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# Detection of *Plasmodium* in *Anopheles arabiensis* using nested-PCR in Jazan region, Saudi Arabia

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## ABSTRACT

The present study was carried out in 26 villages at two Governates (Al-Khobah, and Haroob) in Jazan Region in Southwest Saudi Arabia to identify and detect the presence of *Plasmodium* in *Anopheles arabiensis* using nested-PCR technique. *An. Arabiensis* was identified by PCR and it was the predominant *Anopheles* mosquito in all the collection sites. A total of 257 *An. Arabiensis* females were collected and two samples from two villages (Almuatan and Alsabkha) out of 107 (1.87%) female mosquitoes from Haroob Governate were found positive for the sporozoites of *Plasmodium falciparum*. Similarly, 3 out of 150 (2%) female mosquito samples from Um-alkhameir, AL-Khobah Governate, were also found positive. Around fourfold increase of thesporozoite rate (from 0.61 to 2.0%) in *An. arabiens* is in AL-Khobah Governate has been observed compared to the previous study of 2007-2008. The wide spread of *An. arabiensis* in Jazan region with >90% of the malaria cases caused by *P. falciparum*, along with infectivity rate ranges between 1.87 to 2.0% for *P. falciparum* in Al-Khobah and Haroob Governates, suggests that *P. falciparum* is the most predominant malaria parasite and *An. Arabiensis* is a very efficient malaria vector in the region. It also suggestsmore in-depth researches on the ecology, behavior, and control of *An. Arabiensis* to promote area-specific control programs.

**KEY WORDS:** MALARIA, JAZAN, SAUDI ARABIA, *PLASMODIUM FALCIPARUM*, PCR, *ANOPHELESARABIENSIS*

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## INTRODUCTION

Malaria is an infectious disease caused by the bites of female *Anopheles* mosquitoes infected with *Plasmodium* spp. (Cowman &tCrabb, 2006).There are approximately fifteen *Anopheles* species present in Saudi Arabia, but only four play a major role in parasitic transmission; these species are *Anopheles arabiensis, Anopheles sergentii*, *Anopheles stephensi,* and *Anopheles Superpictus* (Sebai, 1988; Zahar, 1985). *Anopheles arabiensis* has been identified as the primary vector transmitting malaria in Tihama area (southwest of Saudi Arabia and northeast Yemen)(Alsheikh, 2004),andconsidered to be a very efficient transmitting vector in the Afro-tropical area, with large variations in life behaviour including feeding, resting, and breeding(Beiernand Koros, 1991; Coetzee and Le Sueur, 1988).Five species of *Plasmodium* have long been recognized to infecthumans; these species include *Plasmodium falciparum (the most virulent form of malaria with* approximately 90% malaria deaths globally), *Plasmodium vivax* (the most common cause of malaria globally), *Plasmodiummalariae*, *Plasmodiumovale* and *Plasmodiumknowlesi* (WHO,2016).

The annual estimates of WHO in December 2015 for malaria were 214 million cases of and 438 000 deaths, with 3.2 billion people were at risk of malaria transmission (WHO, 2016). The disease remains a considerable threat due to several reasons, such as transmission enabling environments, poverty, lack of awareness, impaired health system infrastructures, political and socioeconomic problems, mass population migration and the emergence of multi-drug resistance (WHO, 2011; WHO, 2014).

In Saudi Arabia, about 5% of the Saudi population is at risk of malaria (approximately 2.4 million people) (Alsheikh, 2011). The disease is restricted to the South Western region of Tihama (Jazan and Asir), where more than 70% of all malaria cases in the country are occurred (El Hassan *et al*, 2015). Moreover, from 2000 to 2014 there were 5522 known locally acquired cases of malaria and around 9936 imported cases (El Hassan *et al*, 2015).

Identifying the presence of sporozoites of human malaria in the salivary glands of potential *Anopheline* vectors is the final step in establishing vector status(Alsheikh, 2004).Moreover, the determination of sporozoite rates has been considered as the most important entomological factor in the epidemiology of human malaria (Wirtz *et al*, 1987).

The intensity of malaria transmission is determined by calculating the entomological inoculation rate (EIR), as a simple estimated parameter, which is the product of the man-biting rate (defined as the number of bites per person per night) and the sporozoite rate (Reisen and Boreham, 1982; Dye, 1986). Although vectorial capacity is also a useful estimator of potential transmission intensity (Garret- Jones and Shidrawi, 1969), it is often difficult to determine with reliability owing to the numerous biological parameters required for its estimation.There are three techniques have been used for identification of *Plasmodium* sporozoites in salivary glands of *Anopheles* female mosquitoes. These include; ordinary microscopy dissection, immunological techniques, and polymerase chain reaction (PCR) (Burkot *et al*, 1984; Beier *et al*, 1988; Alsheikh, 2004).The latter has the potential for detecting and screening malaria parasites, especially in endemic regions, as well as in monitoring the effectiveness of malaria therapy (Moody, 2002; Morassin *et al*, 2002; Gama *et al*, 2007).Al-Maktari and Bassiouny (1999)in Yemen have recorded sporozoitesrate of 0.7% (4/600) for *P. falciparum* in *An. arabiensis* using microscopy method.

In Jazan region, Saudi Arabia, Al-Sheikh (2011) reported 0.61% as sporozoite rate of *P. falciparum* in *A. arabiensis collected in 2007 – 2008* using nested PCR. To the best of our knowledge, since that date no other data in Jazan region or other areas of Saudi Arabia has been published on the determination of sporozoite rate of *P. falciparum* in *A. arabiensis* using nested-PCR. The present study thushas been conducted to detect and identify the infectious *Plasmodium* species inside the malarial vector *Anopheles arabiensis* using nested-PCR techniques comparing previous data.

## MATERIAL AND METHODS

This study was carried out at two small Governates (Al-Khobah, and Haroob) in the Jazan Region in Southwest Saudi Arabia, lies between 16°-12, and 18°-25, latitude north (Alsheikh, 2011), with a total area of about 22,000 km2 and 1.3 million populations (census 2011). Thirty percent of the population concentrated in six major cities, and the remainders living in over 3500 villages (Fig. 1) (Alsheikh, 2011).

Jazan region is situated in the subtropical zone and has average monthly temperatures ranging between 25.8°C in January to 33.4°C in July. The average relative humidity ranges from 55% and 72.5%. The rainy season is started at August through October with a monthly average of 77 and 56.7 mm, respectively (Alsheikh, 2011).

*Anopheles arabiensis* specimens were collected from indoor human dwellings of 26 villages distributed in tow Governates (Al-Khobah and Haroob) from January to December 2015 (Table 1). The two Governates were selected based on the presence of *An. Arabiensis* and the reports of malaria cases.The collection of specimens was performed using Pyrethrum Knockdown (PKD)


FIGURE 1. The map of Jazan region showing its different Governates.

collections as described by WHO (1992). Collected mosquitoes were brought to the National Center for Vector-Borne Diseases in Jazan for morphological and molecular identification, and sporozoite rate determination.

The collected mosquitoes were identified based on morphological features given by Glick (1992) and Mattingly (1956). A total of 257 *An. Arabiensis* females were preserved individually in 1.5 ml plastic tube, labeled,

Table 1. Number and distribution of *Anopheles arabiensis* collected from 26 villages of Alkhobah and Haroob (January – December 2015)

| Serial No. | Al-khobah Governate | | Serial No. | Haroob Governate | |
|---|---|---|---|---|---|
| | Villages | No. of samples | | Villages | No. of samples |
| 1 | Wabrah | 5 | 1 | GaeimMahroog | 13 |
| 2 | GaeimMzubaid | 15 | 2 | Haroob city | 4 |
| 3 | ZahrAljamal | 5 | 3 | Al-muatan | 12 |
| 4 | Um-alturab | 17 | 4 | Al-gahmah | 9 |
| 5 | Al-mujarad | 19 | 5 | Al-sabkhah | 21 |
| 6 | Al-mushbah | 6 | 6 | Al-maseer | 9 |
| 7 | Al-mudeirah | 6 | 7 | Al-zamlah | 11 |
| 8 | Al-mafrag | 5 | 8 | Al-dahmah | 11 |
| 9 | Al-abteiah | 11 | 9 | Al-zahab | 9 |
| 10 | Um-alkhameir | 22 | 10 | Al-kudmy | 8 |
| 11 | Al-garn | 5 | | | |
| 12 | Al-girwaneiah | 6 | | | |
| 13 | Um-alhegil | 7 | | | |
| 14 | Al-rahmaneiah | 7 | | | |
| 15 | Al-jarshab | 8 | | | |
| 16 | Shargan | 6 | | | |
| Total | | 150 | | Total | 107 |

Table 2. Primers used in the detection of sporozoite of *Plasmodium* and the identification of *An. arabiensis*, and PCR conditions

| Species | Primer Name | Sequence (5-3) | PCR Product Size (BP) | PCR Condition |
|---|---|---|---|---|
| *Plasmodium* sp. | rPLU5<br>rPLU6 | CCTGTTGTTGCCTTAAACTTC<br>TTAAAATTGTTGCAGTTAAAACG | 1100 | Initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 60°C for 90 seconds, extension at 72°C for 90 seconds and final extension for 5 minutes |
| *P. falciparum* | rFAL1<br>rFAL2 | TTAAACTGGTTTGGGAAAACC AAATATATT<br>ACACAATGAACTCAATCATGA CTACCCGTC | 205 | Initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 90 seconds, extension at 72°C for 90 seconds and final extension for 5 minutes |
| *P. vivax* | rVIV1<br>rVIV2 | CGCTTCTAGCTTAATCCACAT AACTGATAC<br>ACTTCCAAGCCGAAGCAAAGA AAGTCCTTA | 120 | Initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 90 seconds, extension at 72°C for 90 seconds and final extension for 5 minutes |
| *An. arabiensis* | Universal primer<br>Species specific | GTG TGC CCC TTC CTC GAT GT<br>AAG TGT CCT TCT CCA TCC TA | 315 | Initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 50°C for 60 seconds, extension at 72°C for 60 seconds and final extension for 5 minutes |

capped, and stored at -86 until further investigation. After removing the legs, wings, and abdomen, the mosquito samples were homogenized individually in a mortar and pestle (mini borosilicate glass chamber length 60mm / pestle diameter 9.0mm 3.0Ml, Fisherbrand) in 100 µL of Minimum Essential Media (MEM) (manufactured Euro Clone, UK). The homogenate was saved in -86 degree till next procedure.

## 4- DNA EXTRACTION

DNA was extracted from the stored homogenate using RealLine DNA – Extraction 2 (BIORON Diagnostic, Germany) following the manufacture´s recommendations: 300 µL of lysis Reagent with sorbent (magnetic particles) added to homogenate in 1.5 tubes and placed into the thermo shaker for five minutes at 65°C, 1300 rpm. Then 400 µL of DNA precipitation solution was added to each tube and mixed for 15 seconds in a vortex. Samples were then centrifuged at 13000 rpm for five minutes at room temperature then the supernatant discarded and the pellet was washed twice and driedfor 2-3 minutes at room temperature. Specimen solution used to re-suspend the DNA. The extracted DNA stored at -86°C till next procedure

The morphological identification of *Anopheles arabiensis* has been confirmed by PCR using the procedure described by Scott *et al* (1993), and the primer used and PCR conditions are shown in Table 2. Nested PCR procedures were carried out for detection and identification of *Plasmodium* species as described by Snounou *et al.* (1993a). DNA samples were amplified by oligonucleotide primers obtained from Integrated DNA Technology (Belgium), targeting the *Plasmodium* small subunit ribosomal RNA (ssRNA) genes (Waters and McCutchan, 1989**)** (Table 2). Primer pair's rPLU5 and rPLU6 used to detect *Plasmodium* genus in Primary amplification and species-specific primers rFAL1/rFAL2 (*P. falciparum*) and rVIV1/rVIV2 (*P. vivax*) for nested PCR in 2 separated reactions.

In brief, primary and nested PCR were carried out in total 25 µl reaction volume, each containing 12.5 µl GoTag®G2 green master mix ready to use from Promega and25µM of each primer. Five µl of extracted DNA was used as a sample for the primary amplification and 2 µl of the PCR product for the nested PCR. In each run, negative and positive controls were included. Thermal cycling was done in T100 thermal cycler (Bio-Rad, USA). PCR primers and conditions are shown in Table 2. The PCR products of nested amplification were analyzed by gel electrophoresis (1.5 agarose in Tris-Acetate EDTA buffer) staining with ethidium promide. The visualization was carried out using Gel Doc XR Imaging System (Bio-Rad).


FIGURE 2. Identification of *An. Arabiensis* by PCR.

## RESULTS

A total of 315bp segments of the IGS region of rRNA gene sequences of *An. Arabiensis* were amplified (Figure 2). *An. arabiensis* was found as the predominant *Anopheles* mosquitoes in all the collection sites. All samples which were previously identified morphologically as *An. arabiensis* had beenalso molecularly confirmedby PCR(Fig 2).

Our molecular surveillance covered a total of 26 villages distributed in tow Governates (16 in Al-Khobah and 10 in Horoob) and lasted from January to December 2015. Two samples from two villages (Almuatan and Alsabkha) out of one hundred and seven (1.87%) female mosquitoes collected from HaroobGovernate were found positive for the sporozoites of *Plasmodium falciparum* (Table 3).

Similarly, three out of one hundred fifty (2%) female mosquito samples collected from 16 villages of AL-KhobahGovernate were also found positive. These three positive samples were from Um Alkhameir village. All the positive nested PCR samples that detect *P. falciparum* show band in (205bp) (Fig 3).

## DISCUSSION

*Anopheles arabiensis* is the potential primary vector of malaria in Jazan region. Alsheikh (2004)has identified *An. arabiensis* in the Tihama region (Saudi Arabia and Yemen) using species-specific diagnostic PCR, and showed that it is the only member of *An. gambiae* complex found in the Tihamaregion.In this study, the only detected *Plasmodium* species inthe femaleof *An. Arabiensis* mosquitoes using nested-PCR method is*Plasmodium falciparum,* aresult which coincides with the findings of Alsheikh (2004)who reported that *P. falciparum* represents more than 95% of malaria cases in the Tihama area (including Jazan region).The determination of sporozoites infection in wild *Anopheles* mosquitoes

Table 3. Detection of *Plasmodium* sporozoites from *An. arabiensis* by PCR method

| Governate | No of villages surveyed | Total samples collected | Positive samples | Species detected |
|---|---|---|---|---|
| Horoob | 10 | 107 | 2 (1.87%) | *P. falciparum* |
| AlKhobah | 16 | 150 | 3 (2%) | *P. falciparum* |
| Total | 26 | 257 | 5 (2.3) | *P. falciparum* |

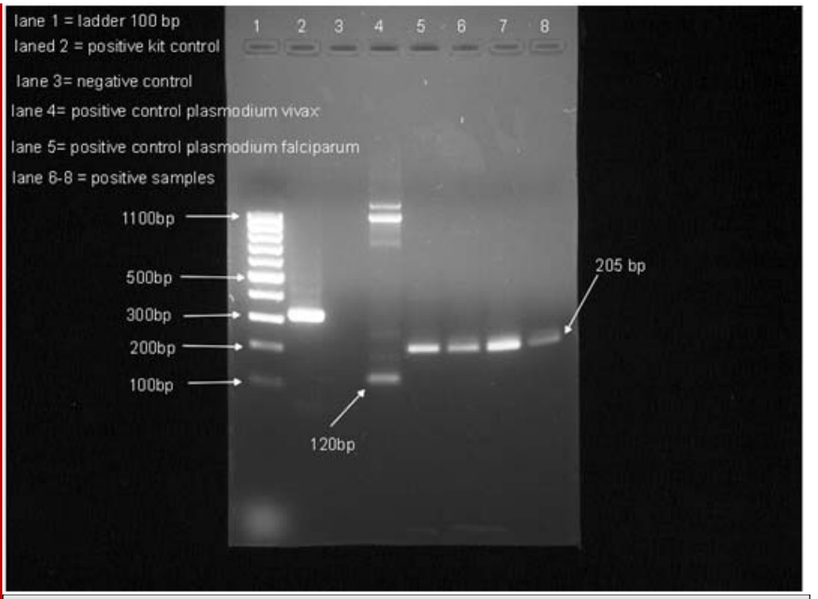


FIGURE 3. Detection of *Plasmodium* species by PCR method in *An. Arabiensis.*

considers an integral part in vector incrimination and malaria transmission dynamics in affected areas and epidemiological research (Alsheikh, 2004).

In this study, Thesporozoite rate of 1.87 %and 2% inHaroob and AlkhobahGovernates, respectively, (Table 3) reinforces the need to intensify the control efforts to compact the vector and reduce malaria transmission. We noticed a fourfold increase in thesporozoite rate of *P. falciparum* in *An. arabiensis in Alkhobah Governate in the present study from 0.61 to 2% compared to the previous study in 2007-2008* (Alsheikh, 2011). This could be attributed to the current war on the border of Yemen, where Alkhobah is located, which hinders the control measures against *An. arabiensis usually used to be conducted in the area before the start of the war.*

Harada *et al.* (2000) have also observed fourfold increase in thesporozoite rate (0.62 to 2.2%) in *Anophelesgambiaes. s.* in Ghana using the PCR method. In Solomon's Islands, *P. falciparum* was detected in 15.2% of *Anophelesfarauti* by PCR technique (Wilson *et al*, 1998).Few previous studies in the Tihamaregion had determined the sporozoite rate of *Plasmodium* species in *Anopheles* mosquitoes. For instance inSaudi Arabia, *Plasmodium* sporozoites had been detected in 0.65% of *An. arabiensis* using nested- PCR(Alsheikh, 2004), and in 0.9 % of 2921 *An. arabiensis* (23 *P. falciparum*, and 2 *P. vivax*) using ELISA method, while *P. falciparum*-sporozoites were detected in *An. sergentii* (2/295) and a single female of *An. algeriensis*in Yemen (Al-Eryani *et al*., 2016).

In East African countries such as Sudan, the *P. falciparum* sporozoite rate in *An. arabiensis* was found to be 4.5% in Sennar State using ELISA technique(Elmahdi *et al*., 2012), and 1.4-15% in eastern part of the country (Hamad *et al*., 2002). While in Ethiopia, it was 0.3% for *P. falciparum* and 0.5% for *P. vivax* (Tirados *et al*., 2006), and in Eritrea, it ranges from 0.54 to 1.3% (Shililu *et al*., 2004). In Asia, the sporozoite rate of *P. falciparum* was higher (10%) in *An. stephensi* from District Shiekhupura in Pakistan (Oneeb *et al*., 2015). Whereas, in various parts of India, sporozoite rates range from 0.012 to 0.2% in *Anophelesannularis* were reported using microscopic method, but the malaria parasite species could not be identified (Dash *et al*., 1982; Gunasekaran *et al*., 1989; Ghosh *et al*., 1985).However, Mahapatra *et al.* (2006) in Keonjhar district, Orissa, India, have detected 3.4% sporozoite rate in *Anophelesannularis* using PCR technique, and have identified the malaria parasite species to be *P. falciparum.*

Infectivity rate of 10.6% *P. falciparum* in *Anopheles gambiae complex* was found by Snounou *et al.*(1993b)in Guinea Bissau using PCR method. Four out of five (80% sporozoite rate) wild caught *Anophelesdirus* were found positive for the sporozoites of *P. falciparum* using PCR method, although they were negative when using ELISA technique (Tassanakajon *et al*., 1993), which reflected the high sensitivity of the PCR method.Variations in sporozoite rates in *An. arabiensis* from a country to another or within the same country could be attributed to the seasonal variations in transmission(Alsheikh, 2004).The wide spread of *Anopheles arabiensis* in Jazan

region with >90% of the malaria cases caused by *P. falciparum*, along with infectivity rate ranges between 1.87 to 2.0%for *P. falciparum*, suggests more in-depth researches on its ecology, behavior, and control to promote area-specific control programs.

## CONCLUSION

The current study indicated the significance of using PCR technology in detecting the presence of *Plasmodium* species in *Anopheles* mosquitoes. Our findings revealed an infectivity rate of *Anopheles arabiensis* ranges between 1.87 to 2.0% for *P. falciparum* in two Governates Haroob and Al-Khobah of the Jazan region, respectively.The wide spread of *An. arabiensis* in Jazan region with >90% of the malaria cases caused by *P. falciparum*, along with infectivity rate ranges between 1.87 to 2.0% for *P. falciparum* in Al-Khobah and Haroob-Governates, suggests that *P. falciparum* is the most predominant malaria parasite and *A. Arabiensis* is a very efficient malaria vector in the region.Further detection for the sporozoite of *Plasmodium* species in *Anopheles* mosquitoes need to be conducted in the rest ofJazan region to determine their infectivity rates by malaria parasites. The importance of detailed knowledge of disease vectors along with their sporozoite rates is of vital importance in the promotion of area-specific control interventions and programs.

## CONFLICT OF INTEREST

The authors declared no conflict of interest.

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