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Prevalence and distribution of human *Plasmodium* infection in Federally Administrative Tribal Areas of Pakistan

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Abstract

About 3.6 million Pashtun and over 1.5 million immigrants from Afghanistan live in the federally administered tribal areas (FATA) on the border between Pakistan's Khyber Pakhtunkhwa Province and southern Afghanistan. Although malaria cases are common in FATA, no detailed studies have yet been performed to reveal the actual status of malaria in the local population and epidemiological data are insufficient to elucidate the actual incidence. A malariometric survey of 691 patients of all ages and genders in seven agencies (districts) in FATA was carried out in 2013 using whole blood samples. Microscopically confirmed positive species were subjected to nested-PCR for the reconfirmation and detection of four species of *Plasmodium* causing human malaria. Of the 626 PCR positive cases, 81.1% were *P. vivax*, 13.8% *P. falciparum* and 4.9% mixed species containing both *P. vivax and P. falciparum*. *P. malariae* and *P. ovale* and were not found in any analysis. Sixty-five microscopic positive samples were identified as negative by PCR. The incidence of *P. vivax* ranged from 10.4% in Orakzai Agency to 22.8% in North Waziristan Agency. The prevalence of *P. falciparum* ranged from 1.3% in Orakzai Agency to 4.7% in North Waziristan, and Khyber Agency had the highest prevalence of 1.7% of mixed species. In FATA, *P. vivax and P. falciparum* are the main causative agents of malaria, while mixed species infections are also prevalent with varying transmission intensities. In addition, Estimates of malaria incidence shows that variation in the incidence, frequency and species composition of malarial parasites is high.

Keywords

Malaria, federally administered tribal areas (FATA), Pakistan, refugees, *P. vivax*, *P. falciparum*

Background

Malaria is a foremost threat to public health, economic growth and development in many countries (Khatoon *et al*. 2010). Around 2.85 billion people globally live at risk of infection, and *Plasmodium vivax* is the main cause of malaria in the human population (Abdullah *et al.* 2013), while *P. falciparum* causes most malaria-induced mortality worldwide (Elyazar *et al.* 2012). Although Africa is the region mainly affected by malaria, the disease remains a major public health problem in other tropical countries such as India, Indonesia, Pakistan, Papua New Guinea and the Amazon region of Latin America (Khan and Khattak 2006).

Along with hepatitis and dengue fever, malaria is considered to be a leading cause of morbidity in Pakistan. It mainly affects less developed districts with poor hygiene that do not have access to health care services (Leslie *et al.* 2009)). In Pakistan, *P. vivax* and *P. falciparum* are the most common malaria parasites. According to the National Malaria Control Program, at least a six-fold increase in *P. falciparum* malaria has been recorded in the past decade, which now encompasses 42% of all malaria cases recorded annually (Rowland *et al.* 2000).

Malaria is a multifactorial disease. As *Plasmodium* has a complex lifecycle that requires an insect vector, different factors control its distribution and abundance (Elyazar *et al.* 2012). Climate change, human migration, drug resistance, agricultural practices and access to health care facilities all affect the abundance and spread of the parasite (Siraj *et al*. 2014).

Malaria transmission is seasonal in Pakistan, and a gradual increase in cases can be noted after the July–August monsoon (Khan and Khattak 2006). *P. vivax* malaria is confined to two peaks per year, the main peak was in late spring as a result relapses of earlier infections and additional peaks occurred in summer and autumn by recent transmissions (Khatoon *et al*. 2010). By contrast, *P. falciparum* malaria show an increasing correlation with August- September temperatures (Rowland

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et al. 2002). The extensive irrigation network throughout the country, agricultural practices and monsoon rains provide a favorable environment for the growth and development of malarial vectors ((Binello *et al.* 2014).

P. vivax and *P. falciparum* account for 64% and 36% of malarial cases in Pakistan, respectively (Zubairi *et al.* 2013). Khyber Pakhtunkhwa has the most positive cases, followed by Punjab (Khattak *et al.* 2013). The mass arrival and continued flight of Afghans contribute to the increase in *P. falciparum* in the refugee camps located in Khyber Pakhtunkhwa, mainly in FATA (Singh *et al.* 1999) (Suleman 1988). About 50% of refugee camps are in FATA, semi-autonomous zones consist of seven parts called "agencies". These areas are operated by tribal laws and are fairly underdeveloped compared with the government-administered districts to the east (Rowland *et al.* 2002). Refugees are more susceptible to malaria due to stress and a low immune status (Bouma *et al.*1996), while the change in the surveillance system has aggravated the prevalence of malaria both in refugees and in the local population (Suleman 1988). The free movement of Afghan refugees across the border between Pakistan and Afghanistan has also facilitated the transmission of malaria in the FATA region in Pakistan. Low economic status and poor health facilities make FATA endemic to malaria (Khatoon *et al.* 2013).

FATA cover about 27,220 km^2 of Pakistan and comprise a population of about 3 million. However, due to increased militancy and violence since 1989 (Nawaz 2009), no detailed studies have been performed to reveal the actual status of malaria in the local population. Further, epidemiological data are insufficient to elucidate the actual incidence of various types of malaria in FATA (Khan and Khattak 2006). In the present study, the first survey of the incidence and distribution of malaria was conducted to provide baseline parasitological information on the population living in FATA.

Materials and Methods

Study areas and sample collection

The current study was conducted in the FATA region (seven federally administered agencies) with the support of the Department of Animal Sciences at Quaid-i-Azam University, Islamabad. Over 3.5 million Pashtun tribes' people and 1.5 million refugees from Afghanistan live in FATA, an area covering 27,500 km^2 on the border between Pakistan's Khyber Pakhtunkhwa province and southern Afghanistan. FATA consist of seven agencies, namely Khyber, Orakzai, Kurrum, Bajaur, Mohmand, North Waziristan and South Waziris-

Fig. 1. Sample sites and distribution of plasmodium species across Tribal Areas Pakistan 2013

tan (Fig. 1). All agencies except Orakzai share a border with Afghanistan (Nawaz 2009)(Khatoon *et al.* 2010).

A detailed malaria survey was performed during the peak transmission season between May and November 2013 and people of all age groups with clinical manifestations of malaria were included. Other parameters such as the sleeping habits of patients, their hemoglobin level and parasite index were also noted. After recording the required information and patient history, whole blood samples were collected in EDTA-coated Vacutainer tubes. This study was approved by the ethical review committee of Quaid-i-Azam University, Islamabad.

Microscopic and molecular analysis

The slide preparation technique was performed to detect the type and stage of the malarial parasite. Thick and thin blood films were prepared on clean glass slides, fixed and stained with Giemsa and were observed at 100X under oil immersion according to WHO guidelines (Tareen *et al*. 2012). For molecular analysis, all samples were transferred to the Parasitology lab at Quaid-i-Azam University, Islamabad in a liquid nitrogen container. DNA was purified from 1 ml of infected venous blood using a commercially available DNA extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A nested-PCR approach of the small sub-unit ribosomal ribonucleic acid (ssrRNA) genes was adopted for the identification of *Plasmodium* species following previously described procedures (Zakeri *et al.* 2010). The amplified PCR products were loaded along with 100 bp DNA ladder using 1.5% agarose gel stained with ethidium bromide. DNA was visualized on an ultraviolet transilluminator and photographed using a gel documentation system (Wealtec, USA).

Results

Overall 691 samples were considered to be positive using microscopy. Of these, 83.2% (n = 508) were identified as *P. vivax*, 15% (n = 5104) as *P. falciparum* and 1.7% (n = 512) as mixed containing both *P. vivax* and *P. falciparum* (Table I).

Table I. Comparison of Microscopy and PCR based Diagnosis

SpeciesC	Microscopy	PCR
Plasmodium vivax	574 ± 7%	502 ± 2.3%
Plasmodium falciparum	108 ± 3	87 ± 0.8%
Mixed*(P.vivax,P.falciparum)*	12 ± 0.6%	31 ± 0.2%
Plasmodium ovale	0	0
Plasmodium malariae	0	0
Negative	0	47 ± 3.7%

These 691 samples along with positive and negative controls were amplified using nested-PCR. PCR identified 81.1% (n = 5508) as *P. vivax*, 13.8% (n = 587) as *P. falciparum* and 4.9% (n = 531) as mixed species. No species of *P. ovale* and *P. malariae* were found in any sample by using both microscopy and PCR.

To correlate the PCR and microscopy results, Cohen's kappa was calculated using SPSS 19.0. The following kappa values were used to categorize the strength of agreement between the nested-PCR and microscopic examination: poor (≤0), slight (.01–.20), fair (.21–.40), moderate (.41–.60), substantial (.61–.80), and almost perfect (.81–1) (Oyedeji *et al.* 2007). The chance adjustment of the kappa statistics indicates that overall agreement in the presence or absence of *Plasmodium* was high (kappa = 50.941) for *P. vivax* (kappa = 50.788) and *P. falciparum* (kappa = 50.987). As many *P. vivax* or *P. falciparum* mono-infections by microscopy were identified as mixed species infections by PCR, agreement on mixed species was low (kappa = 50.571).

Subjects that participated in the study were both male and female aged between 5 months and 70 years and all were local inhabitants of their native localities. However, the prevalence in the male population was quite high, ranging from 76% in Khyber Agency to 73% and 71% in Bajaur and South Waziristan, respectively (Table II).

Of the 626 positive samples by PCR, North Waziristan had the highest number of cases at 114. Bajaur had the second highest number with 104 followed by Khyber Agency at 102. Orakzai Agency had the lowest number of cases at 62. The overall prevalence of *Plasmodium* in Orakzai was 12%,

Table II. Gender and Age wise distribution of plasmodium by location

Locality	Total (mean)	Males (mean)	Females (mean)	Age in years median (mean)
Orakzai Agency	62 ± 4%	43 ± 0.15%	19 ± 0.07%	23 (2–65)
Kurrum Agency	91 ± 3.2%	56 ± 0.2%	35 ± 0.1%	28 (1–68)
Khyber Agency	102 ± 4.7%	78 ± 0.19%	24 ± 0.12%	26 (1–65)
Bajaur Agency	104 ± 4%	76 ± 2%	28 ± 0.25%	22 (0.5–70)
Mohmand Agency	83 ± 3.3%	52 ± 0.17%	31 ± 0.3%	28 (1–65
South Waziristan	70 ± 3.9%	39 ± 0.19%	31 ± 0.23%	29 (0.7–60)
North Waziristan	114 ± 2%	81 ± 0. 35%	33 ± 0.3%	26 (0.5–68)

Table III. Prevalence (%) of Plasmodium infection among individuals presenting with symptoms consistent with malaria, by Agency and Village

			Prevalence			
Agency name	**Village**	**Suspected cases**	***P.vivax***	***P.falciparum***	**Mixed species**	**All species**
Orakzai Agency			**54 (10.4%)**	**7 (1.3%)**	**1 (0.2%)**	**62 (12%)**
	Kalaya	150 ± 2.1%	10%	2%	0%	12%
	Kada	131 ± 1.3%	4.6%	0.8%	0%	5.4%
	Feroz Khel	137 ± 0.5%	14.6%	1.5%	0.7%	16.8%
	Mishti	99 ± 0.93%	12.1%	1%	0%	13.1%
Kurrum Agency			**79 (10.4%)**	**9 (1.1%)**	**3 (0.4%)**	**91 (11.9%)**
	Parachinar	210 ± 2.57%	14.2%	1.9%	0%	16.2%
	Sadda	213 ± 1.76%	15.5%	0.9%	0.9%	17.4%
	Shalozan	201 ± 2.1%	4%	1%	0.5%	5.5%
	Teri Mangal	135± 2%	5.8%	0.7%	0%	6.6%
Khyber Agency			**77 (10.2%)**	**12 (1.6%)**	**13 (1.7%)**	**102 (13.6%)**
	Bara	207 ± 1.3%	9.7%	1.9%	1.9%	13.5%
	Tirah	177 ± 1.6%	11.9%	3.4%	4.5%	19.8%
	Landi kotal	197 ± 1%	15.7%	0.5%	0.5%	16.7%
	Jamrud	170 ± 1.3%	3.5%	0%	0%	3.5%
Bajaur Agency			**89 (11.5%)**	**13 (1.7%)**	**2 (0.3%)**	**104 (13.5%)**
	Khar	230 ± 3.3%	10.9%	2.2%	0%	13%
	Barang	207 ± 3.34%	15%	1.9%	0.5%	17.4%
	Chmarkand	147 ± 1.7%	15.6%	2.7%	0%	18.3%
	Utmankhel	187± 2.5%	5.3%	0%	0.5%	5.8%
Mohmand Agency			**67 (15.4%)**	**11 (2.5%)**	**5 (1.1%)**	**83 (19%)**
	Halimzai	121± 1.83%	14%	3.3%	2.5%	19.8%
	Upper Mohmand	103± 2%	26.2%	1.9%	0%	28.2%
	Barangg Khar	160± 1.47%	13.1%	2.5%	1.3%	16.9%
	Ambar	51± 0.67%	3.9%	2%	0%	5.9%
South Waziristan Agency			**50 (21.5%)**	**16 (6.9%)**	**4 (1.7%)**	**70 (30%)**
	Wana	47±1.3%	27.7%	10.6%	4.3%	42.6%
	Angor Adda	63±0.54%	36.5%	4.8%	3.2%	44.4%
	Ladha	58± 0.35%	8.6%	8.6%	0%	17.2%
	Jandola	65± 0.31%	18.5%	0%	0%	18.5%
North Waziristan Agency			**92 (22.8%)**	**19 (4.7%)**	**3 (0.7%)**	**114 (28.2%)**
	Mirali	107± 1.1%	19.6%	5.6%	0.9%	26.2%
	Miramshah	100±1%	23%	4%	2%	29%
	Datta Khel	113±1.65%	29.2%	6.2%	0%	35.4%
	Jandola	83±1.1%	20.5%	0%	0%	20.5%
Total		**3869**	**13.1%**	**2.2%**	**0.8%**	**16.2%**

*Prevalence was calculated by dividing the number of PCR-positive cases by the number of suspected cases

Table IV. Number of samples PCR-positive for Plasmodium infection, by Agency and village

		PCR positive samples		
Agency	**Village**	***P.vivax***	***P.falciparum***	**Mixed species**
Orakzai Agency		**54 (± 1.7%)**	**7 (± 1.3%)**	**1 (± 1%)**
	Kalaya	15	3	0
	Kada	6	1	0
	Feroz Khel	21	2	1
	Mishti	12	2	0
Kurrum Agency		**79 (± 1.6%)**	**9 (± 1.1%)**	**3 (± 0.7%)**
	Parachinar	30	4	0
	Sadda	33	2	2
	Shalozan	8	2	1
	Teri Mangal	8	1	0
Khyber Agency		**77 (± 2.2%)**	**12 (± 1.6%)**	**13 (± 1.7%)**
	Bara	20	4	4
	Tirah	21	6	8
	Landi kotal	31	1	1
	Jamrud	6	0	0
Bajaur Agency		**89 (± 1.5%)**	**13 (± 1.7%)**	**2 (± 0.3%)**
	Khar	25	5	0
	Barang	31	4	1
	Chmarkand	23	4	0
	Utmankhel	10	0	1
Mohmand Agency		**67 (± 1.4%)**	**11 (± 2.5%)**	**5 (± 1.1%)**
	Halimzai	17	4	3
	Upper Mohmand	27	2	0
	Barangg Khar	21	4	2
	Ambar	2	1	0
South Waziristan Agency		**50 (± 1.5%)**	**16 (± 0.9%)**	**4 (± 1.7%)**
	Wana	13	5	2
	Angor Adda	23	3	2
	Ladha	5	5	0
	Jandola	12	0	0
North Waziristan Agency		**92 (± 2.8%)**	**19 (± 1.7%)**	**3 (± 0.7%)**
	Mirali	21	6	1
	Miramshah	23	4	2
	Datta Khel	33	7	0
	Jandola	17	0	0
Total	**508**	**87**	**31**	

11.9% in Kurrum Agency, 13.6% in Khyber Agency and 13.5% in Bajaur Agency. In Mohmand, North Waziristan and South Waziristan, the overall prevalence of *Plasmodium* was 19%, 30% and 28.2%, respectively (Table III).

In all locations, the predominant species was *P. vivax* followed by *P. falciparum.* However, Khyber Agency had the highest proportion of mixed *P. vivax and P. falciparum* infection, while South and North Waziristan had the highest number of *P. falciparum* infections. *P. falciparum* malaria was lowest in Orakzai Agency along with some regions of Kurrum and Bajaur (Table IV).

Discussion

The prevalence and incidence of malaria can be successfully reduced through passive and active diagnosis (Zoghi *et al.* 2012). The actual and accurate estimate of *Plasmodium* infections can also be helpful in scaling up malaria surveillance and control interventions in Pakistan (Yasinzai *et al*. 2008). Therefore, to achieve and maintain the malaria elimination campaign in Pakistan, the presence and prevalence of malaria parasites were determined in the most endemic areas (FATA) for the first time in the present study, by using light microscopy and nested-PCR methods.

All subjects were sampled in the peak transmission seasons at each location, although sudden rains and abrupt weather changes can alter the peaks for both *P. vivax* and *P. falciparum*. It was confirmed before sampling that subjected patients does not received any chemotherapy so as to avoid any false positive results. Geographic variations in peak periods may also occur (Bouma *et al.* 1996). These effects must be taken into account during the evaluation of relatively small differences in the stated prevalence of malaria and of the proportion of cases of *P. falciparum* and *P. vivax* between regions. Regardless of these limitations, the information displayed here contribute to a more complete and current trend of malaria in the FATA region of Pakistan.

Since the late 1970s, the annual number of malaria cases reported by each of the four provincial health departments has gradually increased. The Punjab province reported a sharp rise until the mid-1980s, a slowdown in the late 1980s and a further increase in the 1990s. Sindh and Khyber Pakhtunkhwa reported a steady increase throughout this period, while Baluchistan reported a rise in the 1990s (Rowland *et al.* 2002). This burden of malaria shifted from the southern and northern districts of Khyber Pakhtunkhwa in the 1980s to the western districts in the 1990s. By the end of the decade, an axis of relatively high malaria prevalence appeared stretching from Chitral and Swat in the north, through Swabi, Mardan, Malakand, to Mohmmand and Khyber along the western boundary with Afghanistan (Kazmi *et al.* 2001). In addition to the north and west, there was also a continuation of relatively high malaria proportions in the south. Kohat, Bannu and Khan continued to report above average incidence for most of these years (Khattak *et al*. 2013). According to recent studies, FATA have the highest incidence of malaria (Khatoon *et al.* 2013).

Our survey indicated that due to the free movement of Afghan refugees, lack of education and access to health care facilities and treatment, Khyber, Bajaur and Waziristan all had the highest proportion of both *P. vivax* and *P. falciparum*. In addition to the high *P. vivax* incidence in Khyber Agency, it also had the highest proportion of mixed infections, while North Waziristan had the highest number of *P. falciparum* cases. The recent internal displacement has thus contributed to the high incidence of malaria in Pakistan. In 2009, residents of several districts in FATA fled war conditions to settle in nearby districts and cities. This extensive cross-border movement of susceptible populations may have transformed the distribution of malaria in the country (Khattak *et al.* 2013). The overall prevalence in males is high, as they are more likely to work outside and not be as well covered as females, leading to an increased number of infected Anopheles bites.

The changing meteorological factors, resulting in increasing vectorial capacity and basic reproductive rate of *Anopheles sinensis* which may lead to further malaria re-emergence in these areas. The increase in *P. vivax* malaria could be explained by the high transmissibility of *P. vivax*, which is correlated to its typical biological features, including the presence of hypnozoites and shorter sporogony and instant appearance of gametocytes (Woyessa *et al.* 2012). Drug resistance and the refugees dwelling here from Afghanistan, a country endemic for *P. falciparum*, is a leading cause of falciparum malaria in the corresponding regions (Zoghi *et al.* 2012).Thus, chloroquine resistance and the free refugees' movement have accounted for the increasing prevalence of *P. falciparum* in all the areas sharing borders with Afghanistan, as observed in this study.

False diagnosis can also lead to incorrect or incomplete treatment, especially in countries such as Pakistan where chloroquine is used to treat *P. vivax*, but is known to be ineffective against *P. falciparum* (Woyessa *et al.* 2012). In our study, we emphasized that the microscopic examination of blood smears depends on skilled and experienced examiners; therefore, it is vital to improve the accuracy of malaria diagnostic techniques especially to determine species-specific parasitemia and to assist with diagnostic method procurement decisions in a more professional fashion. This would also ease the calculation of the ratio of mixed *Plasmodium* species infection and the monitoring of patients receiving anti-malarial treatment.

Conclusion

The lack of published documentation from international agencies about what has been tried, what has worked and what has failed leaves a significant gap in our knowledge of malaria control in complex emergencies. It is concluded from the present study that in the local area the prevalence of malarial parasites is still high due to the continuous influx of refugees, unfortunate hygienic conditions and poor drainage systems, lack of education, use of presumptive treatments and unavailability of artemisinin-based combination therapy. The findings of this study will be helpful to set up an emergent anti-malarial campaign to prevent and control the spread of malaria in FATA with an emphasis on improving species diagnosis and the availability of artemisinin-based combination therapy for the treatment of *P. falciparum.*

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Competing interests
The authors declare that they have no competing interests.

References

Abdullah N.R., Barber B.E., William T., Norahmad N. A., Satsu U.R., Muniandy, P.K., Ismail Z., Grigg J.M., Jelip J., Piera K., Seidlein L.V., Yeo T.W., Anstey N.M., Price R. N. Auburn S. 2013. *Plasmodium vivax* Population Structure and Transmission Dynamics In Sabah Malaysia. *PloS One*, 8, 178–200. DOI:10.1371/journal.pone.0082553

Binello N., Brunetti E., Cattaneo F., Lissandrin R., Malfitano A. 2014. Oligohydramnios in a pregnant Pakistani woman with

Plasmodium Vivax malaria. *Malaria Journal*, 13, 1–4. DOI: 10.1186/1475-2875-13-156

Bouma M., Dye C., Van der Kaay H. 1996. *Falciparum* malaria and climate change in the Northwest Frontier Province of Pakistan. *Bulletin of the World Health Organization*, 74, 413–421

Elyazar I.R., Gething P. W., Patil A.P., Rogayah H., Sariwati E., Palupi N.W.,Hay S. I. 2012. *Plasmodium vivax* malaria endemicity in Indonesia in 2010. *PloS One*, 7, e37325. Doi:10.1371/journal.pone.0037325

Kazmi J.H., Pandit K. 2001. Disease and dislocation: the impact of refugee movements on the geography of malaria in NWFP, Pakistan. *Social Science and Medicine*, *52*, 1043–1055. DOI: 10.1016/S0277-9536(00)00211-2

Khan H., Khattak A.M. 2006. A study of prevalence of malaria in adult population of DI Khan, Pakistan. *Biomedica*, *22*, 99–104

Khatoon L., Baliraine F.N., Bonizzoni M., Malik S.A., Yan G. 2010. Genetic structure of *Plasmodium vivax* and *Plasmodium falciparum* in the Bannu district of Pakistan. *Malaria Journal*, *9*, 112. DOI:10.1186/1475-2875-9-112

Khatoon L., Jan M.I., Khan I.U., Ullah F., Malik S.A. 2013. Genetic diversity in human malarial parasites of Khyber Agency Pakistan. *Acta Parasitologica*, 58, 564–569. DOI:10.2478/s11686-013-0181-3

Khattak A.A., Venkatesan M., Nadeem M.F., Satti H.S., Yaqoob S., Khatoon M.S., Plowe C. 2013. Prevalence and distribution of human *Plasmodium* infection in Pakistan. *Malaria Journal*, *12*, 297. DOI:10.1186/1475-2875-12-297

Leslie T., Kaur H., Mohammed N., Kolaczinski K., Ord R.L., Rowland M. 2009. Epidemic of *Plasmodium falciparum* malaria involving substandard antimalarial drugs, Pakistan, 2003. *Emerging Infectious Diseases*, 15, 1753.DOI: 10.3201/eid1511.090886

Nawaz, S. 2009. FATA-a Most Dangerous Place: Meeting the Challenge of Militancy and Terror in the Federally Administered Tribal Areas of Pakistan. Center for Strategic and International Studies, Washington, DC 2600 USA

Oyedeji S.I., Awobode H.O., Monday G.C., Kendjo E., Kremsner P. G., Kun J.F. 2007. Comparison of PCR-based detection of *Plasmodium falciparum* infections based on single and multicopy genes. *Malaria Journal*, *6*1, 112. DOI:10.1186/1475-2875-6-112

Prybylski D., Khaliq A., Fox E., Sarwari A.R., Strickland G. T. 1999. Parasite density and malaria morbidity in the Pakistani Punjab. *The American Journal of Tropical Medicine and Hygiene*, 61, 791–801

Rowland M., Mahmood P., Iqbal J., Carneiro I. and Chavasse D. 2000. Indoor residual spraying with alpha cypermethrin controls malaria in Pakistan: a Community-randomized trial. *Tropical Medicine and International Health*, 5, 472–481. DOI: 10.1046/j.1365- 3156.2000.00581.x

Rowland M., Rab M.A., Freeman T., Durrani, N., Rehman N. 2002. Afghan refugees and the temporal and spatial distribution of malaria in Pakistan. *Social Science and Medicine*, 55, 2061–2072. DOI: 10.1016/S0277-9536(01)00341-0

Siraj A., Santos-Vega M., Bouma M., Yadeta D., Carrascal D. R., Pascual M. 2014. Altitudinal changes in malaria incidence in highlands of Ethiopia and Colombia. *Science*, 343, 1154–1158. DOI: 10.1126/science.1244325

Suleman M. 1988. Malaria in Afghan refugees in Pakistan. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 82, 44–47. DOI: 10.1016/0035-9203(88)90257-X

Tareen A. M., Rafique M., Wadood A., Qasim M., Rahman H., Shah, S., Pirkani G. 2012. Malaria burden in human population of Quetta, Pakistan. *European Journal of Microbiology and Immunology*, 2, 201–204.DOI: 10.1556/EuJMI.2.2012.3.5

Singh B., Sung K.L., Matusop A., Radhakrishnan A., Shamsul S.S., Cox-Singh J., Balbir T.S., Albino B., Janet C.S., Georges S., Shukri A.M., Hasan A.R.1999. A genus and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *American Journal of Tropical Medicine and Hygiene*, 60, 687–692

Woyessa A., Deressa W., Ali A., Lindtjorn B. 2012. Prevalence of malaria infection in Butajira area, south-central Ethiopia. *Malaria Journal*, 11, 84. DOI:10.1186/1475-2875-11-84

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

Zakeri S., Kakar Q., Ghasemi F., Raeisi A., Butt W., Safi N. 2010. Detection of mixed *Plasmodium falciparum* and *P. vivax* infections by nested-PCR in Pakistan, Iran and Afghanistan. *Indian Journal of Medical Research*, 132, 31–5

Zoghi S., Mehrizi A.A., Raeisi A., Haghdoost A.A., Turki H., Safari R. 2012. Survey for asymptomatic malaria cases in low transmission settings of Iran under elimination programme. *Malaria Journal*, 11, 126. DOI: 10.1186/1475-2875-11-126

Zubairi A.B.S., Nizami, S., Raza A., Mehraj V., Rasheed A.F., Ghanchi N. K., Beg M. A. 2013. Severe Plasmodium vivax Malaria in Pakistan. *Emerging Infectious Diseases*, 19, 1851. DOI:10.3201/eid1911.130495

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