Determination of Microsporidia Infection in Larvae and Adult Anopheles Mosquitoes (Diptera: Culicidae) Northwest Ethiopia

Awoke Minwuyelet (awokeminwuyelet5@gmail.com)
Debre Markos university Ethiopia
Yibeltal Aschale
Debre Markos University
Getnet Atnafu
Debre Markos University

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Determination of Microsporidia Infection in Larvae and Adult *Anopheles* Mosquitoes (Diptera: Culicidae) Northwest Ethiopia.

Awoke Minwuyelet[1] , Yibeltal Aschale[2], and Getnet Atenafu[1]

[1]Department of Biology, College of Natural and Computational Science, Debre Markos University, Debre Markos, Ethiopia

[2]Department of Medical Laboratory Sciences, College of Health Sciences, Debre Markos University, Debre Markos, Ethiopia.

Corresponding author: Awoke Minwuyelet

Email: awokeminwuyelet5@gmail.com

Tel: +251915852223

Email addresses

AM: awokeminwuyele5@gmail.com

YB: yibea33@gmail.com

GA: getnet.atenafu@gmail.com

Abstract

Background: Microsporidia are a diverse group of obligate intracellular, spore forming parasites that infect all phyla of invertebrates. Microsporidia are known to infect a wide variety of animals including mosquitoes. A few studies showed that microsporidia infection of mosquitoes is associated with larval mortality or reduced adult fecundity and lifespan. The aim of this study is to determine microsporidia infection in field collected Anopheles larvae and reared adults.

Methods: Anopheles larvae were collected from the field and analyzed from February to April 2022. The 4th stage larvae, after determining the species identity, they were dissected to extract their midgut while the early stage larvae were transported to entomological laboratory for reareing the adult in the insectary room. Similarly, after determining the species identity they were dissected to extract their midgut. Then two thin smears for each dissected larvae and/ adult were made. Fixed preparations were stained with Giemsa and modified Ziehl-Neelsen staining techniques to detect the microsporidia. Descriptive statistics and independent t-test were used to analyze the data.

Results: *Anopheles gambiae complex* was identified as the most predominant species from field collected larvae and insectary reared adults. From the total 258 larvae and 258 adult *An.gambiae complexes* tissue smears examined, microsporidia were detected in 2.7% [7/258, (95%, CI: 0.8-5)] and 1.2% [3/258, (95%, CI: 0.01-2.7)] of larvae and adult respectively. The mean density of microsporidia in larvae and adults were 64.9 (± 23.4 SD) and 36 (± 8.5SD) respectively. The difference of microsporidia density between infected larvae and adults was statistically significant (F=1.77, P= 0.02).

Conclusion: Significant level of microsporidia infection was detected using light microscopy. Further microsporidia identification down to the genus and species level needs to determine its ultrastructure characteristics and the comparative analysis of small subunit rRNA sequence data.

Keywords: Microsporidia, Anopheles, Larvae, Adult, light microscopy.

Introduction

Mosquitoes are the most significant groups of insects that can transmit infectious human diseases: Malaria, Chikungunya, dengue diseases, lymphatic filariasis and others to humans which impose an enormous burden on sub-Saharan Africa. Of them, malaria is considered as an important public health burden, especially in sub-Saharan Africa, with an estimated 241 million cases and 627,000 deaths reported worldwide in 2020 [1]. This malaria burden needs more attention to search new strong protecting measures to control malaria transmission.

The chemical insecticides used in vector protection, to reduce the damage caused by pathogens and pests in agricultural fields, pose many long-term threats and risks to living things due to their harmful side effects and development of insecticide resistance [2, 3]. These are a strong indication that current control measures are insufficient and additional novel strategies to control *Anopheles* mosquitos' population or their capacity to transmit *Plasmodium* parasites are needed.

Mosquitoes are vectors of many disease-causing viruses and parasites and even carry and transmit multiple pathogens in a single host, creating numerous opportunities for interactions among vertebrate hosts, vectors, and pathogenic organisms [4-6]. These interactions can occur on multiple levels and may ultimately affect transmission patterns and disease pathogenesis. Reports strengthen that various microsporidia species found in mosquitos [7-9].

Microsporidia are a diverse group of obligate intracellular, spore forming parasites that infect all phyla of invertebrates and vertebrates animals. They are single celled eukaryotic microorganisms that have small genomes in the size, range of prokaryotic cells and are now thought to be highly evolved fungi [10]. Microsporidia are apparently simple and are all very small, ranging between 1-4 µm and their content is very unconventional compared with other eukaryotes [11, 12].

Microsporidia are ubiquitous pathogens that are found in terrestrial and aquatic ecosystems worldwide [13]. Currently, more than 1300 species of these microorganisms have been described in the literature [14]. Of them, 14 species are known to infect humans, around 150 species have been recognized to parasitize 14 genera of mosquitoes [15, 16]. Based on this evidences, it has been stated that all of the mosquito species could be possibly the host of at least one microsporidium agents [16]**.** Spores are the only stage that can exist outside a living host cell and

they are the primary vehicles for horizontal and vertical transmission between and within the host [10, 17].

During microsporidia development, some species exhibit simple life cycles involving only one sporogonic sequence called monomorphic forms (eg: *Anncaliia and Vavraia)* produce only one spore type responsible for oral (horizontal) transmission. Commonly there is no leading to ovarian infection in female hosts which results mildly pathogenic to mosquitoes, generally producing low larval mortality [18, 19].

While polymorphic forms (eg; *Amblyospora* and *Parathelohania*) have a complex life cycle and produce different types of spores [19]. These include elements of asexual and sexual reproduction, the formation of multiple spore types in various stages of the host (commonly there is an obligatory intermediate copepod host in their life cycle) and separate developmental sequences leading to vertical and horizontal transmission. These microsporidia generally exhibit higher levels of host specificity. These genera have at least one phase of development that naturally kills larval hosts during the former stadium. Despite the fact, they do not cause acute mortality or detectable morbidity in adult female hosts but they can transmit infections vertically. Mortality in larvae results from destruction of various host tissues and subsequent depletion of essential energy reserves necessary for pupation [20-22]. The production of entomopathogenic toxins has never been recognized. However, all of which are pathogens where virulence is primarily associated with larval mortality or reduced adult fecundity and lifespan.

Likewise, currently the effects of microsporidia on the development of disease-causing organisms in mosquitoes have been studied mainly for malaria parasites for developing novel strategies to control mosquito populations or their capability to transmit *Plasmodium* parasites [23, 24]. Presumably by the means of a sharp reduction in the longevity and fecundity of infected adult survivors and infected anophelines have a reduced capacity to transmit malaria.

Microsporidian infections are most readily detected in late stage (4th instar) larval mosquitoes where heavy concentrations of spores from the fat body, midgut or gastric caecae. Otherwise, light infections also found in adult hosts from fat body, midgut, ovary, gastric caeca and salivary gland. Microsporidia can be detected microscopically from samples of mascerated tissues or whole specimens of infected mosquito. Best results are obtained with infected tissues from live

hosts that are air-dried smears and fixed with 100% methanol and stained modified Zihl-Neelsen and giemsa solutions to examine by bright field optics [17]. Even though, these procedures and techniques will effectively identify and diagnose most microsporidian infections in mosquitoes, definitive identification of a particular isolate to the species level will require an examination of detailed ultrastructure of life stages [12], especially the spore and comparative analysis are commonly detected by using rRNA sequence data [11, 25].

Therefore, the search for natural mosquito-associated symbionts with the ability to reduce vector competence has been a growing interest. Given the relevance of microsporidia to vector control combined with recent reports of microsporidia infections in *Anophelines* mosquitoes, this area requires investigation.

Materials and methods

Study design and period

A cross-sectional entomological study was carried out for the determination of microsporidia infection among the common *Anopheles* species known to transmit malaria in Ethiopia, from February to April 2022.

Study area and Sample Size

Anopheles larvae were collected using standard dippers from Gozamen District, Denba Villages which is 20km far from Debre Markos town which is located in western part of Ethiopia. It is geographically located at 10020'N37043'E with an average altitude of 2446meter above sea level. It has conducive weather conditions with 1380 mm average annual rainfall and 180 ^{o}c average annual temperature.

Sample Size determination

To recruit the number of mosquito sample, the minimum number of ***Anophelines*** mosquitoes was calculated using two population proportions determination formula $n = z^2 (p1q1+p2q2)/d^2$: with the following assumptions: the previous study prevalence microsporidia (P1) of 2.6% from larvae of *Anopheles* mosquitoes in Western Siberia [26], and (P2) 17.3% from newly emerged

adults (from field-caught larvae) in Ghana [27], 95% confidence level, and 5% margin of error. Accordingly, the minimum sample size (n) was found to be 258 for both, larvae and adult.

Characterization of larval habitats

Physical parameters: during larval collection parameters like larvae density estimated by calculating the number of mosquito larvae per dip, PH, temperature and depth of breeding area and clear of any vegetables were screened.

All Anopheles larvae were sorted from culicine larvae and counted. Larval density was determined by taking the average number of mosquito larvae from the total dips taken at specific habitats. Anopheles larvae were then sorted into early stages (1st and 2nd instars) and late stages (3rd and 4th instars) and counted and recorded. The late stages were isolated for species identification and dissection but early stage larvae were transported to Debre Markos University insectary laboratory for rearing to adult.

Identification of Anopheles mosquito larvae

Third and fourth instar larvae which were collected from the field were transported to Debre Markos University entomology laboratory. A drop of Absolute Methanol on the petri dish was added to kill the larvae found on it. Then a drop of Normal Saline mounting medium was placed on a clean microscopic glass slide. Then each fresh died larva was mounted on a slide and identified morphologically using the identification key of Gillies and Coetzee [28] under a dissection microscope. Immediately after identification of the species, dissected and covered other slides and allowed to crash by adding pressure and thin smear microscopy was done by using giemsa and modified Ziehl-Neelsen staining procedure for determination of microsporidia infection from midgut.

Likewise, emerging adult Anopheles mosquitoes were put in holding cages and fed with 10% sugar solution from cotton wool pads. The adult holding room temperature was measured daily. Approximately 270 *An. gambiae complex* mosquitoes were reared to the adult stage, then after identification of species using standard morphological keys [28], isolated adult *An. gambiae complexes* were dissected for determination of microsporidia infection from midgut using giemsa stain and modified Ziehl-Neelsen techniques.

Midgut Dissection of Mosquitoes and Examination by light microscopy

Field collected larvae and reared adult mosquitoes were anesthetized for one minute with Absolute methanol in an anesthetizing chamber. After identification of the species of mosquito, the wings and legs were removed; the *An.gambiae complex* was then placed on a clean microscope slide with a drop of normal saline. Then grasp the thorax using forceps until you detach the terminalia (7th abdominal segment). While gently pressing the thorax, a dissecting needle was used to pull the terminal end of the mosquito in such a way that the midgut can be pulled out of the abdomen with it. Once midgut was detached from the terminalia, it made a thin smear on the slide and transferred a few drops to make another smear and allowed to air dry. The smears are then fixed with 100% absolute methanol and stained with 10% Giemsa and Modified Zihl-Neelen. Finally, the stained slides were allowed to air-dry; both giemsa and modified Ziehl-Neelsen stains were examined at a magnification of 100×. Infection was detected in images by observation of spores in tissue smears [29].

Giemsa stain

This stain revealed microsporidia with suboptimal morphology. The spores appeared blue in color though some remained unstained. There was poor differentiation from background and other inflammatory debris. In two cases the darkly stained belt could be identified, which helped in the preliminary diagnosis [30].

Modified Ziehl-Neelsen stain

The spores appeared bright red against a bluish background. Some spores did not take up the stain and appeared blue, but all of them showed a thick band-like nucleus at one pole. This stain helps in enhanced detection of the spores as bacteria and other tissue structures appear blue. Here the spores could be identified even in low magnification [29].

Quality control

To maintain the quality of results, a test procedure was performed in accordance with standards. Giemsa and modified Zihl-Neelsen solution were checked using a known negative microsporidia

parasite. In addition, randomly selected slides were re-examined by an expert microscopist to check the discrepancy in detection of microsporidia parasites.

Data management and analysis

Data were collected and then entered and analyzed using the Statistical Package for Social Sciences (SPSS version 23). Simple descriptive statistics and table were used to explain physical parameters, prevalence rate of smear positive microsporidia. Independent t test was used to show the mean difference of microsporidia density between infected larvae and adult, *P* value less than 0.05 used as statistically significance.

Result

Demographics of larvae collected area

When the larvae were collected, the average larvae density was five, PH and temperature of the breeding site was 7.4 and 24.5 ^{o}c respectively. Similarly the depth of the breeding site was 35cm and clear of any vegetables. Whereas, the average temperature for reared adults was 24 ^{0}c ±2.5 and 45% ±15.5 humidity was measured.

Anopheles larvae species composition

Anopheles mosquitoes identified from the study site are shown in Table 1 and 2. In total, 265 late instar Anopheles mosquito larvae were morphologically identified belonging to 3 species. From the total Anopheles larval species, *An. gambiae complex*, *An. christyi*, and *An. pharoensis* were identified from the study site. *Anopheles gambiae complex* constituted 97.4% and *An. pharoensis is* 1.9% of all identified larvae in the study site.

Table1. Total number of Anopheles larvae identified from study site (February–April 2022).

Anopheles species identified	Number of larvae collected	Number of larvae examined	Number of larvae infected
An. gambiae complex	258 (97.4%)	258	7

An. pharoensis	5 (1.9%)	5	0
An. christyi	2 (0.7%)	2	0
Total	265(100%)	265	7

Similarly, 270 reared adult Anopheles mosquitoes were morphologically identified belonging to 3 species. From the total Anopheles adult species, *An. gambiae complex, An. pretoriensis,* and *An. pharoensis* were identified from the study site. *Anopheles gambiae complex* constituted 95.6% and *An. pharoensis is* 2.9% of all identified larvae in the study site (see in Table 2).

Table 2. Total number of Anopheles adult identified from study site (February–April 2022).

Anopheles species identified	Number of adult reared	Number of adult examined	Number of adult infected
An. gambiae complex	258 (95.6%)	258	3
An. pharoensis	8 (2.9%)	8	0
An. pretoriensis,	4 (1.5%)	4	0
Total	270 (100%)	270	3

Microsporidia prevalence

From the total 258 larvae and 258 adult *An.gambiae complexes* tissue smears examined, microsporidia were detected from 2.7% [7/258, (95%, CI: 0.8-5)] and 1.2% [3/258, (95%, CI: 0.01-2.7)] of larvae and adult respectively.

Level of parasitaemia of microsporidia from the detected smear was also counted per 100 fields. The mean density of microsporidia in larvae and adults were 64.9 (± 23.4 SD) and 36 (± 8.5SD) respectively. According to the independent t-test, the difference of microsporidia density between infected larvae and adults was statistically significant (F=1.77, P= 0.02). From this, the level of parasitaemia was higher in the larvae stage than the adult stage.

Discussion

Studies of the Anopheles-associated microsporidians have been shown to interfere with the infection and development of *Plasmodium* [31, 32]. According to the report some microsporidia spp like *Nosema stegomyia* disrupt the development of the oocysts in *An. gambiae,* attributed to midgut degradation and consequent disruption of *Plasmodium* binding [31], while others like *Vavraia culicis* can impaired the development of *Plasmodium* which has been associated with host innate immune priming [33].

In this study, the prevalence of microsporidia was 2.7% [7/258, (95%, CI: 0.8-5)] and 1.2% [3/258, (95%, CI: 0.01-2.7)] in larvae and adult *An.gambae complex* respectively. A study conducted in the field sampled Anopheles larvae mosquitoes in Mwea and Mbita in Kenya reported 5% microsporidia prevalence using fluorescence microscopy. Similarly, a study conducted in Busia and Mbita in Kenya collected adult *An. arabiensis* detected by molecular method showed the prevalence of microsporidia were 1% and 4% respectively [34].

Whereas, a similar study conducted in Mwea and Ahero in Kenya from field collected adult *An. arabiensis* showed that prevalence of microsporidia were 10% and 15% respectively [34] and other studies conducted elsewhere showed that epizootics of lethal meiospore infections in larval mosquitoes have been reported to be as high as 80–90%. Prevalence rates of horizontally acquired infections in copepods range from 40–80% and up to 60% in larval mosquitoes [16]. In northern climates, overwintering occurs in copepods and diapausing mosquito eggs. The low prevalence of microsporidia in our study might be due to the limitation of our diagnostic method which might result false negative and in addition the majority of infections were acquired by 2nd and 3rd instars during the 1st 3-week of exposure, but we were collecting the whole larvae stages that might reduce microsporidia infection.

Level of parasitaemia of microsporidia from the detected smear was also counted per 100 fields. Based on this, the mean difference of microsporidia density in infected larvae and adults were 64.9 (± 23.4 SD) and 36 (± 8.5SD) respectively. However, a study conducted in Kenya reported a heavier level of microsporidia density [34]. Other study conducted in Western Siberia, regarding the ecology and epizootology of microsporidia in malarial mosquitoes larvae of both sexes of larvae stated that microsporidia infection rate is much higher in the male larvae (77.8%),

than that in the female larvae (22.2%) [26].). In contrast with this, our observation shows that the Microsporidia parasites are commonly found in mosquitoes in some and more likely heavier in larvae and killed during pupation but lower in adults and not fatal. But, in our study the microsporidia density was low; this might be due to the specificity of the detection method used and smear integrity during dissection.

The *Anopheles gambiae complex* was the predominant species in the current study of the study site. This is in line with other studies in parts of Ethiopia [35-37] and other African countries [34, 38].

Limitations of the study

The major limitation of this study is that the prevalence of microsporidia was determined solely by microscopic examination of thin tissue smears. This may underestimate the prevalence of microsporidia and its density. Similarly, species of mosquitoes was identified in using keys; however, there are drawbacks to morphological species identification.

Conclusion and recommendations

Two point seven and one point two percent of microsporidia were detected from *An. gambiae complex* larvae and adult respectively. Higher microsporidia density was found in larvae than adult. *Anopheles gambiae complex* was the predominant species and incriminated as the main microsporidia host in the study area.

The causative agent is diagnosed as a member of the phylum microsporidia. Further identification down to the genus and species level needs to determine its ultrastructural characteristics and the comparative analysis of small subunit rRNA sequence data. It is also necessary to understand the detail of the components of the transmission cycle.

Author Contributions

AM conceived and designed the majority of the experiments. AM and GA collected mosquitoes and AM carried out the microscopy to detect microsporidia infection. AM and YA analyzed the data and all authors wrote the manuscript.

Conflict of Interest

The authors declare that there is no potential conflict of interest

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