Original Article

Pfcrt Gene in *Plasmodium falciparum* Field Isolates from Muzaffargarh, Pakistan

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Abstract

Background: The aim of the study was to identify the prevalence of different species of *Plasmodium* and haplotypes of *pfcrt* in *Plasmodium falciparum* from the selected area.
Methods: Overall, 10,372 blood films of suspected malarial patients were examined microscopically from rural health center Sinawan, district Muzaffargrah, Pakistan from November 2008 to November 2010. *P. falciparum* positive samples (both whole blood and FTA blood spotted cards) were used for DNA extraction. Nested PCR was used to amplify the *pfcrt* (codon 72–76) gene fragment. Sequencing was carried out to find the haplotypes in the amplified fragment of *pfcrt* gene.
Result: Over all slide positivity rate (SPR), *P. vivax* and *P. falciparum* positivity rate was 21.40 %, 19.37 % and 2.03% respectively. FTA blood spotted cards were equally efficient in the blood storage for PCR and sequencing. Analysis of sequencing results of *pfcrt* showed only one type of haplotype S_{agt}VMNT (AGTGTAATGAATACA) from codon 72–76 in all samples.
Conclusion: The results show high prevalence of CQ resistance and AQ resistant genes. AQ is not recommended to be used as a partner drug in ACT in this locality, so as to ward off future catastrophes.

Keywords: Malaria, *pfcrt*, Resistance, Pakistan

Introduction

Globally 106 countries from tropical and subtropical are endemic for malaria (WHO 2010). These are in tropical or subtropical zones with are suitable breading places far anopheline mosquito. The regions included in this area are Asia, Africa, Islands of South, West and Central Pacific Ocean, Latin America, Turkey and certain Caribbean Islands (Lamar et al. 2007). In the areas of malaria transmission, epidemics of malaria have been leading cause of high morbidity and mortality. Complex epidemics of malaria have been observed in Kenya (May 1999–August 1999, Burundi (September 2000–May 2001), South Sudan (June 2003–November 2003), in two areas of Ethiopia (July 2003–February 2004) and India (January 2005–March 2005) (Checchi et al. 2006, Kumari 2009).

In 3rd world countries including Pakistan, malaria has always been major public health problem and it accounts for more than 95% of regional burden (Huda and Zamani 2009). The main reason of malariogenic potential in Pakistan is extensive agricultural practices, vast irrigation network, monsoon rains and poverty (WHO 2005). Annually, about 500000 episodes of malaria infection occur (Yasinzai and Kakharsulemankhel 2008) in rural areas, were associated with more people live below poverty line (38.65%) than urban areas (22.39%).

The main-stay in the control of malaria is

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the increasing frequency of drug resistant parasites especially *P. falciparum* (Gama et al. 2010).

First reports of resistance against CQ came from South America (Moore and Lanier 1961), South East Asia (Harinausta et al. 1965). Later, it reached to Africa. Across the world, CQR strain of *P. falciparum* originated from at least six locations independently: in Java, Philippines, Papua New Guinea, South East Asia and two locations in South America (Wootton et al. 2002, Chen et al. 2003). In Pakistan, the resistance of *P. falciparum* to CQ was first detected in 1981 from Sheikhupura and National Institute of Malaria Research and Training (NIMRT) revealed that R1 level of CQ resistance was widespread in Pakistan (frequency ranging from 30–84%) (Shah et al. 1997). Chloroquine was used as a first line treatment in Pakistan from 1950–2007 (Asif 2008). In the district Muzaffargarh, response of *P. falciparum* to chloroquine was found out during the time period of 2003–2005. RI was 29.36 %, and RII was 29.41 % (Rana 2009).

In 1990s, chloroquine resistant *P. falciparum* spread widely in Pakistan (Rab et al. 2001). Factors involved in the spread of drug resistance are drug use practices, drug half life, transmission intensity, clone multiplicity, parasite density, host immunity and genetic basis of drug resistance (Hasting and Watking 2006, Pongtavornpinyo et al. 2008). Resistance against many antimalarials has arisen in the same geographic areas. These areas have low transmission rate, low immunity level and higher parasites biomass (White and Pongtavornpinyo 2003). Use of substandard medicines can also be involved in the emergence of drug resistance (Leslie et al. 2009).

With the advancement in molecular biology and isolation of CQ resistance clone (Dd2) helped in identification of molecular markers in *P. falciparum* associated with drug resistance (Jiang et al. 2008). In 1990, Thomas Wellem along with his team performed a cross between HB3, a chloroquine sensitive (CQS) and Dd2, a chloroquine resistant (CQR) strain (Wellem et al. 1990). It was found that the main determinant of CQ resistance is a locus on chromosome 7 of *P. falciparum* (Wellem et al. 1991). It was further noted that it is a 38 Kb fragment (10 genes) (Su et al. 1997). Fidock et al. (2000) reported that a highly polymorphic 3.1 Kb gene (composed of 13 exons) is involved in resistance. Martin and Kirk (2004) reported that it is a 48.6 kDa protein of 424 amino acids long. It forms 10 transmembrane domains. The protein is located on digestive vacuole and it has transporter-like properties. It was called *P. falciparum* chloroquine resistant transport gene (*pfcrt*) and considered as a member of drug metabolite transporter super family (Tran and Saier 2004). In the past, SVMNT (ser-val-meth-asn-thr) allele carrying parasites had been reported twice, in 1996–1997 from Africa and in 2004 from Tanzania (Alifrangis et al. 2006). Three common haplotypes of *pfcrt* are CVMNK (CQS, present in all geographic regions), CVIET (CQR found in Africa and South East Asia), SVMNT (CQR, S_{agt}VMNT is present in some countries of Asia and S_{tct} VMNT is found in South America) (Wootton et al. 2002, Ursing et al. 2006). The high level of resistance to AQ metabolite desethylamodiaquine (DEAQ) is found to be associated with the presence of *pfcrt* codon 72–76 haplotype SVMNT. It replaces CVMNK found in CQS strain (Wootton et al. 2002, Warhurst et al. 2003, Sa et al. 2009, Beshir et al. 2010). In oocyte system, competition of drugs with CQ was checked by mutated *pfcrt*. Drug quinine and AQ at normal concentration and mefloquine and primaquine at higher concentration were able to compete with CQ for transport by mutated *pfcrt* while piperaquine and artemisinin did not interact with *pfcrt* (Martin et al. 2009).

Keeping in view the situation of malaria,

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this study was designed to identify the chloroquine resistant genes of *P. falciparum* which convert a chloroquine sensitive parasite (CQS) to chloroquine resistant (CQR) type parasite.

Materials and Methods

Selection of Area for Sampling

Muzaffargarh is located at latitude 30.06˚ longitudes 71.02˚, between the two rivers, Chenab in the East and Indus in the West. For present study, the area of Muzaffargarh was selected on the basis of high endemicity. Data obtained by the curtsy of Directorate General Health Punjab, Pakistan (through personal communication) reported "1388 laboratory confirmed cases" in Punjab were with 15 % in district Muzaffargarh in 2006. In 2007, total number of positive malarial cases was 1903, again highest positive cases (7.5%) were observed in Muzaffargarh.

Sample Collection

Blood samples were collected from individuals presented with fever, persistent headache and malaria like symptoms. Patient's consent was obtained prior to sampling, finger prick blood was used for slide preparation. Thick and thin blood smears were prepared and stained by Giemsa stain (3% solution in 7.2 pH phosphate buffer) (WHO 1991). Two ml blood of *P. falciparum* positive patients was collected in EDTA vacutainers and spotted on Flinders Technical Associates (FTA) classic cards (Whatman) by micropipette according to manufacturer's instruction. Vacutainers were stored at 4 ˚C and cards were stored at room temperature till use for DNA extraction. Sampling was done at Rural Health Centers Muzaffargarh from November, 2008 to November, 2010 (25 months) as shown in Fig. 1.

DNA Extraction

For amplification of *pfcrt* gene, DNA was eluted both from FTA cards and whole blood. *P. falciparum* positive samples (by microscopic review and RDTs method). FTA Cards contain chemicals that lyses (removes) blood cells, denature proteins and protect nucleic acids from nucleases, oxidation and UV damage. Captured nucleic acid is ready for downstream applications in less than 30 min and is stable for years at room temperature. DNA was eluted according to manufacturer's instructions (Whatman). While from whole blood, 200 µl of sample was used for DNA extraction with the QIA amp midi kit, as described by the manufacturer (Qiagen). Extracted DNA was analyzed by using 1 % agarose gel.

Amplification of *pfcrt* Gene Fragment

Two-step nested PCR was done to amplify *pfcrt* gene fragment of 145 base pairs. Primers were designed, using DNA sequence of *P. falciparum* chloroquine resistant strain Dd2/Indochina (by Pub Med accession number AF030694). The sequences of primers used in round 1 and round 2 are given in Table1.

Preparation of PCR Reaction Mix for Round 1 (*pfcrt*)

The PCR mixture contained 1X PCR buffer (Promega), 2.0 mM $MgCl_2$ (Promega), 0.2 mM dNTPs, 0.25 µM each primer, 1.25 units, 0.5 units Taq polymerase (Promega) and 5 µl template DNA. The total reaction volume was 50 µl.

Cycling conditions for PCR were: one cycle of 95 ˚C for 5 minutes followed by 30 cycles of 95 ˚C for 10 seconds, 57 ˚C for 30 seconds and 72 ˚C for 30 seconds, followed by a final extension at 72 ˚C for 10 minutes.

PCR Reaction Mix Preparation for Round 2 (*pfcrt*)

The PCR mixture contained 1X PCR buffer (Promega), 2.0mM $MgCl_2$ (Promega), 0.2 mM dNTPs, 0.25 µM each primer, 0.5 units Taq polymerase (Promega) and 5 µltemplate DNA.

The total reaction volume was 50 µl.

Cycling conditions for PCR were: one cycle of 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, 48 °C for 30 seconds and 65 °C for 30 seconds, followed by a final extension at 65 °C for 3 minutes.

Amplified PCR products of each gene fragment were electrophoresed using 3 % agarose gel and visualized on UV. PCR products were cleaned using Exonuclease I (BioLabs Inc) and sequenced in both directions using Big Dye® Terminator system.

Statistical Analysis

Results of sequencing were analyzed by the programme of Applied Bio-system (sequence scanner V, 0.1).

Results

Sampling was carried out in Muzaffargarh, Pakistan from November 2008 to November 2010 (25 Months), in order to find out the malaria frequency. During this period, 10372 blood films of suspected malarial patients were prepared and examined. Their positivity rates for the infection of *Plasmodium* (both *falciparum* and *vivax*), *P. vivax* and *P. falciparum* were 2220, 2009 and 211 respectively. In this duration, slide positivity rate (SPR), *P. vivax* positivity rate % (VPR) and *P. falciparum* positivity rate (FPR) were 21.40 %, 19.37 % and 2.03 % respectively. The difference in *P. vivax* (90.49%) and *P. falciparum* (9.51%) infection was found highly significant (x^2= 1456, P< 0.001).

Extracted DNA

Samples positive for *P. falciparum* (211) were used for DNA extraction. Genomic DNA is shown in Fig. 2, 3.

Amplification of *pfcrt* (codon 72–76)

All the 211 *P. falciparum* positive samples were amplified using polymerase chain reaction. Amplification was carried out of the extracted DNA from whole blood and from FTA blood spotted cards.

***pfcrt* (codon 72–76) Amplification (by using disc of blood spotted FTA cards)**

Amplification by using the disc (2mm) was also conducted by using punched disc from FTA blood spotted cards. Product of (145bp) was visible as show in Fig. 4.

Similar PCR results were obtained using DNA extracted from whole blood and FTA blood spotted cards which showed that FTA cards were found equally efficient for storage of blood for PCR reaction.
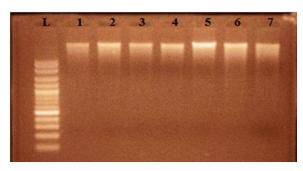
Sequencing Result

Sequencing results were analyzed by using Applied-Biosystem VI software. It showed only one type of haplotype sequence S_{agt} VMNT (AGTGTAATGAATACA) from codon 72–76 in all samples. No wild type CVMNT or other drug resistant (mutant) haplotype was found. Fig. 5 is depicting sequencing results of *pfcrt* (codon 72–76) with forward primer.

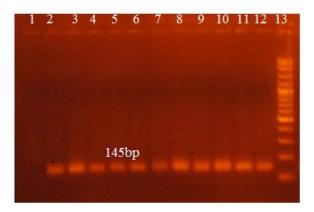**Table 1.** Primers used for amplification of *pfcrt* gene fragment of 145 base pairs

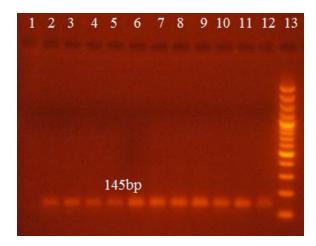| PCR Round	Primer	Primer Sequence (5'–3')	Tm
1st Round	Crtd- A (forward primer)	5'- GGTGGAGGTTCTTGTCTTGGTA -3'	57.5 °C
	Crtd- B (reverse primer)	5'- GACCTATGAAGGCCAAAATGACTG-3'	56.7 °C
2nd Round	Crtd- C (forward primer)	5'- TGTGCTCATGTGTTTAAACTT -3'	50.06 °C
	Crtd –D (reverse primer)	5'- CAAAACTATAGTTACCAATTTT -3'	46.10 °C

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Fig. 1. Map of district Muzaffargarh showing RHCs

Fig. 2. Gel electrophoresis picture showing extracted genomic DNA of falciparum positive malarial patients (Lane L shows 100bp ladder, lane 1–7 show genomic DNA)

Fig. 3. Gel electrophoresis picture showing amplified *pfcrt* gene fragment (145bp) (by using DNA extracted from the whole blood). Lane 1 and 2 show negative and positive controls respectively, lanes 3 to 12 show patient samples, lane 13 shows 100bp DNA Marker

Fig. 4. Picture of gel electrophoresis showing amplified *pfcrt* gene fragment (145bp) (by using disc of blood spotted card). Lane 1 and 2 show negative and positive controls respectively, lanes 3 to 12 show patient samples, lane 13 shows 100bp DNA Marker
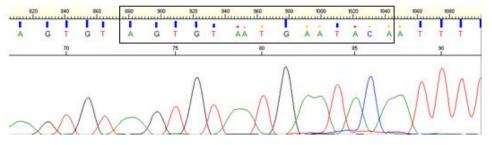

Fig. 5. Sequencing results of *pfcrt* (codon 72–76)

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Discussion

Present findings showed that overall SPR observed in Muzaffargarh, Pakistan was 21.40 %. According to the report of Ministry of Health, (2006) malaria was the second most prevalent disease in Pakistan (Murtaza et al. 2009). Akin figures of SPR (19.7% and 19.40%) were reported from Punjab and Khyber Pakhtunkhwa (Idris et al. 2007, Mohammad et al. 2010), which were respectively 1.64 % and 2.00 % less than present study. SPR reported from India has shown the percentages of 25.2 % and 24.54 % (Dhiman et al. 2010, Panigahi et al. 2011), which were 3.80% and 3.14% higher than in current study.

Although malaria is rife worldwide but the maximum numbers of devastations caused by it are in Sub-Saharan Africa (WHO 2010). In Africa 30.20 %, 27.07 % and 22.60 % SPR was reported from Uganda, Ethiopia and Tanzania (Jensen et al. 2009, Yohannes and Haile 2010, Mubi et al. 2011) and these figures indicated an enhancement of 8.80 %, 5.67 % and 1.20 % respectively than the figures of the current study. These differences may be due to geographical, climatic, economic and cultural variations.

In the current study, *P. vivax*, was the predominant specie of this region. The overall infection caused by *P. vivax* and *P. falciparum* was 90.49 % and 9.51 % respectively, and the difference between the infection of *P. vivax* and *P. falciparum* was highly significant (^{2}= 1456, P< 0.001). Similar figures were collected from Muzaffarabad, Azad Kashmir and Khyber Pakhtunkhwa, occurrence of *P. vivax* infection (90.40% 92.20%, 92.20%) was far more than that caused by *P. falciparum* infection (9.6%, 7.8% and 7.8%) (Jan and Kiani 2001, Jamal et al. 2005, Jalal-ul-din and Ally 2006). Similarly higher incidence of *P. vivax* infection (72.4%) than *P. falciparum* infection (24.1%) was reported from Baluchistan province (Idris et al. 2007). Some other figures from the same prov-

ince showed higher incidence of *P. vivax* infection (58.9%, 51.8%, 88.6%, 72.3%) than *P. falciparum* infection (41%, 48.1%, 11.3%, 27.6%) (Yasinzai and Kakarsulemankhel 2008). Infections caused by *P. vivax* were also reported more than those of *P. falciparum* from some areas of Sind (Mahmood et al. 2005, Atif et al. 2009). *P. vivax* infection (11.70%, 75%) was less than *P. falciparum* infection (88.29%, 85%) in some areas or Indian (Dhiman et al. 2010, Panigrahi et al. 2011). Similarly, *P. falciparum* (90%, 70%, 90.7% 68.53%) was predominant specie in some African regions (Pinto et al. 2000, Yohannes and Haile 2010, Lemma et al. 2011).

Currently, parasites strains are exhibiting resistance to all major classes of drug present in the field and some of them have been withdrawn from use because of their ineffectiveness. Unfortunately we are unaware that where the next generation of antimalarial will come from (Grimberg and Mehlotra 2011). Core objective of the study was the scrutiny of mutant and wild types of *pfcrt* (codon 72–76). *pfcrt* gene fragment of 145bp was amplified in all positive *P. falciparum* (211) samples. Results showed 100 % S_{agt} VMNT (ser-val-meth-asn-thr). No S_{tct}VMNT haplotype or wild type (CVMNK) was observed. Origin of S_{agt}VMNT had been hypothesized from Mato Grasso, Brazil (Vieira et al. 2004) and was reported from Guyana, Peru, Suriname, Venezuela (Contreras et al. 2002, Cortese et al. 2002, Peek et al. 2005, Mehlotra et al. 2008, Griffing et al. 2010). Clinical resistance to chloroquine (CQ) and amodiaquine (AQ) was found associated with mutations in *pfcrt* gene (Picot et al. 2009, Beshir et al. 2010). Sa et al. (2009) correlated high level resistance to AQ metabolite desethylamodiaquine (DEAQ) in tests (*in vitro*) with the presence of SVMNT haplotype of *pfcrt*. Mehlotra et al. (2008), Zakeri et al. (2008), and Beshir et al. (2010)

detected high prevalence of this haplotype in the parasitic population of Brazil, Papua New Guinea, Laos, Afghanistan and Iran. Esmaeili et al. (2008) also reported very high prevalence of K76T mutation in an endemic district of Iran. These results were also supported by the study carried out (in vitro) on natural *P. falciparum* isolate, laboratory reference strain, parasites transfected with allelic replacement and progeny of genetic crosses (Sidu et al. 2002, Sa et al. 2009). In the past, SVMNT (ser-val-meth-asn-thr) allele carrying parasites had been reported twice, in 1996–1997 from Africa and in 2004 from Tanzania (Alifrangis et al. 2006). Three common haplotypes of *pfcrt* are CVMNK (CQS, present in all geographic regions), CVIET (CQR found in Africa and South East Asia), SVMNT (CQR, S_{agt} VMNT is present in some countries of Asia and S_{tct} VMNT is found in South America) (Wootton et al. 2002, Ursing et al. 2006). In the area of Muzaffargarh *P. vivax* was pre-dominant specie so CQ exposure was higher. It may be the main reason of high prevalence of spreading CQR parasite (having *pfcrt* "SVMNT") in this area. Similar findings were reported from India, mutant *pfcrt* "SVMNT" haplotype was endemic in those areas, where *P. vivax* was dominant specie (Mallick et al. 2012).

Conclusion

Prior to everything else, appropriate diagnosis of *Plasmodium* and its species is highly necessary. A treatment initiated without proper diagnosis not only increases the probabilities of drug resistance, but also invites complications. Malaria controlling policies should be decentralized: made and implemented at province and district levels. Proper understanding and careful evaluation of local epidemiological pattern and the other aspects of malaria can produce base-line data in planning task for devising control strategies. The drug resistance issue is likely to

pose long term challenges for us. Hybrid of conventional and neo drugs can help to conserve the efficiency of valuable anti malarial medicines, but choosing the right combination partners will continue to require the study of presently known and emerging mechanism.

The results show high prevalence of CQ resistance and AQ resistant genes. Excessive presence of CQ and AQ resistant genes in plasmodium population isolated from the people of Muzaffargarh suggest establishing the base line for monitoring of mutations. This is evident now that in this area, CQ or AQ will not be effective for the victims of *P. falciparum* infection. AQ is not recommended to be used as a partner drug in ACT in this locality, so as to ward off future catastrophes.

Acknowledgements

References

Alifrangis M, Dalgaard MB, Lusingu JP, Vestergaard LS, Staalsoe T, Jensen ATR, Enevold A, Rønn AM, Khalil IF, Warhurst DC (2006) Occurrence of the Southeast Asian/ South American SVMNT haplotype of the chloroquine-

resistance transporter gene in *Plasmodium falciparum* in Tanzania. J Infect Dis. 193(12): 1738–1741.

Asif SA (2008) Departmental audit of malaria control programme 2001–2005 North West Frontier Province (NWFP). J Ayub Med Coll Abbottabad. 20(1): 98–102.

Atif S, Farzana M, Naila S, Abdul F (2009) Incidence and pattern of malarial infection at a tertiary care Hospital of Hyderabad. World J Med Sci. 4(1): 9–12.

Beshir K, Sutherland CJ, Merinopoulos I, Durrani N, Leslie T, Rowland M, Hallett RL (2010) Amodiaquine resistance in *Plasmodium falciparum* malaria in Afghanistan is associated with the *Pfcrt* SVMNT allele at codons 72 to 76. Antimicrob Agents Chemother. 54(9): 3714–3716.

Checchi F, Cox J, Balkan S, Tamrat A, Priotto G, Alberti KP, Zurovac D, Guthmann JP (2006) Malaria epidemics and interventions, Kenya, Burundi, southern Sudan, and Ethiopia, 1999–2004. Emerg Infect Dis. 12: 1477–1485.

Chen N, Kyle DE, Pasay C, Fowler EV, Baker J, Peters JM, Cheng Q (2003) *pfcrt* allelic types with two novel amino acid mutations in chloroquine-resistant *Plasmodium falciparum* isolates from the Philippines. Antimicrob Agents Chemother. 47(11): 3500–3505.

Contreras CE, Cortese JF, Caraballo A, Plowe CV (2002) Genetics of drug-resistant *Plasmodium falciparum* malaria in the Venezuelan state of Bolivar. Am J Trop Med Hyg. 67(4): 400–405.

Cortese JF, Caraballo A, Contreras CE, Plowe CV (2002) Origin and dissemination of *Plasmodium falciparum* drug-resistance mutations in South America. J Infect Dis. 186(7): 999–1006.

Dhiman RC, Chavan L, Pant M, Pahwa S (2010) National and regional impacts of climate change on malaria by 2030.

Curr Sci. 101(3): 372–383.

Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos L (2002) Mutations in the *Plasmodium falciparum* digestive vacuole transmembrane protein *Pfcrt* and evidence for their role in chloroquine resistance. Mol Cell. 6(4): 861–871.

Gama B, de Oliveira N, de Souza J, Santos F, de Carvalho L, Melo Y, Rosenthal P, Daniel-Ribeiro C, Ferreira-da-Cruz M (2010) Brazilian *Plasmodium falciparum* isolates: investigation of candidate polymorphisms for artemisinin resistance before introduction of artemisinin-based combination therapy. Malar J. 9(1): 355–359.

Griffing S, Syphard L, Sridaran S, McCollum AM, Mixson-Hayden T, Vinayak S, Villegas L, Barnwell JW, Escalante AA, Udhayakumar V (2010) *pfmdr1* amplification and fixation of *pfcrt* chloroquine resistance alleles in *Plasmodium falciparum* in Venezuela. Antimicrob Agents Chemother. 54(4): 1572–1579.

Grimberg BT, Mehlotra RK (2011) Expanding the Antimalarial Drug Arsenal-Now, But How? Pharmaceuticals. 4(5): 681–712.

Hastings IM, Watkins WM (2006) Tolerance is the key to understanding antimalarial drug resistance. Trends Parasitol. 22(2): 71–77.

Huda A, Zamani G (2009) The progress of roll back malaria in Eastern Mediterranean region over the past decade. East Mediterr Health J. 14: 82–9.

Idris M, Sarwar J, Fareed J (2007) Pattern of malaria infection diagnosed at Ayub Teaching Hospital Abbottabad. J Ayub Med Coll Abbottabad. 19(2): 35–36.

Jalal-ud-din SAK, Ally SH (2006) Malaria in children: study of 160 cases at a private clinic in Mansehra. J Ayub Med Coll Abbottabad. 18(3): 44–45.

Jan A, Kiani T (2001) Haematozoan para-

sites in Kashmiri refugees. Pak J Med Res. 40(1): 10–12.

Jensen T, Bukirwa H, Njama-Meya D, Francis D, Kamya M, Rosenthal P, Dorsey G (2009) Use of the slide positivity rate to estimate changes in malaria incidence in a cohort of Ugandan children. Malar J. 8(1): 213.

Kumari R, Joshi P, Lal S, Shah W (2009) Management of malaria threat following tsunami in Andaman and Nicobar Islands, India and impact of altered environment created by tsunami on malaria situation of the islands. Acta Trop. 112(2): 204–211.

Lamar J, Martschinske R, Tetreault G, Doud C (2007) Navy Medical Department Pocket Guide to Malaria Prevention and Control. Technical Manual NEHC-TM PM 6250.1

Lemma H, San-Sebastian M, Lofgren C, Barnabas G (2011) Cost-effectiveness of three malaria treatment strategies in rural Tigray, Ethiopia where both *Plasmodium falciparum* and *P. vivax* co-dominate. Cost Eff Resour Alloc. 9: 2–10.

Leslie T, Kaur H, Mohammed N, Kolaczinski K, Ord RL, Rowland M (2009) Epidemic of *Plasmodium falciparum* malaria involving substandard antimalarial drugs, Pakistan, 2003. Emerging Infect Dis. 15(11): 1753–1759.

Mahmood K (2005) Malaria in Karachi and other areas in Sindh. Pak Armed Forces Med J. 55: 345–348.

Mallick P, Joshi H, Valecha N, Sharma S, Eapen A, Bhatt R, Srivastava H, Sutton P, Dash A, Bhasin V (2012) Mutant *pfcrt* "SVMNT" haplotype and wild type pfmdr1 "N86" are endemic in *Plasmodium vivax* dominated areas of India under high chloroquine exposure. Malar J. 1(11): 16.

Martin RE, Kirk K (2004) The malaria parasite's chloroquine resistance trans-
porter is a member of the drug/ metabolite transporter superfamily. Mol Biol Evol. 21(10): 1938–1949.

Martin RE, Marchetti RV, Cowan AI, Howitt SM, Bröer S, Kirk K (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Sci. 325(5948): 1680–1682.

Mehlotra RK, Mattera G, Bockarie MJ, Maguire JD, Baird JK, Sharma YD, Alifrangis M, Dorsey G, Rosenthal PJ, Fryauff DJ (2008) Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria parasite *Plasmodium falciparum*. Antimicrob Agents Chemother. 52(6): 2212–2222.

Moore DV, Lanier JE (1961) Observations on two *Plasmodium falciparum* infections with an abnormal response to chloroquine. Am J Trop Med Hyg. 10(1): 5–9.

Mubi M, Janson A, Warsame M, Martensson A, Kallander K, Petzold MG, Ngasala B, Maganga G, Gustafsson LL, Massele A (2011) Malaria rapid testing by community health workers is effective and safe for targeting malaria treatment: randomized cross-over trial in Tanzania. Plos one. 6(7): e19753.

Muhammad W, Khan A, Faiz-ur-Rehman Ali A, Irshad S (2010) Slide positivity rate in clinically suspected malaria cases. J Med Sci. 18(2): 101–103.

Murtaza G, Memon IA, Memon AR, Lal M, Kallar NA (2009) Malaria morbidity in Sindh and the *Plasmodium* species distribution. Pak J Med Sci. 25(4): 646–649.

Panigrahi B, Kerketta A, Mohapatra A, Hazra R, Parida S, Marai N, Kar S, Mahapatra N (2011) Effect of construction of an irrigation canal on malaria situation in two primary health centers of Dhenkanal district of Orissa, India. Trop Biomed. 28(1): 76–84.

Peek R, Van Gool T, Panchoe D, Greve S, Bus E, Resida L (2005) Drug resistance and genetic diversity of *Plasmodium falciparum* parasites from Suriname. Am J Trop Med Hyg. 73(5): 833–838.

Peter BB (2001) Drug Resistance in Malaria. WHO/CDS/CSR/DRS/2001.4, World Health Organization.

Picot S, Olliaro P, De Monbrison F, Bienvenu AL, Price RN, Ringwald P (2009) A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in falciparum malaria. Malar J. 8: 89.

Pinto J, Sousa C, Gil V, Goncalves L, Lopes D, do Rosario V, Charlwood J (2000) Mixed-species malaria infections in the human population of Sao Tome island, West Africa. Trans R Soc Trop Med Hyg. 94(3): 256–257.

Pongtavornpinyo W, Yeung S, Hastings I, Dondorp A, Day N, White N (2008) Spread of anti-malarial drug resistance: mathematical model with implications for ACT drug policies. Malar J. 7(1): 229–240.

Prashant KM, Hema J, Neena V, Surya KS, Alex E, Rajendra MB, Harish CS, Patrick LS, Aditya PD, Virendra KB (2012) Mutant *pfcrt* "SVMNT" haplotype and wild type *pfmdr1* "N86" are endemic in *Plasmodium vivax* dominated areas of India under high chloroquine exposure. Malar J. 11: 16.

Punjab Strategic Plan for Malaria Control (2005) National Malaria Control Programme, Ministry of Health, Government of Pakistan. Strategic Plan. 15–21.

Rab M, Freeman T, Durrani N, De Poerck D, Rowland M (2001) Resistance of *Plasmodium falciparum* malaria to chloroquine is widespread in eastern Afghanistan. Ann Trop Med Parasitol. 95(1): 41–46.

Rana SM (2009) Treatment responses of uncomplicated *falciparum* malaria to different antimalaria drugs by mono and combination therapy in Punjab, Pakistan. [PhD thesis]. Department of Zoology, University of the Punjab Lahore, Pakistan.

Sá JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, Wellems TE (2009) Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. Proc Natl Acad Sci. 106(45): 18883.

Sandie M, Isabelle M, Rachida T, Collins S, Pembe IM, Xavier I, Françoise B, Brigitte L, Jean-François M, Parfait A, Leonardo KB, Antoine B (2012) Molecular monitoring of *Plasmodium falciparum* drug susceptibility at the time of the introduction of artemisinin-based combination therapy in Yaoundé, Cameroon: Implications for the future. Malar J. 11: 113.

Shah I, Rowland M, Mehmood P, Mujahid C, Razique F, Hewitt S, Durrani N (1997) Chloroquine resistance in Pakistan and the upsurge of falciparum malaria in Pakistani and Afghan refugee populations. Ann Trop Med Parasitol. 91(6): 591–602.

Sidhu ABS, Verdier-Pinard D, Fidock DA (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Sci. 298(5591): 210–213.

Su X, Kirkman LA, Fujioka H, Wellems TE (1997) Complex polymorphisms in an 330 kDa protein are linked to chloroquine-resistant *Plasmodium falciparum* in Southeast Asia and Africa Cell. 91(5): 593–603.

Tran CV, Saier MH (2004) The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/ metabolite transporter

superfamily. Microbiol. 150(1): 1–3.

Ursing J, Zakeri S, Gil J, Bjorkman A (2006) Quinoline resistance associated polymorphisms in the *pfcrt*, pfmdr1 and pfmrp genes of *Plasmodium falciparum* in Iran. Acta Trop. 97(3): 352–356.

Vieira PP, Urbano FM, Alecrim MG, Alecrim WD, da Silva LHP, Sihuincha MM, Joy DA, Mu J, Su X, Zalis MG (2004) *pfcrt* Polymorphism and the spread of chloroquine resistance in *Plasmodium falciparum* populations across the Amazon Basin. J Infect Dis. 190(2): 417–424.

Warhurst DC (2003) Polymorphism in the *Plasmodium falciparum* chloroquine-resistance transporter protein links verapamil enhancement of chloroquine sensitivity with the clinical efficacy of amodiaquine. Malar J. 2: 31.

Wellems TE, Panton LJ, Guzman IY, do Rosario VE, Gwadz RW, Walker-Jonah A, Krogstad DJ (1990) Chloroquine resistance not linked to mdr-1 like genes in a *Plasmodium falciparum* cross. Nature. 345: 253–255.

Wellems TE, Walker-Jonah A, Panton LJ (1991) Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. Proc Natl Acad Sci. 88(8): 3382–3386.

White N, Pongtavornpinyo W (2003) The denovo selection of drug–resistant malaria parasites. Proc R Soc Lond B Biol Sci. 270(1514): 545–554.

Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, Magill AJ, Su X (2002) Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. Nature. 418(6895): 320–323.

World Health Organization (2005) World malaria report. Geneva.

World Health Organization (2010) World malaria report. Geneva.

Yasinazi MI, Kakarsulemank JM (2008) Incidence of Human Malaria Infection in Northern Hilly Region of Balochistan Adjoining with NWFP, Pakistan: District Zhob. Pak J Biol Sci. 11(12): 1620–1624.

Yohannes M, Haile M (2010) The potential of *in situ* rain water harvesting for water resources conservation on malaria transmission in Tigray, Northern Ethiopia. Momona Ethiopian J Sci. 2(2): 49–63.

Zakeri S, Afsharpad M, Kazemzadeh T, Mehdizadeh K, Shabani A, Djadid ND (2008) Association of *pfcrt* but not pfmdr1 alleles with chloroquine resistance in Iranian isolates of *Plasmodium falciparum*. Am J Trop Med Hyg. 78(4): 633–640.