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Determination of microsporidia infection in larvae and adult *anopheles* mosquitoes (Diptera: Culicidae) Northwest Ethiopia

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Abstract
Microsporidia are a diverse group of obligate intracellular, spore-forming parasites that infect invertebrates including mosquitoes. A few studies showed that microsporidia infection of mosquitoes is associated with larval mortality or reduced adult fecundity and lifespan. This phenomenon might be used to protect against vector-borne disease transmission. This study aimed to determine microsporidia infection in field-collected Anopheles larvae and reared adults.
Anopheles larvae were collected from the field and analyzed from February to April 2022. After determining the species identity of the 4th stage larvae, they were dissected to extract their midgut while the early-stage larvae were transported to an entomological laboratory for rearing the adult in the insectary room. Similarly, after determining the species identity of adult female *Anopheles* mosquitoes, they were dissected to extract their midgut. Then, two thin smears for each dissected larvae and/ adults were made. Fixed preparations were stained with Giemsa and modified Ziehl-Neelsen staining techniques to detect the microsporidia. Descriptive statistics and an independent t-test were used to analyze the data. *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 adults *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 adults respectively. The mean density of microsporidia in larvae and adults was 64.9 (± 23.4 SD) and 36 (± 8.5SD) respectively. The difference in 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 in the genus and species level is needed.

Keywords: Microsporidia, *anopheles*, larvae, adult, light microscopy

Introduction
Mosquitoes are the most significant groups of insects that can transmit different 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 and lymphatic filariasis are transmitted by *Anopheles* mosquitoes. Among them, malaria is considered 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].
The chemical insecticides were used to protect vector borne diseases which caused by pests in agriculture and pathogens for humans. Insecticides have been pose many long-term threats and risks to living things due to their harmful side effects and the development of insecticide resistance [2, 3]. Those 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. Biological control methods are the current alternative control methods for vector [4].
Biological controls of vectors are the use of natural vector predators, bacteria, viruses, protozoa, fungi and bacterial toxins or botanical compounds that can support control vector populations. Example, using *Bacillus thringnisisis* that can poison mosquito larvae, the use cat fish to eat up mosquito larvae in pond can eradicate the mosquito population, or reducing breeding rates by introducing sterilized male tsetse flies have been shown to control vector populations and reduce infection risks [4, 5]. Likewise, different parasitic, viral and fungal infections of mosquitoes delay pathogen transmission of vector-borne diseases.
Mosquitoes can carry and transmit multiple pathogens in a single host, infections creating numerous opportunities for interactions among vertebrate hosts, vectors, and pathogenic organisms [6-8]. These interactions can occur on multiple levels and may ultimately affect transmission patterns and disease pathogenesis. Reports strengthen that various microsporidia species are found in mosquitos [9-11].
Microsporidia are a diverse group of obligate intracellular, spor e-forming parasites that infect all phyla of invertebrates and vertebrates animals. They are single-celled eukaryotic microorganisms that were once considered protozoans or protists but are now known to be highly evolved fungi [12]. Microsporidia are simple and are all very small, ranging between 1-4 µm and their content is very unconventional compared with other eukaryotes [13, 14].
Microsporidia are cosmopolitan pathogens that are found in terrestrial and aquatic ecosystems worldwide [13]. Nowadays, more than 1300 species of these microorganisms have been described [15]. Of them, around 150 species have been recognized to parasitize 14 genera of mosquitoes [16, 17]. Based on this evidence, it has been stated that all the mosquito species could be possibly the host of at least one microsporidium agent [17]. 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 [12, 18].
Microsporidia in the mosquitoes have two distinct developmental forms based on their life cycles and host-parasite relationships. Some generals like *Anncaliia* and *Vavraia* only need a single host with a sporononic sequence and produce only one spore which is more virulent but not host specific. They are responsible for oral (horizontal) transmission [19, 20]. While

others like *Amblyospora*, *Parathelohania* and *Edhazardia* have a complex life cycle and produce different types of spores by asexual and sexual reproduction [20]. These multiple spore types are produced asexually from the copepod host and sexually from the mosquito host during their life cycle. These are less virulent but highly host specific and separate developmental sequences leading to vertical and horizontal transmission. During parasite development, mortality in larvae results from the destruction of various host tissues and subsequent depletion of essential energy reserves necessary for pupation [21-23].

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 [24, 25]. 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. While, light infections are also found in adult hosts from the fat body, midgut, ovary, gastric caeca, and salivary gland. Microsporidia can be detected microscopically from samples of mascerated tissues or whole specimens of an 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 [18].

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.

2. Materials and methods

2.1 Study design and period

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

2.2 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 the 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.

2.3 Sample Size determination

To recruit the number of mosquito samples, 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 adults.

2.4 Characterization of larval habitats: Physical parameters: during larval collection parameters like larvae density were 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 adults.

2.5 Identification of *Anopheles* mosquito larvae and Adults

The 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 fresly 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 the 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.

2.6 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].

2.6.1 Giemsa stain

After 258 tissue smears fixed with absolute methanol, the dried fixed smears were stained with 10% giemsa solution for 40 minutes. This stain revealed microsporidia with suboptimal morphology. The spores appeared blue though some remained unstained. There was poor differentiation between background

and other inflammatory debris. This staining more commonly used to the preliminary diagnosis [30].

2.6.2 Modified Ziehl-Neelsen stain

Likewise, after 258 tissue smears were fixed with absolute methanol, the dried fixed smears were stained with Carbone fucshion for 20 minutes. After decolorization by 1% acid alcohol for 5 minutes, finally counter stain with methylene blue for 3 minutes. During examination of the stained smear, 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. Compare to Giemsa stain, during this stain the spores could be well identified and enhanced detection of the spores and other tissue structures appear blue [29].

2.7 Quality control

To maintain the quality of results, a test procedure was performed per 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 the detection of microsporidia parasites.

2.8 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 tables were used to explain physical parameters and the prevalence rate of smear positive microsporidia. An independent t-test was used to show the mean difference of microsporidia density between infected larvae and adults. Given a *P* value less than 0.05 was used as statistically significant.

3. Result

3.1 Parameters of larvae collected area

When the larvae were collected, the average larvae density was five; PH and temperature of the breeding site were 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.

3.2 Anopheles larvae species composition

Anopheles mosquitoes identified from the study site are shown in Tables 1 and 2. In total, 265 late-instar *Anopheles* mosquito larvae were morphologically identified as 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 (Table 1).

Table 1: A total number of *Anopheles* larvae were identified from the 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 as 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 (Table 2).

Table 2: A total number of anopheles adults were identified from the 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

3.3 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.

The 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.

4. Discussion

Studies of the effect of microsporidia on development of Plasmodium in infected *Anopheles* have been shown the negative strong correlation [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 [17]. 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 in 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.

The level of parasitaemia of microsporidia from the detected smear was also counted per 100 fields. Based on this, the mean difference in microsporidia density in infected larvae and adults was 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 mosquito's 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].

5. 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.

6. Conclusion and recommendations

Two-point seven and one point two percent of microsporidia were detected from *An. gambiae complex* larvae and adults 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.

7. 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.

8. Conflict of Interest

The authors declare that there is no potential conflict of interest

9. References

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