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Morphological and Molecular Identification of Malaria Vector in Three Sites in Kordofan Region with Exposure of Plasmodium Falciparum as Malaria Parasite

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Abstract: ***Background: The aim of this study is to identify the malaria vector and the parasite in three towns in the region of Kordofan, namely Elobeid, Alfula and Kadugli. Methods: The samples of Anopheles arabiensis vector were collected and identified morphologically and genetically. Blood samples from patients were collected on filter paper and Plasmodium species was determined by nested PCR amplification. Results: The results showed that Anopheles arabiensis was the main vector of malaria in Kordofanregion while Plasmodium falciparum was the dominant parasite. Conclusion: The present data suggest that the molecular identification of Anopheles aribiensis and nested PCR detection of Plasmodium falciparum can be useful complement to microscopical diagnosis.***

Keywords: Plasmodium faciparum, malaria, Anopheles arabiensis

1. Introduction

The malaria vectors *Anopheles gambiae* and *Anopheles arabiensis* have recently become the subject of intensive molecular geneticstudies to determine gene flow patterns and population structure (Lehmann et al. 1996; Besansky et al. 1997; Lanzaro et al. 1998; Donnelly et al., 1999; Simard et al., 2000). The objective of these studies has been to estimate processes that influence the spread of genes, such as those that confer the resistance of insecticides between populations. These studies have used microsatellite loci or mitochondrial to calculate statistics such as Wright's (1978) and Slatkin's (1995), which measure differentiation between populations based on the inter population component of the total genetic variation. Anopheles is a genus of mosquito (Culicidae). There are approximately 484 recognized species: while over 100 can transmit human malaria, only 30-40 commonly transmit parasites of the genus Plasmodium that cause malaria which affects humans in endemic areas. *Anopheles gambiae* is one of the best known, because of its predominant role in the transmission of the deadly species - *Plasmodium falciparum* (Wikipedia, 2010). *An. arabiensis* tend to increase towards the end of the rainy season and during the dry season as have been observed in northern Nigeria and Tanzania (White et al., 1972). *An. arabiensis* is found resting both indoors and outdoors and feeds on cattle and humans (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).Problems in classical Anopheles mosquito's taxonomy include not only strong morphological similarity between species, but also pronoun morphological variation within species. *An.arabiensis* in Sudan from different sites in Khartoum, showed a 500bp banding pattern indicating the amplification of An. Arabiensis DNA in PCR reaction (Abushama, 1999). Fadl, (2002) stated that all amplification based on two ribosomal sets of primers successfully gave a product of 500bp in all samples indicating the specific amplification of *An. arabiensis* leading to suggestion of a predominant distribution of *An. arabiensis* in Northern Sudan.). *Plasmodium falciparum* malaria is a major cause of mortality and morbidity, particularly in endemic areas of sub-Saharan Africa (Adel, et. al.2011). Usually only *P.falciparum* can cause death during an acute stricken ofmalaria. Other species of malaria parasites infect animals but are not normally passed on to humans. The parasite of malaria is very small and can only be seen by a microscope. They can be observed by examination a stain blood smear from infected person. Their numbers vary according to intensity of infection. If there are only a few numbers of parasites, it may be necessary to spend several minutes examining a blood smear under microscope before one can be observed. Using the microscope, it is also possible to identify the species of the parasite that is present; a trained worker can determine whether a patient has been infected by *Plasmodium falciparum* or other species (WHO, 1992).

2. Methods

2.1 The Study Area

For the purpose of the study, the survey covered three sites in Kordofan region chosen as follows: Alfula site in the east of Kordofan region with a population of 127462; Kadugli site in the south of the region with a population of 105252 and Elobeid site in the north of the region with a population of 157505. The bulk of the building survey in the towns were straw huts, mud houses and virtually, stone construction.

2.2 Entomological Studies of the Anopheles Vector

2.2.1 Sampling Techniques
Sampling of mosquitoes was carried at each of the three sites. Ten huts and mud houses not sprayed before close to breeding sites were randomly chosen from each site for adult mosquito sampling. Three mosquito workers assisted throughout the experimental period.

2.2.2 Sampling of Adult Mosquitoes
Populations of adult mosquitoes were sampled by two methods: Knock-Down Collection (KDC) also known as Spray Sheet Collection and sucking tubes (ST) method. For each sampling method, records of the numbers caught were made.

2.2.3 Species Morphological Identification
On arrival at the laboratory the mosquitoes were identified according to the length and shape of palps. Anopheles arabiensis mosquitoes were identified using the identification key by AbdElnur (1998) which used morphological features of the adult. After identification, each individual sample of Anopheles arabiensis adult was transferred to a labeled eppendorf tube. Samples to be used later for DNA extraction were preserved in one ml of 70% ethanol and kept at4°C.

2.2.4 Molecular Techniques for Identification
Polymerase chain reaction (PCR) technique was used to amplify mosquito DNA using specific primers to identify Anopheles arabiensis mosquitoes collected from the three study sites.

2.2.5 DNA Extraction for Anopheles arabiensis
Genomic DNA was extracted from individual specimens according to the phenol chloroform method of Richard and Frank (2002). Two hundred (200) of Anopheles arabiensis mosquito samples from the three sites were used.

The procedure used for DNA extraction was as follows: Each sample was put into 1.5 ml eppendorftube, 500µlSTE buffer (0.1MNACL, 0.5MTrisHCL, 0.001 M EDTA) and 2.5µl of 10 ml of stock solution proteinase K were added and mixed well. After that 25µl of 20% of sodium dodcylesulphate (SDS) solution were added and incubated at 37º C overnight. An equal volume of solution of phenol, chloroform and isoamyl alcohol (PCI) in ratio of 25:24:1 was added to the mixture and incubated at room temperature for five minutes. Then the samples were centrifuged for 5 minutes at 7000 rpm. The aqueous layer was removed carefully with a micropipette and transferred toa clean tube. The aqueous layer was re-extracted with PCI. After that, an equal volume of chloroform and iso-amyl alcohol (CI) solution in a ratio 24:1 was added and then incubated at room temperature for 3 minutes. The mixture was centrifuged for 5 minutes at 7000 rpm. The aqueous layer was removed carefully with a micropipette and transferred to a clean tube; the aqueous layer was re-extracted again with PCI. Fourty five (45) µl of 2M Nacl and double and the same volume cold absolute ethanol were added to precipitate the DNA. The mixture was then incubated overnight at -20°C. Ethanol was decanted and the pellet was dried and the pellet was re-suspended in 20µ of 1XTE buffer (0.01 M TrisHCl, Ph8, 0.001MEDTA).

2.2.6 PCR Primers for Anopheles Species
PCR primers, methods used to produce genomic libraries and obtain the r DNA containing phage clones. All sub clones are in the plasmid p Blue script (strata gene) and were sequenced by standard dideoxy methods, modified for double stranded templates (Sanger, 1977). The 3′ end of the 28S gene is contained in a 0.8 Kb Hind 111 fragment that was sequenced in both species. The first PCR primer chosen, Aº, consisted of a 20 bp 28S gene sequence shared by both species (Table3.1). The 5º end of the Hind III fragment contained the start of the intragenic spacer region which provided the first set of species–specific PCR primer sequences ($A_a$and Ag), located about 0.5 Kb from Aº. A second set of species–specific primer ($A_a$and Ag) was chosen from intragenic spacer sequences located approximately 800 bp from the A_a.

Table 1: DNA sequences of primers for Anopheles species-distinguishing assays based on polymerase chain reaction (PCR).

Primers	Specific Primers DNA Sequences (5' to 3')
Aº	ATGCCTGAACGCCTCTAAGG
A_a	CAAGATGGTTAGTTACGCCAA

Reaction Conditions
Genomic DNA from single mosquitoes was prepared as described by Collins et al. (1987). PCR reagents were obtained as a kit from Perkin–Elmer Cetus. Optimal condition for PCR reaction was determined empirically. Conditions for most reactions were as follows: X1 PCR buffer, 1.5 Mm $MgCl_2$, 1.5 mMdNTPs, 0.4µM of each of the primers, 2.5 µl Ampitaq, 2.0 µl DNA and 13.0 µl sterile water in a total volume of 25µl. Anopheles arabiensis identified PCR cycling consisted of melting at 94º C for 3 minutes followed by 35 cycles of 94º C for one minute, 55º C for one minute, 72º C for 2 minutes followed by 70º C for 5 minutes and a final hold at 4º C

2.3 Gel Electrophoresis for Anopheles araboensis

An amount of 0.2 gram agarose were added to 1X TBE (18 ml water to 2 ml 10 xTBE); the gel was put in a water bath until the gel dissolved, then 0.5 ml of ethidium bromide were added to the gel solution. Agarose gel was poured in a horizontal electrophoresis apparatus casting tray, a comb was inserted in the liquid gel and the agarose was left to solidify. The solution of 1X TBE was poured in The gel apparatus, five microliters of each PCR product were mix.

The gel apparatus, five microliters of each PCR product were mix with 3 µl of loading buffer (glycerol, bromophenol blue andwater in a ratio of 1:1:8). A marker DNA ladder was also loaded for determination of the amplified DNA fragment size. The electrophoresis apparatus was connected to a power supply at a voltage of 80 V and run for one hour. After completion of the run, the gel was observed under ultraviolet light to determine whether the PCR product was amplified. Selected gels were photographed using a gel documentation UVITECmachine.

2.4 Parasitological Survey of Plasmodium falciparum

A survey was undertaken in Elobeid city between the period 2010 and 2011 to evaluate whether Plasmodium falciparum was endemic in the area. The study of the parasite and its percentage among the population of the city was determined by taking 200 blood samples in 3mm what Mann filter papers.

2.5 Filter Paper Genomic DNA Extraction from Plasmodium falciparum

Preparation of Plasmodium falciparum genomic DNA was made from the blood collected on 3mm Whatmann filter paper. The dry blood on filter paper was treated by the phenol-chloroform method described by Wooden et al. (1992). The extracted DNA from each sample was used immediately for PCR and the remaining DNA portion was stored at -20°C in appropriately labeled storage tubes. DNA extraction solutions were prepared in the form of EDTA (0.5 M pH 8.0), lysis buffer (pH 7.0 and pH 8.0), TE buffer (pH 7.0 and pH 8.0), phenol chloroform isoamyl alcohol (25:24:1), Sodium Dodcyl Sulphate (SDS), 3M sodium acetate (pH7.0). Absolutean 70%, ethanol was chilled at -20 and a solution of: proteinase K enzyme was prepared and refrigerated.Clinical samples of blood drops on filter paper were used to isolate genomic DNA using the Phenol – chloroform extraction method. Using a sterile puncture (70% ethanol cleaned), 2 to 4 pieces from dry blood drops on the Whatmann paper were punctured and put separately on eppendorf tubes. Lysis buffer (500 µl STE, 20 µl SDS, 5 µl Proteinase K) was added to each piece of paper and incubated over night at -54º C. The samples were centrifuged at 12000 rpm for one minute. The supernatant from each sample was transferred to a clean tube. 500 µl of PCI (phenol, chloroform, isoamyl alcohol) were added, vortexed and centrifuged at 12000 rpm for 5 minutes. The upper layer was transferred into a new eppendorf tube and 45µl sodium acetate and one ml absolute ethanol were added, incubated on ice for 15 minutes and then centrifuged at 1000 rpm for 10 minutes. Ethanol was decanted and the pellet was air dried. For washing the pellet, 200µl of 70% ethanol were added, the mixture vortexed and centrifuged at 12000 rpm for 10 minutes. Ethanol was decanted and the pellet was dried for 2-3 hours. The pellet was then dissolved in 50µl TEbuffer.

2.6 Nested PCR for Detection of Plasmodiumfalciparum

Plasmodium species DNA was detected by nested PCR amplification of small sub-unit ribosomal RNA gene (ssrRNA) using the primers and cycling parameters described by Snounou et al. (1993). The species-specific primers were designed to hybridize to the genes coding for only one of the two ssrRNA types present in the Plasmodium genome. The PCR reactions were carried as described below.

1x PCR buffer, 10 µM enhancer solution, 1.5 mMdNTPs were mixed in a PCR tube. Two µl of the purified template DNA was used for the mixture of the first reaction (outer) in which the fragment spanned by rPLU5 (5′CCTGTTGTTGCCTTAAACTTC3′) and rPLU2 (5′ TTAAAATTGTTGCAGTTAAAACG). To the mixture, 12.3µL sterile water and 2.5 units ampliTaq polymerase were added. The total volume of each of the reactions was 25 µl. Two µl of aliquot from the product of the first PCR reaction was then used as a template in the (inner) nested reaction. The species-specific primer pair for detection of P. falciparum rFAL1 (5′TTAAACTGGTTTGGGAAAACCAAATATATT3′) and rFAL2 (5′ACACAATGAACTCAATCATGACTACCCGTC3′), was used. PCR cycling for Plasmodium falciparum consisted of positive control and negative control for each set of amplification reactions. The PCR assays were performed using a Techno USA DNA Thermal Cycler. The cycling parameters for the first amplification reaction were as follows: 95º C for 5 minutes followed by 25 cycles of 58º C for 2 minutes, 72º C for 2 minutes, 94º C for one minute, 72º C for 5 minutes and a final hold at 4º C. The entire above program was repeated for the inner reaction, species specific amplification reaction, using 30cycles.

2.7 Gel Electrophoresis for Plasmodiumfalciparum

One % of agarose gels were made by adding one gram of molecular grade agarose to 1X TB buffer (54 ml water to 6 ml 10X TBE). The gel was put in a water bath until the gel dissolved. 0.5 ml of ethidium bromide solution was added to the gel solution. Agarose gel was poured in a horizontal electrophoresis apparatus (casting tray) and a comb was inserted in the gel and left to solidify. A solution of 1X TBE was poured in the electrophoresis apparatus, five µl of each PCR product were mixed with 3 µl of loading buffer (glycerol, bromophenol blue and water in the ratio 1:1:8). A marker DNA ladder was also loaded for determination of the amplified DNA size. The electrophoresis apparatus was connected to the power supply at voltage of 80 V and run for one hour. After completion of the run, the gel was observed under ultraviolet light to determine whether PCR product was amplified. Selectedgel was photographed using a gel documentation UVITEC machine as before.

3. Results

Anopheles Species Morphological Identification

The mosquito samples collected from the three sites in Kordofan region were identified according to the external feature of wing spots, leg shape, length of maxillary palps and abdominal end model.

Sampling of AdultMosquitoes

A total of 905 *Anopheles arabiensis* were collected from the three study sites, 801from Elobeid, 50 from Alfula and 54 from Kadugli. All the samples were morphologically An. arabiensis (Table 1) and no other vector of malaria was detected in the study sites.

Table 1: Mosquito samples collected from the three study areas using knock down collection (KDC)

Date	Site	No. of houses being sprayed	No. of Anopheles Arabiensis collected
4-9-2010	Elobeid	10	87
5-8-2010	Elobeid	10	51
6-8-2010	Elobeid	10	76
15-8-2010	Elobeid	10	101
16-8-2010	Elobeid	10	97
17-8-2010	Elobeid	10	98
26-6-2010	Alfula	10	50
28-8-2010	Kadugli	10	54
26-7-2011	Elobeid	10	66
14-8-2011	Elobeid	10	102
15-8-2011	Elobeid	10	123

Detection of *Anopheles arabiensis* usingPCR

PCR was used as the reference standard method for the detection of the samples being collected from the study areas. From all the samples only 100 samples were amplified using primer A° and Aa. The characteristic 500 bp banding pattern indicating the amplification of *An. arabiensis*DNA in PCR reaction appeared. All the samples from the three sites have the same band size. Figure (1.0) shows the result of amplification products of PCR using rDNA gene-based primer A° and $A_a$with template DNA from Elobeid site. Figure (2.0) shows the template DNA from Kadugli and Figure (3.0) shows the template DNA from Alfula.

4. Molecular Identification

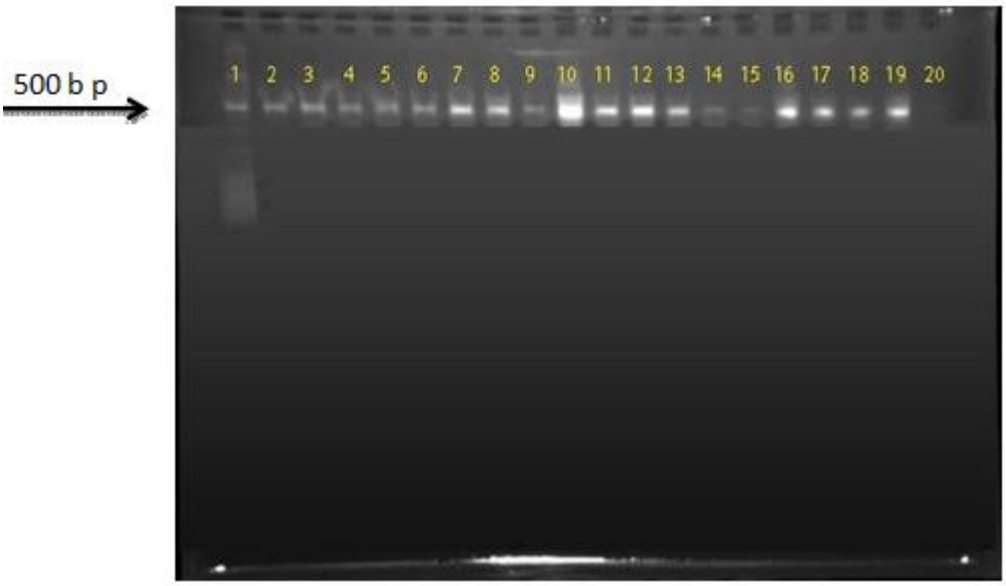

Figure 1: Amplification products of PCR using rDNA gene- based primer A° and Aawith template DNA from Elobeid site

- The marker sample is in lane 1
- The mosquitos' samples are in lane 2-18 O The positive control sample is in lane 19 O The negative control sample is in lane 20 O The amplified band is 500 bp in size

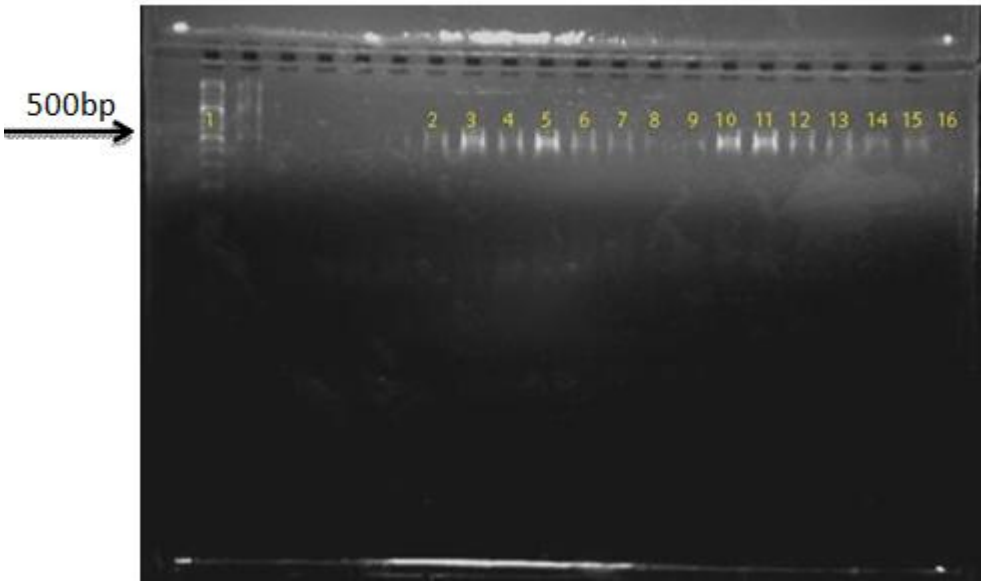

Figure 2: Amplification products of PCR using rDNA gene- based primer A° and $A_a$with template DNA from Kadugli

- The marker is in lane 1
- The samples are in lanes 2-15
- The negative control is in lane 16 O The amplified band is 500 bp

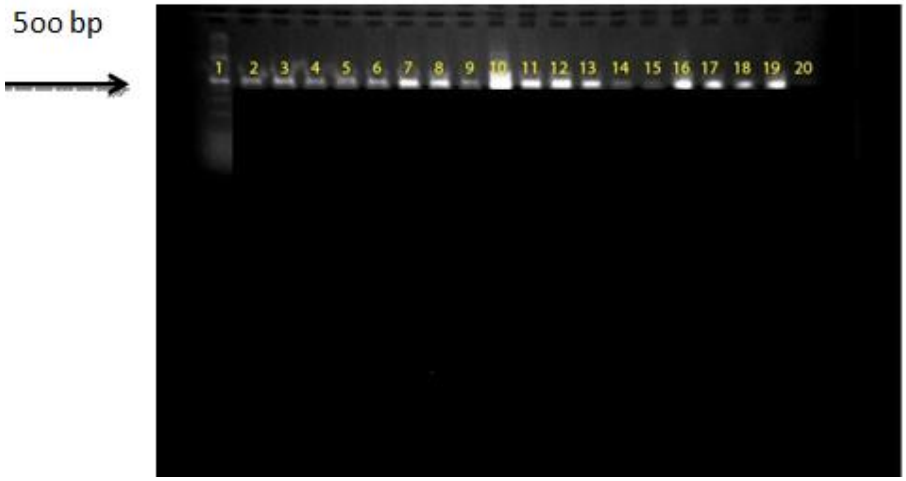

Figure 3: Amplification products of PCR using rDNAgene-based primer A° and $A_a$with template Anopheles DNA from Alfula

- The marker sample is in lane 1
- The Anopheles samples are in lane 2-18 O The positive control is in lane 19
- The negative control is in lane 20 O The amplified band is 500 bp

Detection of Plasmodiumfalciparumusing nestedPCR

The prevalence of malaria parasite in the three study sites was detected using PCR reaction. All the positive samples had the same base pair indicating the amplification of 205 pb band. This result confirmed the result of the microscopy, however, the mixed infections samples were not examined by PCR (Figure4.0).

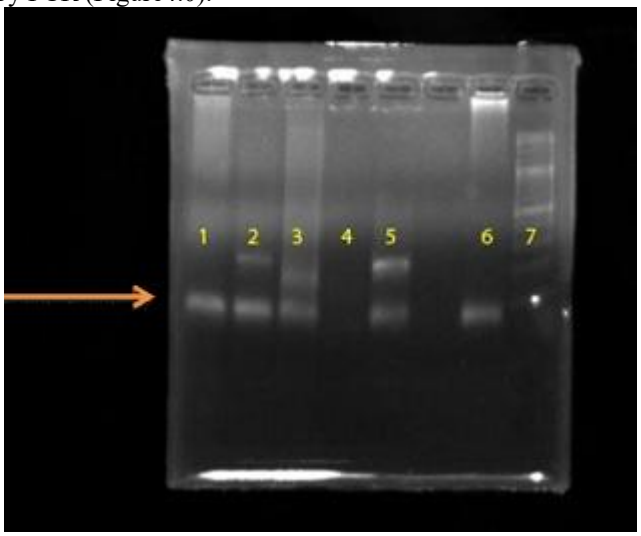

Figure 4: Detection of Plasmodium falciparum using nested PCR

- The positive control is in lane 1
- The Plasmodium falciparum samples are in lanes 2,3,5 and 6
- The negative control is in lane 4
- The marker is in lane 7
- The amplified band is 205 bp

5. Discussion

The large number of *Anopheles arabiensis* collected from Elobeid site does not indicate that the density of prevalence of the vector is high in this site, but can be explained by the fact that the collection was repeated several times during two years. However in areas of Kadugli and Alfula collection was done only in one year. The target of entomological survey is to confirm that the vector of malaria was established in the three study sites according to the phenotyping and genotyping survey. The results show that morphologically, and genetically, all the samples were *Anopheles arabiensis* mosquitoes and no other species were present in the study population except for the other non-vectors of malaria which was excluded. The prevalence and distribution limits of the *Anopheles arabiensis* have remained extremely stable since the time of the first available data instead of the big environmental changes that have occurred, like the Scheme of Storing water from Bara town. Previous studies indicated that *Anopheles arabiensis* prevailed in some localities in arid savanna zones (Coetzee et. al., 2000), but also was the prevalent species in some forest zones. Previous studies of the relationship between transmission of malaria and the density of *Anopheles arabiensis* revealed that transmission occurs mainly during the rainy season, which peaks in August, September and October but also there was transmission during the dry season. Why there is transmission during the dry season? It was observed that the continuous artificial breeding sites in the Elobeid town were because of the behavior of the people who used to store drinking water in containers like stand-tanks, underground tanks and barrels. Although no artificial breeding sites were reported in Sudan (Omer, 1986. Omer et.al. 1970), these containers may be suitable places for breeding of *Anopheles arabiensis*. This observation was supported by Bruce-Chwatt (1957) Choumara (1961) and Visser (1965) who reported the observation of continuous breeding by *Anopheles arabiensis* during extended dry periods in parts of Africa where surface water are scarce indicating it can exploit containers habitats.

The evidence of malaria stricken being widely distributed in the beginning of rain fall at July, but malaria infection started to decline and be in low level. At the end of June to the beginning of August the infection of malaria increased gradually untilled the beginning of September through October where the rate of malaria infections reached the beak, and most of the patients come to the hospitals have the same symptoms. Also the people in the town were suffering from repeated malaria symptoms in less than two weeks, although they completed the dose of malaria according to the doctors' prescription.

The result of detecting the parasite by PCR in this study, indicate that *Plasmodium falciparum* is responsible of all the infection in the Elobeid, however when recently the laboratories began to use ICT for detection of malaria parasite they discovered mixed infections of *Plasmodium falciparum* with *Plasmodium vivax*. Even with this detection of mixed infection, until now the main parasite in the area is *P. falciparum*. The symptoms of malaria are always the same: fever, chills, vomiting, diarrhea and headaches; but some people in the town feel fatigue and tiredness in the whole of the body without any fever. This directs to suggestion of prevalence of new types of malaria in the town.

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