

THE USE OF NESTED-PCR TO DETECT THE PRESENCE OF *PLASMODIUM* IN *ANOPHELES ARABIENSIS* IN JAZAN REGION, SAUDI ARABIA

By

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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 Governorate were found positive for the sporozoites of *Plasmodium falciparum*. Similarly, 3 out of 150 (2%) female mosquito samples from Um-alkhameir, AL-Khobah Governorate, were also found positive. Around fourfold increase of the sporozoite rate (from 0.61 to 2.0%) in *An. arabiensis* in Al-Khobah Governorate 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 Governorates, suggests that *P. falciparum* is the most predominant malaria parasite and *An. Arabiensis* is a very efficient malaria vector in the region. It also suggests more in-depth researches on the ecology, behavior, and control of *An. Arabiensis* to promote area-specific control programs.

Keywords: Malaria, Jazan, Saudi Arabia, *Plasmodium falciparum*, PCR, *Anopheles arabiensis*.

Introduction

Malaria is an infectious disease caused by the bites of female *Anopheles* mosquitoes infected with *Plasmodium* spp. (Cowman and Crabb, 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* (Zahar, 1985; Sebai, 1988). *Anopheles arabiensis* has been identified as the primary vector transmitting malaria in Tihama area (southwest of Saudi Arabia and northeast Yemen) (Alsheikh, 2011), and considered to be a very efficient transmitting vector in the Afro-tropical area, with large variations in life behaviour including feeding, resting, and breeding (Coetzee and Le Sueur, 1988; Beiern and Koros, 1991). Five species of *Plasmodium* have long been recognized to infect humans; these species include *Plasmodium falciparum* (the most vir-

ulent form of malaria with approximately 90% malaria deaths globally), *Plasmodium vivax* (the commonest cause of malaria globally), *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* (WHO, 2016).

The annual estimation for malaria from December 2015 was 214 million cases 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 was at risk of malaria (approximately 2.4 million people) (Alsheikh, 2011). The disease was 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 of sporozoites of human malaria in the salivary glands of potential *Anopheles* vectors is the final step in establishing vector status (Alsheikh, 2011). Besides, the determination of sporozoite rates was 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 was also a useful estimator of potential transmission intensity (Garret-Jones and Shidrawi, 1969), but, 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, 2011). 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 recorded sporozoites rate of 0.7% (4/600) for *P. falciparum* in *An. arabiensis* using microscopy method.

In Jazan region, Al-Sheikh (2011) reported 0.61% as sporozoite rate of *P. falciparum* in *An. arabiensis* collected in 2007 - 2008 using nested-PCR. To the best of the

authors 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 *An. arabiensis* using nested-PCR. The present study is thus conducted to detect and identify the infectious *Plasmodium* species inside the malaria vector *Anopheles arabiensis* in Jazan region using nested-PCR techniques comparing to the previous data.

Materials and Methods

Study site: 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).

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).

Mosquito samples collection: *An. arabiensis* specimens were collected from indoor human dwellings of 26 villages distributed in tow Governorates (Al-Khobah and Haroob) from January to December 2015 (Tab. 1). The two Governorates 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) 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.

Mosquito examination: Mosquitoes were identified based on diagnostic morphological features (Mattingly and Knight, 1956; Taai *et al*, 2017). A total of 257 *An. arabiensis* females were preserved individually in 1.5ml plastic tube, labeled, capped, and stored at -86 until further investigated. After

removing legs, wings, and abdomen, the mosquito samples were homogenized individually in a mortar and pestle (mini borosilicate glass chamber length 60mm/ pestle di-

ameter 9.0mm 3.0ml, Fisherbrand) in 100µL of Minimum Essential Media (MEM) (manufactured Euro Clone, UK). Homogenate was saved in -86 degree till next procedure

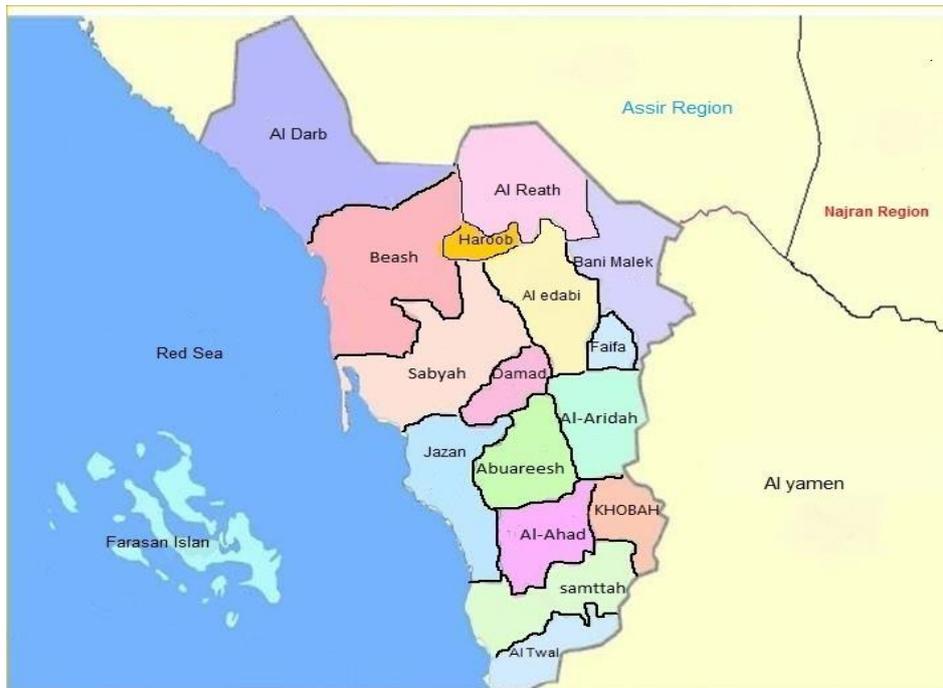


Fig 1: Map of Jazan region showing its different Governorates

DNA extraction: DNA was extracted from the stored homogenate using RealLine DNA – Extraction 2 (BIORON Diagnostic, Germany) following the manufacturer’s recommendations: 300µL 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, 1300rpm. Then 400µL 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 dried for 2-3 minutes at room temperature. Specimen solution used to re-suspend the DNA. The extracted DNA stored at -86°C till next procedure

Molecular detection; a- Mosquito identification: Morphological identification of *An. arabiensis* was confirmed by PCR using the procedure described by Scott *et al* (1993), and the primer used and PCR conditions. **b- *Plasmodium* detection:** Nested PCR proce-

dures 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). Primer pair's rPLU5 & rPLU6 used to detect *Plasmodium* in Primary amplification and species-specific primers rFAL1/rFAL2 (*P. falciparum*) and rVIV1/ rVIV2 (*P. vivax*) for nested PCR in 2 separated reactions (Tab. 2).

Primary and nested PCR were carried out in total 25µl reaction volume, each containing 12.5µl GoTag®G2 green master mix ready to use from Promega and 25µM of each primer. Five µl of extracted DNA was used as a sample for primary amplification and 2µl of PCR product for the nested PCR. In each run, negative & positive controls were included. Thermal cycling was done in T100 thermal cycler (Bio-Rad, USA).

PCR primers and conditions. PCR products of nested amplification were analyzed by gel electrophoresis (1.5 agarose in Tris-Acetate EDTA buffer) staining with ethidium promide. Visualization was done using Gel Doc XR Imaging System (Bio-Rad).

Results

A total of 315bp segments of IGS region of rRNA gene sequences of *An. Arabiensis* were amplified (Fig. 2). *An. arabiensis* was found as the predominant *Anopheles* mosquitoes in all the collection sites. All sam-

ples which were previously identified morphologically as *An. arabiensis* had been also molecularly confirmed by PCR (Fig 2). The molecular surveillance covered a total of 26 villages distributed in tow Governates (16 in Al-Khobah and 10 in Haroob) and lasted from January to December 2015. Two samples from two villages (Almuatan and Al-sabkha) of one hundred and seven (1.87%) female mosquitoes collected from Haroob Governorate were positive for sporozoites of *P. falciparum* (Tab. 3).

Table 1: *Anopheles arabiensis* collected from 26 villages of Al-khobah and Haroob (January to December 2015)

Serial No.	Al-khobah Governorate		Serial No.	Haroob Governorate	
	Villages	No. of samples		Villages	No. of samples
1	Wabrah	5	1	Gaeim Mahroog	13
2	Gaeim Mzubaid	15	2	Haroob city	4
3	Zahr Aljamal	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 detection of *Plasmodium*'s sporozoite and identification of *An. arabiensis*, and PCR.

Species	Primer Name	Sequence (5-3)	PCR Product size (BP)	PCR Condition (Initial denaturation)
<i>Plasmodium</i> sp.	rPLU5	CCTGTGTGCTTAAACTTC	1100	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 then extension for 5 minutes
	rPLU6	TTAAAATTGTTGCAGTAAAAACG		
<i>P. falciparum</i>	rFAL1	TTAAACTGGTTTGGGAAAACC	205	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 then extension for 5 minutes
	rFAL2	AAATATATT ACACAATGAACTCAATCATGA CTACCCGTC		
<i>P. vivax</i>	rVIV1	CGCTTCTAGCTTAATCCACAT	120	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 then extension for 5 minutes
	rVIV2	AACTGATAC ACTTCCAAGCCGAAGCAAAGA AAGTCCTTA		
<i>An. arabiensis</i>	Universal primer	GTG TGC CCC TTC CTC GAT GT	315	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 then extension for 5 minutes
	Species specific	AAG TGT CCT TCT CCA TCC TA		

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

Governorate	No of villages surveyed	Total samples	Positive %	Species detected
Haroob	10	107	2 (1.87%)	<i>P. falciparum</i>
Al-Khobah	16	150	3 (2%)	<i>P. falciparum</i>
Total	26	257	5 (2.3)	<i>P. falciparum</i>

Three out of one hundred fifty (2%) female mosquito samples collected from 16 villages of Al-Khobah Governorate were

positive, mainly from Um-Alkhameir village. All positive nested PCR samples of *P. falciparum* showed band in (205bp) (Fig 3).



Fig 2: Identification of *An. Arabiensis* by PCR.

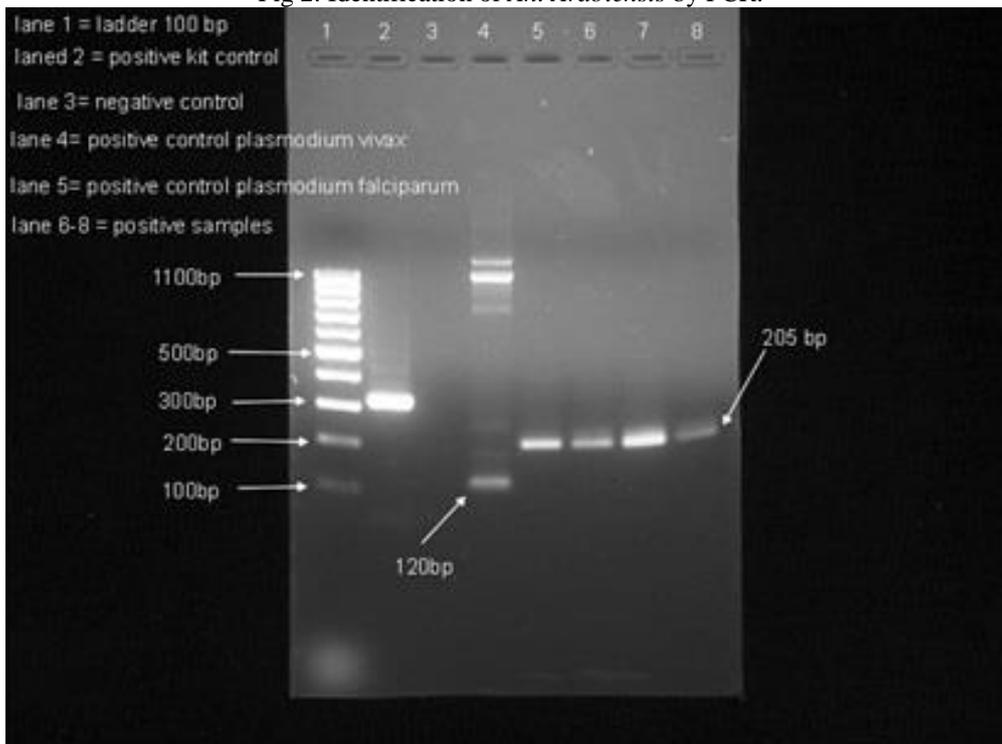


Fig 3: Detection of *Plasmodium* species by PCR method in *An. arabiensis*.

Discussion

Anopheles arabiensis is the potential primary vector of the malaria in Jazan region. Alsheikh (2011) 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 Tihama Region.

In the present study, only detected the *Plasmodium* species in female of *An. arabiensis* using nested-PCR method was *P. falciparum*, a result which agreed with Alsheikh (2011) who reported that *P. falciparum* represents more than 95% of malaria cases in the Tihama Region (including Jazan Region). The determination of sporozoites infection in wild *Anopheles* mosquitoes considers an integral part in vector incrimination and malaria transmission dynamics in affected areas and epidemiological study (Alsheikh, 2011).

In this study, sporozoite rate of 1.87% & 2% in Haroob and Alkhobah Governorates, respectively, reinforces the need to intensify the control efforts to compact the vector and reduce malaria transmission.

It was noticed a fourfold increase in sporozoite rate of *P. falciparum* in *An. arabiensis* in Alkhobah Governorate in the present study from 0.61 to 2% compared to the previous study in 2007-2008 (Alsheikh, 2011). This could be attributed may be to the ongoing war on the border of Yemen, where Alkhobah is located, which hinders the proper control measures against *An. arabiensis* usually used to be conducted in the area.

Harada *et al.* (2000) reported fourfold increase in the sporozoite rate (0.62 to 2.2%) in *Anopheles gambiae* S.S. in Ghana using PCR method. In Solomon's Islands, *P. falciparum* was detected in 15.2% of *An. farauti* by PCR technique (Wilson *et al.*, 1998).

Few previous studies in the Tihama Region had determined the sporozoite

rate of *Plasmodium* species in *Anopheles* mosquitoes. For instance in Saudi Arabia, *Plasmodium* sporozoites had been detected in 0.65% of *An. arabiensis* using nested-PCR (Alsheikh, 2011), 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), in Ethiopia, it was 0.3% for *P. falciparum* and 0.5% for *P. vivax* (Tirados *et al.*, 2006), and in Eritrea ranged 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 ranged from 0.012 to 0.2% in *An. annularis* 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). But, Mahapatra *et al.* (2006) in Keonjhar district, Orissa, India, have detected 3.4% sporozoite rate in *Anopheles annularis* using PCR technique, and have identified the malaria parasite species to be *P. falciparum*.

Infectivity rate of 10.6% *P. falciparum* in *An. gambiae* complex was reported by Snounou *et al.* (1993b) in Guinea Bissau using nested-PCR method. Four out of five (80% sporozoite rate) wild caught *Anopheles dirus* 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, 2011).

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*, 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 *Plasmodium* species in *Anopheles* mosquitoes. The outcome results revealed an infectivity rate of *Anopheles arabiensis* ranged between 1.87 to 2.0% for *P. falciparum* in two Governorates; Haroob and Al-Khobah of the Jazan Region, respectively.

The wide spread of *An. arabiensis* in the 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 Governorates, suggested that *P. falciparum* is the most predominant malaria parasite and *An. 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 of the Governorates of the Jazan 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.

Compete of interest: The authors declared no compete of interest.

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