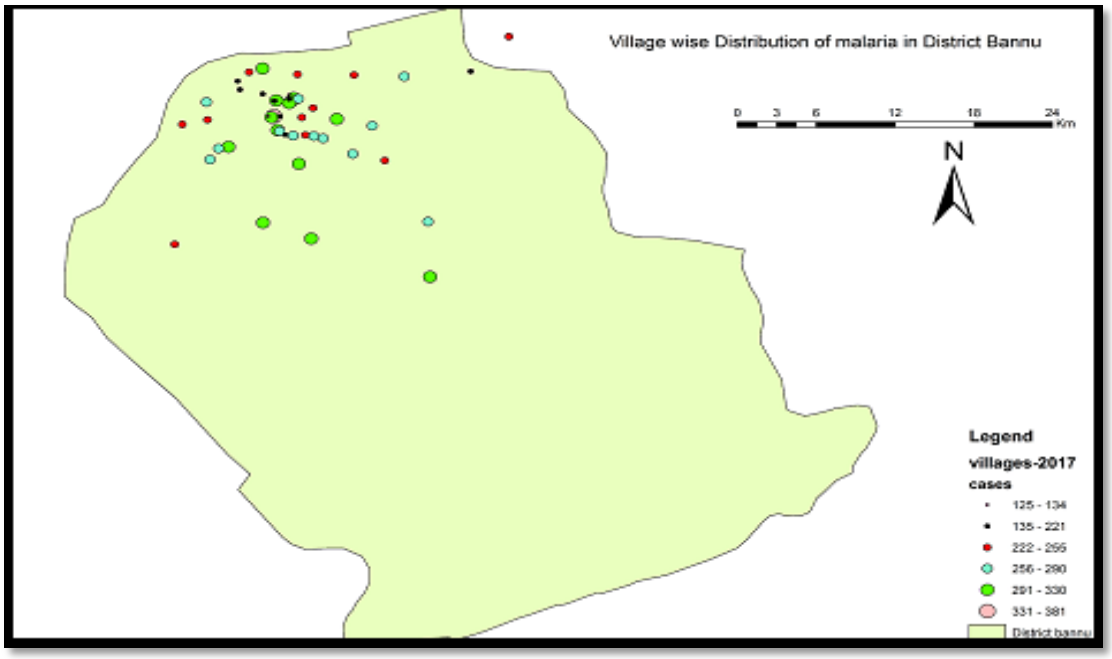

Figure 3.3: Spatio-temporal distribution of malaria in 2016.

3.9. Spatio-temporal Distribution of Malaria in 2017

The Maximum values of incidence was found in Mandew 381 cases and Kausar Fateh Khel (330), followed by Kot Qalandar (301) and Mama Khel (300) villages amongst all 41 villages in 2017 shown in Table 3.6. The incidence of malaria was lowest in Gari Sher Ahmad, Mandan and Khander Khan Khel, 125, 134, and 192 cases respectively. The Incidence rate of malaria Mandan and Mama Khel is due to river, rain pools, drainage pools, irrigation streams drainage system, man mad ponds, marshlands and artificial water ponds. The overall result shows that malaria in 2016 was high as compared to 2017.

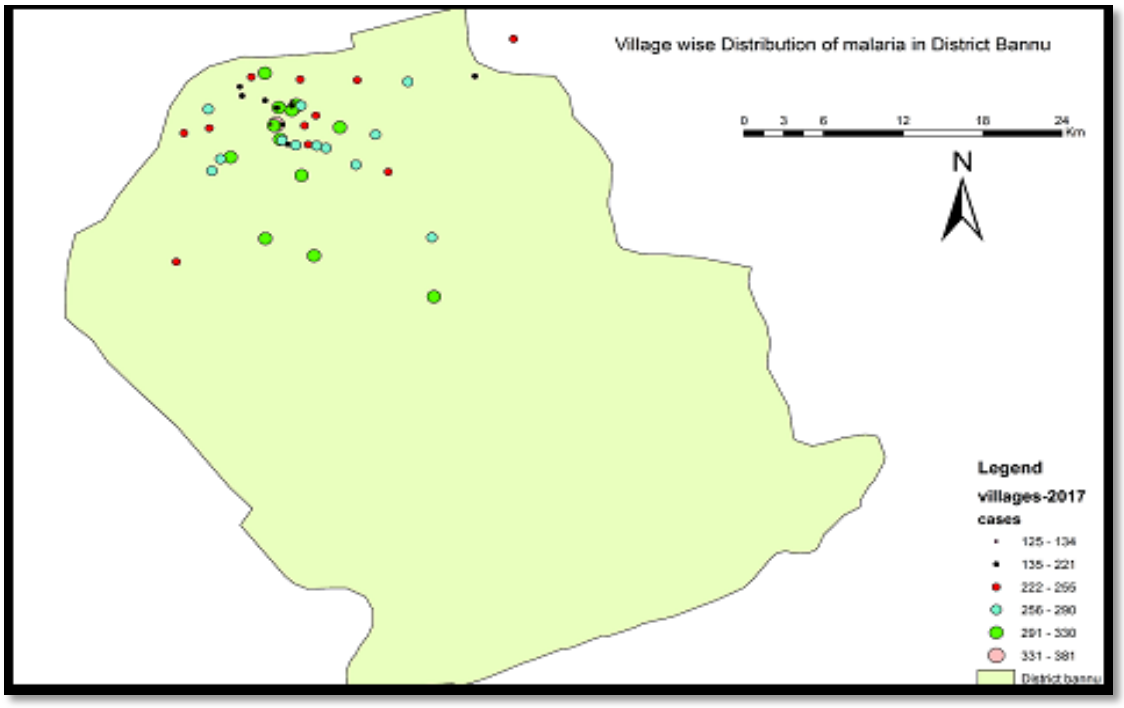

Figure 3.4: Spatio-temporal distribution of malaria in 2017.

Table 3.6: Spatio-temporal distribution of malaria in last two years (2016-2017)

Villages	Years			Villages	Years		
	2016	**2017**	**Total**		**2016**	**2017**	**Total**
Chi Gari	310	200	510	**Mira Khel**	230	285	515
Momand khel	370	290	660	**Baka Khel Wazir**	309	252	590
Baka khel Wazir	361	255	616	**Shahbaz Azmat Khel**	345	295	640
Mumbati Barakzi	300	278	578	**Kala Khel Masti Khan**	345	245	590
Mandoori Patal Shah	289	280	569	**Kotka QamarZaman**	430	330	760
Jhando Khel	374	290	664	**Ghari Sher Ahmad**	320	220	540
Domail	306	286	592	**Gharib Abad**	378	310	688
Multani Landidak	388	300	688	**Nasir Abad**	280	298	578
Tap Takhti Khel	399	314	713	**Kakki**	490	330	820
Esaki	360	230	552	**Fatima Khel**	308	134	442
Jani Khel	329	192	521	**Nurar**	389	276	665
Hassan Khel Esaki	333	235	568	**Ghori wala**	301	255	556
Mandew	389	309	298	**Bazar Ahmad Khan**	387	281	668
Amandi Umar Khan	265	230	495	**Badda Mir Abas Khan**	391	209	600
Inayat Mitha Khel	280	125	405	**Zalim Khel**	378	275	653
Khawaja Mad Mandan	321	196	507	**Hujrim Khel**	333	233	566
Kotka Sadder Din	335	300	635	**Bannu Township**	383	232	615
Sokari	390	221	611	**Sarai Nurang**	307	287	594
Lalozai Surrani	361	299	660	**Gandi Chouk**	391	300	691
Koti Sadat	378	255	633	**Kamali Banda**	375	234	609
Bharat	397	301	698				

3.10. Inverse Distance Weighting Interpolation Method (IDW) for the Representation of Malaria Incidence

The spatial analyst tool IDW were used for two years (2016-2017) to interpolate the result of malaria prevelence in the study area, due to this IDW it showed that the probability of getting malaria is higher in villages (household) neighboring another villages with a high malaria prevalence and decreased with the increased in distance. In the present study 41 villages of known malaria cases were used to interpolate prevalence of unmeasured location, the closest villages have a high risk for the malaria then those further away.

The highest disease incidence has occurred in the in the West side of Banuu due to the presence of highly endemic villages like Jani khel, Multani Landidak, Nurar and due to Mandew the disease will be spread to the neighboring villages in near future. Due to these villages the disease will be spread to the neighboring villages because of visiting of these villages. The disease prevalence in 2017 is high in the center of the District. The red colour in maps illustrated the area (villages like Multani landidak, Takhte khel and Sari nurang) having large number of malaria cases and it is the hub of the risk. The disease radiate from this village to the surrounding neighborhood villages, but the disease incidence decreases when we go to the left and right side (Periphery) of the Ditrict. This model showed that the disease incidence decreases when we go further away from the disease hub area. The reasons for the high prevalence of malaria in these villages are due to the presence of marshy and stagnant water. So, high population of mosquitoes is likely to occur in such poor sanitary conditions. Another reason for the high incidence of this disease is due to the rice fields and dates dropping to the stagnant water producing favorable environmental conditions for mosquito breeding. Environmental factors (Land use, irrigations, water reservoir), demographic issues (gender, males has more exposure than females to mosquitos working in the fields, household size, density of houses or high populations of villages are also the possible reasons.

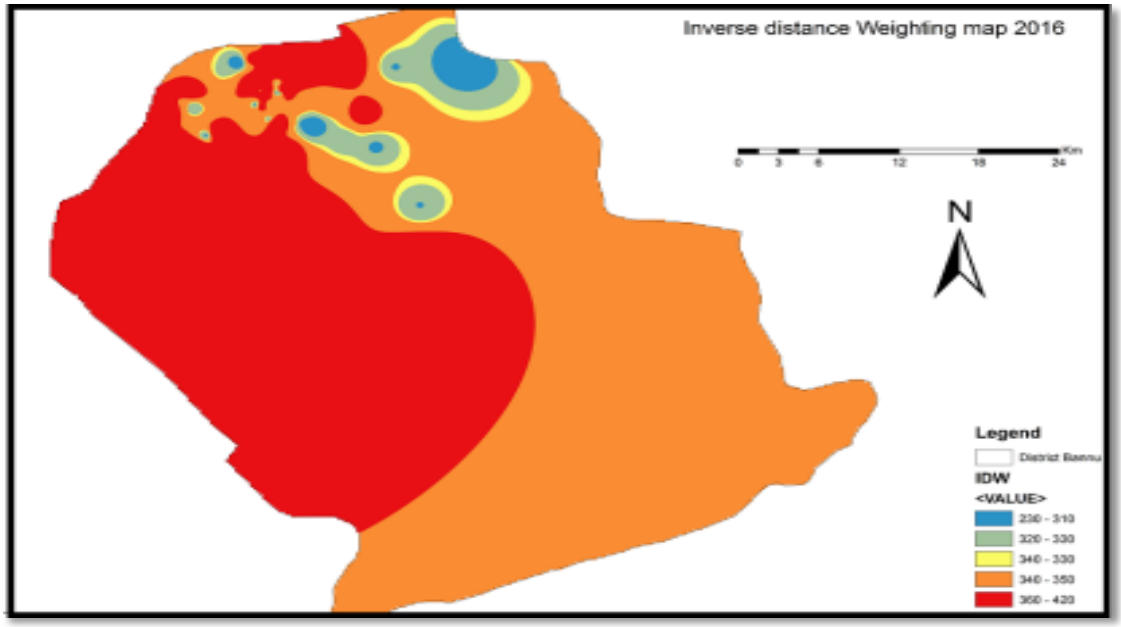

Figure 3.5: Inverse distance weighting interpolation method (IDW) for the representation of malarial incidences 2016

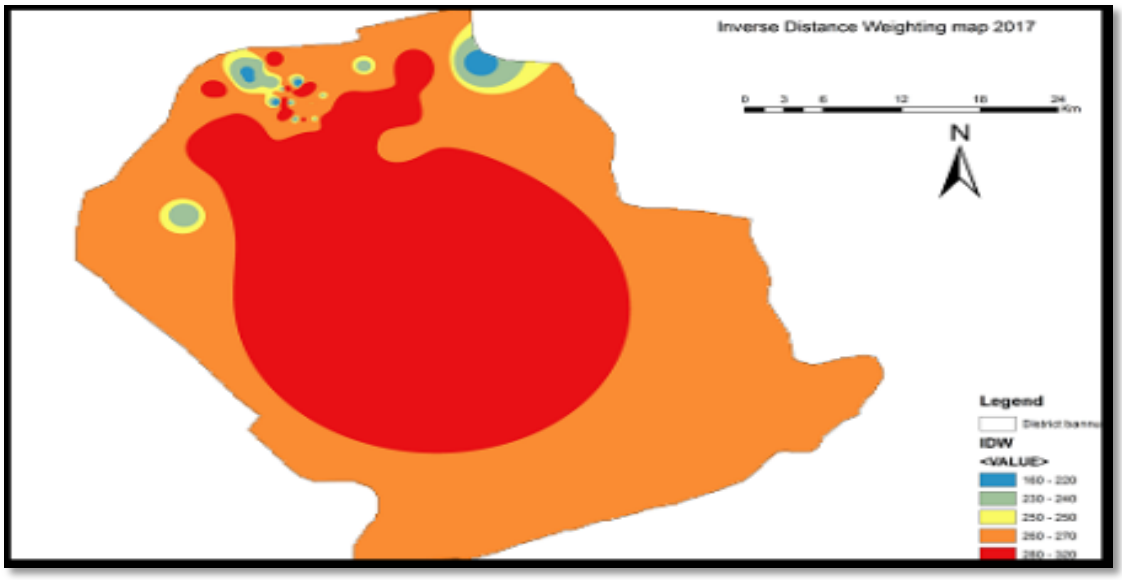

Figure 3.6: Inverse distance weighting interpolation method (IDW) for the representation of malarial incidences 2017

3.11. Microscopy and PCR Confirmation of the Genus *Plasmodium*

The total of 500 clinically diagnosed cases from different villages of District from Jan-July 2018 was examined through microscopy and PCR. Out of these, 281(56.2%) were found positive for *Plasmodium* while the remaining 219 (43.8%) were detected negative microscopically. Similarly the 500 samples were examined for the presence of *Plasmodium* of species specific nested PCR. Out of these 315 (63%) were positive detected by PCR and 185 (37%) were negative.

Table 3.7. Comparison between microscopy and PCR of the genus *Plasmodium*

Diagnostic technique	**Total blood samples**	**Positive**	**Negative**
Microscopy	500	281 (56.2%)	219 (43.8%)
PCR	500	315 (63%)	185 (37%)

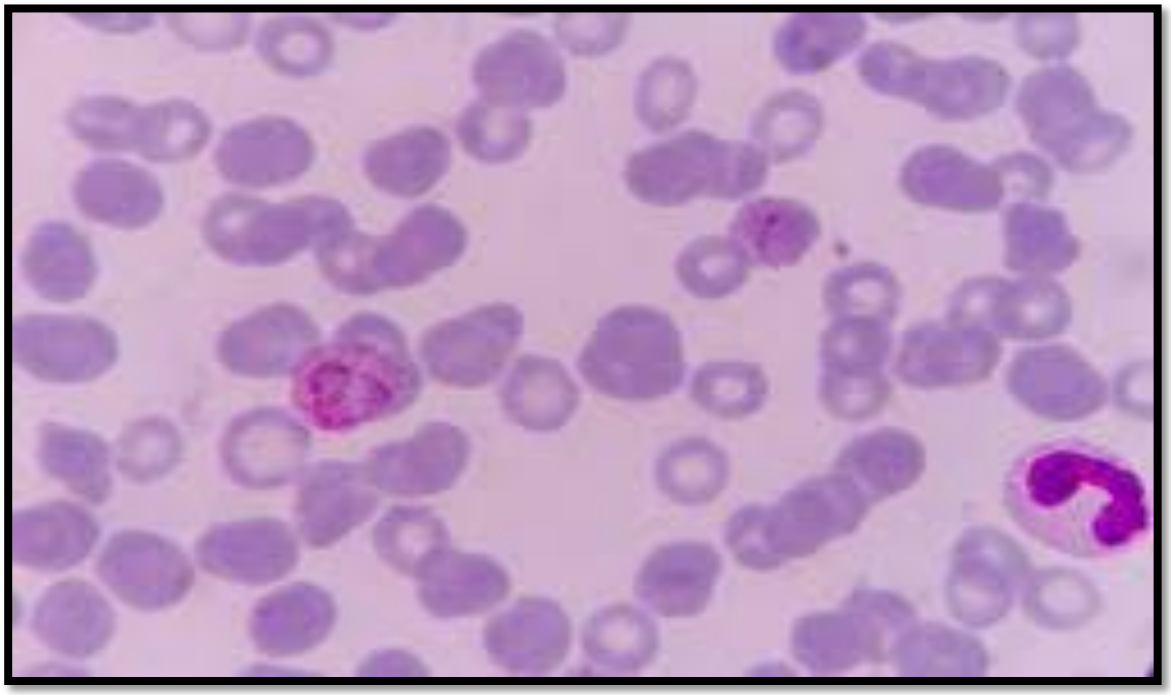

Figure 3.7: Schiozont stage of the *P. vivax* under microscope at 100X magnification.

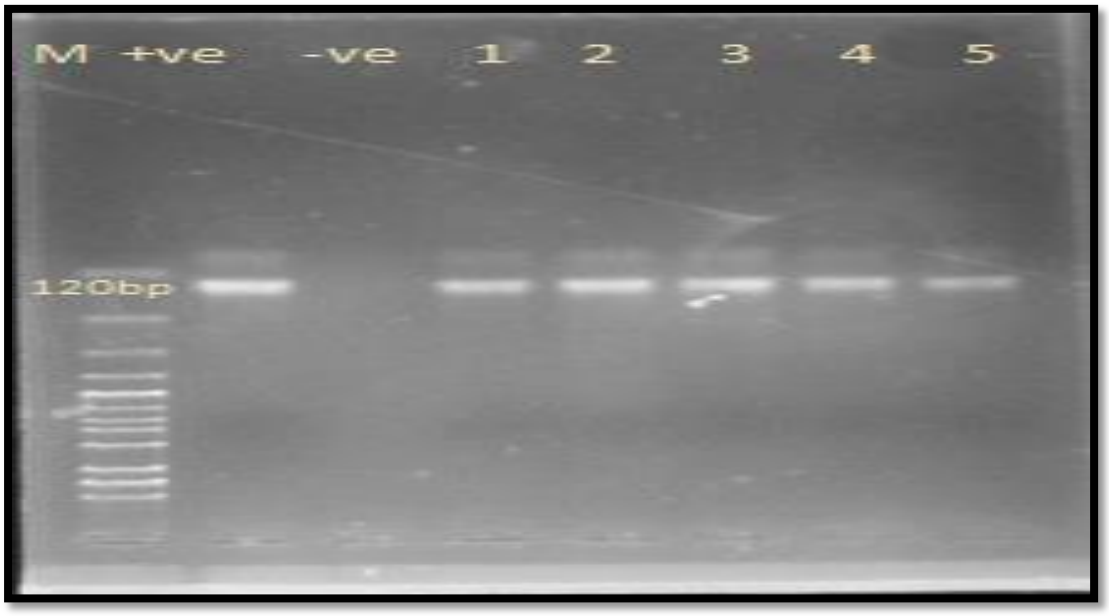

Figure 3.8: Line 1-5 are the 120bp amplicon representing the length for *P. vivax*, Lanes M - 100bp DNA Step Ladder (Promega) while in line +ve and –ve shows negative and positive control.

3.12. Comparison between Microscopy and PCR

The *Plasmodium* species were detected in 63% (315 of 500) examined by using PCR and in 56.2% of stained smears by microscopy (281 of 500) as 281 species of *P. vivax.* Identification at the species level was achieved by nested PCR for all 315 specimens compared to 281 specimens determined positive by microscopy. The total 500 samples were examined for the presence of *Plasmodium* of species specific nested PCR. Out of this 500 sample, 315 were detected positive by PCR for *P. vivax* while no sample was positive for *P. falciparum, P. ovale, P. Malaria, which* indicates that all the species in Bannu in 2018 were *P. vivax.* The total negative samples detected by microscopy were 219 and by PCR were 185.

Table 3.8. Comparison between microscopy and PCR of the *Plasmodium* species

Species	**Microscopy**	**PCR**
P. vivax	273	315
P. falciparum	0	0
Mix infection	0	0
P. malariae	0	0
P. ovale	0	0
Negative cases	219	185
Total cases	500	500

DISCUSSION

Malaria is also referred as the king of diseases (Tangpukdee *et al*., 2009), a prominent health problem worldwide (Ahmad *et al*., 2016). It is common diseases in Pakistan but its incidence rate cannot be assessed accurately due to the scarce epidemiological data from various region of Pakistan (Khadim *et al*., 2002). The current study was conducted to determine the prevalence of malarial infection in Bannu.

The previous two years (2016-2017) a total of 193419 of blood film were examined microscopically in different Health Care Center of Bannu. Within the last 2 years (2016-2017) the average blood examination rate (BER) was 7.8%, while average slide positivity rates (SPR) was 16.43%. Compare to 2017 there was a significant rise in blood examination rate 8.05% and slide positivity rate 16.43% in 2016. In 2017, the average BER was 7.5%, while the average SPR was 13.18%. According to the Government Health Centers the average annual parasitic incidence (API) in the last 2 years was recorded at 14.83%. The APR recorded was higher in 2016 1.32% than 2017 which is 0.9%. There was a significant decline in examined and confirmed cases of malaria in 2017 as compared to 2016. There was a successive reduction in the prevalence of malaria in 2017. Regarding the identified *Plasmodium* species, both the species of *Plasmodium* were reported in each year in the study area. During the recorded two years the rate of *P. falciparum* was 3.08%, while *P. vivax* was 95.01%, which clearly indicate the *P. vivax* dominancy in the study area. The AR of *P. falciparum*, *P. vivax* and mix infection in 2016 was 2.81%, 94.67% and 2.50 % respectively, while these in AR in 2017 was 2.66%, 95.45% and 1.10% respectively. *P. falciparum* was decreasing, but *P. vivax* was increasing which that there was a trend shift from *P. falciparum* to *P. vivax* in the study area. The AR of mix infection show significant decrease 2.50% in 2016 to 1.10% in 2017.

The present study also based upon the month wise incidence of malaria. *P. vivax* infection most common in the month of august followed by September and July while the lowest incidence rate was observed in the month of February which was followed by December and January respectively. *Plasmodium falciparum* and mix infection was high

in the month of November where total 123 and 122 positive cases were reported with a rate of 0.4% while the lowest prevalence ratio for *P. falciparum* was observed in April 0.08% and for mix infection was found in June 0.07% cases.

The *P. falciparum* infection reaches to its peak in the month of June up to September and then after relapse from April to June in Pakistan (Molineaux, 1998). The peak value of *P. vivax* was noted during the month of October and lowest in the month of March while infection with *P. falciparum* shows the peak value in the month of October and the lowest value in the month of March (Yasinzai and Kakarsulemankhel, 2008). *P. falciparum* malaria was investigated for the mode of presentation and seasonal variations by Balli and Samiullah (2001) at CMH Multan. The high rate of *P. falciparum* prevalence in the month of August to November was studied by Balli *et al*. (2001).

The highest prevalence of malaria in the month of August and lowest in the November was studied by Yasinzai and Kakarsulemankhel (2007) in Mustang and Khuzdar area of Balochistan. Most of literature showing the peak incidence of malaria in the month from August to November indication the monsoon season as a suitable time for the mosquito development and survival which is linked directly with malaria transmission.

The two major species of *Plasmodium* are responsible for the transmission of malaria *P. vivax* and *P. falciparum* in Pakistan. In the current study species wise distribution was also studied and the result shows that infection caused due to *P. vivax* was too much higher than that of *P. falciparum* in Bannu. The average percentage of infection for the last two years (2016-2017) was found to be 95.01%, 3.08% and 1.8% for *P. vivax*, *P. falciparum* and mix infection respectively. No cases with *P. malariae* and *P. ovale* were observed in the current study. A similar study was conducted by Khattak *et al*. (2013) showing a high prevalence of *P. vivax* 76% followed by *P. falciparum* 18% and 6% was mix infection. Similarly another study was conducted by Shah *et al*. (2016) reported 821 cases in the general population of lower Dir in which 19.5% was reported as malaria positive. Out of these 30.1% positive cases of the infection was due to *P. vivax*

while only 9.4% was due to *P. falciparum* and no cases of mix, *P. malariae* and *P. ovale* were found in this study.

The similar study was conducted by Khan *et al*. (2013) in Bannu, notified that overall malaria infection of 27.1% with 22 .6% , 3.04% and 1.46% positive cases for *P. vivax*, *P. falciparum* and mix infection, respectively which is similar to our current study. Our study indicates that *P. vivax* is the most prevalent specie in District than *P. falciparum*, the possible reason for that might be due to its ability to produce gametes under hot temperate conditions and its great distribution (Khan *et al*., 2013). Species distribution of malaria was also studied by Asif (2008) according to their result, 73.5% case were due to *P. vivax*, 21.5 % *P. falciparum* and 5 % were mixed infected. Species distribution of malaria was also investigated among the studied individual at Ayub Teaching Hospital Abbotabad.

The high rate of infection was caused by *P. vivax* 72.4% which were followed by *P. falciparum* 21.4%, while the remaining 3.44% were mixed infection, according to the analysis out of the total 7.27 % positive cases. Similarly, in another meteorological survey population was investigated by Khattak *et al*. (2013) noticed the highest rate of *P. vivax* 76% cases than *P. falciparum* 18% and mix 6% positive cases. A contrast result of our finding was also seen by Farooq *et al.* (2008) at CMH Khuzdar in Balochistan where maximum rate of infection was caused by *P. falciparum* 69% cases *P. vivax* 24% and 7% mix infection was also reported in their study. Species wise malaria prevalence was also studied by Sheikh *et al*. (2005) according to their result a total of 34.85% patient was infected by malaria with 66.8% *P. vivax*, 30.7% *P. falciparum* and 3.29% were mix infection. Similarly according to the Study conducted by Tareem *et al*. (2012) a high rate of infection 81.66 % was caused by *P. vivax and* 18.34% by *P. falciparum* showing similarity to our current study but there was no mix infection.

The gender wise incidence was also evaluated in infected people of Bannu. In the current study a high rate of *Plasmodium* infection was found in males as compared to females. The average incidence of malaria in Bannu was seen to be higher in males 50.8% than in females 49.1 %. Similarly, study conducted by Ibrahim *et al*. (2014) in the District Buner show a high rate of malarial infection in males 69.79% than in females 30.72%.

The high prevalence rate was also noted by Khan *et al*. (2014) in FATA. A same rate of infection as 94.11% in males and 93.33% in females was also noted by Daud *et al*. (2014) which is similar to our current study. The study conducted by Tareen *et al*., (2012) in Quetta, Balochistan also shows that malaria infection is more common in male as compared to females. According to the Khan *et al*. (2013) males were more infected with malaria as compared to female in the general population of Bannu. Another study conducted by Shah *et al*. (2016) in District Swat concluded that out 385 cases 27.53% patient were males and 16.01% were females. Males are more susceptible to malaria as compared to female because they spend most of their time outside the home working in the fields and participated in different activities as they getting more chances for biting mosquitoes which is the reason behind the high rate of infection in males, secondly females are traditionally more covered as compared to males and mostly does not go outside so they decreasing the chances of biting anopheles mosquitoes. Change in environmental conditions also influences the incidence of malaria. Rainy and dry season affect the prevalence of malaria.

The rain season provides mosquito breeding habitat, which increases the chances of malaria transmission according to Madhavana *et al*. (2001). High temperature shortens the generation period that the increase mosquito population as increases the chances of malaria transmission (Bouma *et al*., 1996). Seasonal variation of malaria was also noted in the current study. In the whole study *P. vivax* is the most dominant species in Bannu and its high rate of incidence was noted during Summer season (June- August) and Autumn season (September-November) respectively. Khan *et al*. (2013) also studied the association between malaria incidence and seasonal variation. They reported the high

prevalence of *P. vivax* infection from April to September and *P. falciparum* from August to September.

The rate of parasitemia is high in the rainy season as compared to the dry season and peck value of morbidity and mortality in the rainy season was reported by Khan *et al*. (2013). Malaria prevalence with respect to seasonal variations was also studied by Khan *et al*. (2014). According to their study high rate of *P. vivax* was most common in the month of August and September while *P. falciparum* was common in the month of October, November and December which was similar to our current study.

The current study also showed the age wise incidence of malaria was also studied, the age group <14 15864 (61.64%) were highly affected, followed by 5-15 years old 7133 (27.7%) but >5 years old 5776 (22.05%) were least affected. Similar to our present study conducted by Yamir *et al*. (2017) showed that the age groups, >20 years 411 (55.5%) were highly affected, followed by 16–20 years old 135 (18.2%) but from 10 to 15 years old 39 (5.2%) were the least affected. The possible reason might be due to responsibility of these age groups for caring for the family and hence, the probabilities of staying outdoors for a longer period.

The present study based upon 500 samples collected from Bannu KP in 2018. *Plasmodium* was detected in 63% (315 of 500) samples examined by using nested PCR and in 56.2% of stained smears by microscopy (281 of 500). The negative samples were 219 detected by microscopy and 185 by PCR. The present study showed that nested PCR was more specific as compared to microscopy, allowing the detection of *Plasmodium* in cases with low parasitemia. In all instances, specimens that were PCR positive and microscopy negative were collected from symptomatic patients with a history of travel to malaria areas of endemicity.

The similar study was conducted by Coleman *et al*. (2006) which showed that a total of 698 samples that had non-concordant PCR and microscopy results when initially tested were retested by both microscopy and PCR and the performance of each assay calculated by assuming that the initial test result with each method was correct. The agreement between first and second test results was significantly better for PCR 91.9% than for microscopy 58.5%. This data clearly indicated that PCR was the more specific method. The possible reasons are that PCR is the most advance and reliable technique as compared to microscopy. Tests based on PCR for species specific *Plasmodium* genome are more sensitive and specific than microscopy detecting as few as 10-fold sensitive than microscopy. Other reasons may be due to small sample size, negative false results.

CONCLUSION

The current study suggests that most of the plane areas of Bannu with higher temperature are among the most malaria affected areas, especially in the summer and autumn seasons. The poor hygienic condition, absence antimalarial sprays, use of irrigated land, sharing of house with livestock, improper diagnosis, high temperature of the area and load shading play role in the spread of malaria in Bannu. Village Kausar Fateh Khel and Ismail Khel and Salema Sikander Khel are suffering from malaria the most in the near future, because of its increasing favorable condition for malaria vector breeding. The current study showed that *P. vivax* is the dominant species of malaria in Bannu, while *P. falciparum* introduced in Bannu in 2016 due to movement of Afghan refugees.

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