Molecular Identification of *Plasmodium* species in Anopheline vector in Al Adwa village, Aswan Governorate, Egypt

A Thesis

Submitted to Faculty of Medicine, Ain Shams University for partial fulfillment of M.D. degree in Basic Medical Sciences (Medical Parasitology)

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Protocol

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Malaria is a vector-borne serious illness caused by infection with *Plasmodium* species. More than 3 billion individuals live in endemic areas, which are mainly in tropical areas of the world **(White et al., 2014)**.

In 2013, WHO report confirmed that there are still 97 countries and territory with ongoing malaria transmission while 7 countries in the prevention of reintroduction phase, making a total of 104 countries and territories in which malaria is considered endemic **(WHO, 2013)**.

Five species of malaria parasites affect humans all of these belong to the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. *P. falciparum* and *P. vivax* are the most prevalent. Malaria due to *P. falciparum* is the deadliest form and it predominates in Africa. *P. vivax* has a wider distribution and can develop in the *Anopheles* mosquito vector at lower temperatures and to survive at higher altitudes and in cooler climates. It also has a dormant liver stage, the hypnozoite that enables it to survive during periods of absence of *Anopheles* mosquitoes, such as during winter months **(Sandeu et al., 2012)**.

Malaria is transmitted from one person to another by the bite of female mosquitoes of the genus *Anopheles*. There are about 400 different species of *Anopheles* mosquitoes, but only 30 of these are vectors of major importance **(Blandin and Levashina, 2004).**

The distribution of Malaria is usually determined by the presence of its mosquito vector. Climatic factors such as

humidity, rainfall and temperature control the development of both malaria parasites and vectors **(Rahman et al., 2006)**.

Malaria transmission ultimately depends upon the sporozoite rate of mosquitoes **(Lee et al., 2002)**. Determining the presence of malaria sporozoites in *Anopheles* mosquitoes caught in the wild is an important factor in epidemiologic studies of malaria-endemic areas **(Mahapatra et al., 2006)**.

Out of the twelve *Anopheles* species present in Egypt, only five are known to be malaria vectors. *Anopheles pharoensis* proved to be the most important all over Egypt, especially in the Delta. *Anopheles sergenti* proved to be the primary vector in the Oases of the Western Desert, *Anopheles multicolor* in Faiyoum, *Anopheles stephensi* in the Red Sea Coast and *Anopheles superpictus* in Sinai **(Wassim, 2014)**.

In 1943, a major malaria epidemic occurred in Egypt associated with the spread of *A. arabiensis* from Sudan along the Nile Valley **(Malcolm et al., 2009)**.

Between 1982 and 1991, *Plasmodium vivax* and *P. falciparum* were reported in 7 governorates: Port Said, Suez, Sharkia, Menofia, Beni Suef, Fayoum and Aswan. However, between 1992 and 2001, malaria was recorded only in Fayoum, which was categorized as a high-risk area for malaria during the last 2 decades **(Hassan et al., 2003)**.

Recently in May 2014, the Centre for Disease Control and Prevention (CDC) received information that 19 locally transmitted malaria cases have been reported in Al-Adwa village in Aswan Governorate. The Egyptian Ministry of Health and local government and health authorities have engaged an intensive malaria control program in the affected village. They have recently completed active surveillance involving screening and treating if needed, of all villagers for malaria.

The risk of localized outbreaks of malaria cases in Egypt due to infection of local anopheline mosquitoes by imported cases does exist. Several factors contribute to this risk including the continuous movement of people between Aswan governorate and Sudan and the influx of large populations from Africa and Asia to Cairo and other cities for educational and religious purposes **(Hassan et al., 2003)**.

In 2010, Menegon and his colleagues reported that malignant malaria is common in Sudan with antimalarial drug resistance mainly in *P. falciparum* **(Menegon et al., 2010)**. With global warming and the continuous increase in temperature, it is possible that *A. gambiae* expands its range of distribution to Egypt. This situation may be further complicated by the presence of *A. sergentii* in Aswan and Toshka **(Shoukry et al., 2011)**.

Also, in regions where malaria has been locally eliminated, as in Egypt, but the vectors persist; there is a theoretical but small risk of localized outbreaks that could

increase under this climate change with the probability of emergence/re-emergence of the disease. Very few sporadic cases of malaria are diagnosed in Egypt **(El-Bahnasawy et al., 2010)**.

The detection of *Plasmodium* sporozoites in anophelines has relied on dissection and microscopic examination of mosquito salivary glands. Although this method is referred to as the "gold standard," it is not convenient for examining large numbers of samples because it is very laborious and samples need to be tested when fresh. Moreover, it requires experienced microscopists for accurate identification yet cannot differentiate species **(Li et al., 2001)**.

The enzyme linked immunosorbent assay (ELISA) using monoclonal antibodies targeting the circumsporozoite protein (CSP) have been introduced as an alternative to microscopy **(Stoffels et al., 1995)**. Sensitivity and specificity of CS-ELISA is high and parasite quantification is also possible by this method **(Hasan et al., 2009)**. Nevertheless, ELISA does not only detect the sporozoites in the salivary glands, but also detects CSP in other mosquito tissues. This results finally in an overestimation of the sporozoite rate, even if only the head-thorax part of the mosquito is used **(Fontenille et al., 2001)**.

A rapid dipstick immuno-chromatographic assay (Vec-Test™ Malaria) also showed rapid and accurate means for detection of different *Plasmodium* species, however,

specimen with loads as high as 400 sporozoites could give negative result **(Appawu et al., 2003)**.

Polymerase chain reaction (PCR) has proven to be a more sensitive method for the diagnosis of all four species of human malaria parasites **(Demas et al., 2011)** and useful tool than the dipstick assay to determine the malarial infection rate in mosquitoes **(Moreno et al., 2004)**. It can detect as few as 10 sporozoites compared to 200–400 for CS antigen detection **(Demas et al., 2011)**.

Aim of the work

This study will be conducted in Al-Adwa village, Edfu, Aswan Governorate, Egypt to determine the *Plasmodium* transmitting vectors species composition, infection rates and their feeding preferences whether anthropophilic or zoophilic through blood meal analysis.

Plan of the work

Study area:

Al-Adwa village, Edfu, Aswan Governorate, Egypt. Aswan Governorate is along the southern part of the Nile River Coordinates 23.59°North 32.82°East.

Methods

Study design: This study is considered pilot exploratory study

Mosquito sampling: Outdoor trapping will be conducted twice (December 2014, May 2015) for 5 consecutive nights at selected field sites using CDC light traps. Traps will be set in the evening around 5:00 p.m and collected the following morning around 08:00h a.m for approximately 12 h. trap period.

GPS readings (Garmin Oregon 550t handheld Global Positioning System), a text description of each collection site will be recorded as well as digital photographing to help identifying the sites when returning for follow-up and re-sampling.

Morphological identification of mosquitoes: Anopheline mosquitoes will be identified using the morphological keys of **Gilles and De Meillon (1968)**.

Mosquito processing: The head and thorax will be separated from the abdomen from individual mosquito to isolate only sporozoite-infected (salivary glands) mosquitoes. Abdomens, wings and legs of dried female *Anopheles* will be removed to reduce the risk of detection of sporozoites from parts of the body other than the salivary glands. Abdomens of blood fed mosquitoes will be used for blood meal analysis.

Detection of *Plasmodium* spp. by polymerase chain reaction (PCR):

1- DNA extraction from mosquitoes head & thorax using QIAamp DNA mini Kit (Qiagen).
2- screening all samples for detection of genus *Plasmodium* using conventional PCR, *Plasmodium*

small subunit ribosomal RNA (SSUrRNA) will be detected using genus primers RPLU5/RPLU6 established by **Snounou et al. (1993)**. Then positive samples will be processed for species identification by real time PCR.

Reading and Statistical analysis of results.

Identification of blood meal origin: DNA will be isolated from only engorged abdomens to be used in the blood meal diagnostic analysis. A multiplex PCR targeting Cytochrome b will be used using primers established by **Kent and Norris (2005)**. This assay can identify the blood meals from five mammal species: cow, human, pig, goat and dog.

Ethical clearance:

This protocol was approved by The Egyptian Ministry of Health and Population.

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