Hindawi
Psyche: A Journal of Entomology
Volume 2023, Article ID 2806034, 10 pages
https://doi.org/10.1155/2023/2806034

Research Article

Evaluation of Pathogenicity of Entomopathogenic Oomycetes *Lagenidium giganteum* and *L. ajelloi* against *Anopheles* Mosquito Larvae

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Received 7 November 2022; Revised 31 January 2023; Accepted 4 February 2023; Published 27 February 2023

Academic Editor: Kathirvelu Baskar

Malaria is a mosquito-borne life-threatening parasitic disease of humans and the leading cause of mortality and morbidity in sub-Saharan Africa. Despite the major efforts made towards malaria control, it is facing challenges of development of parasite resistance towards antimalarial drugs coupled with *Anopheles* vector resistance towards insecticides being used in control. There is, therefore, a need to develop complementary control strategies that are economical and environmentally friendly. Biological control using entomopathogenic fungi against the immature malaria mosquito vectors presents an untapped opportunity. This study sought to isolate and characterize entomopathogenic oomycetes *Lagenidium giganteum* and *L. ajelloi* from wild *Anopheles* larvae from Ahero rice fields in western Kenya and test their pathogenicity against laboratory-reared *Anopheles gambiae* larvae. Laboratory-reared *A. gambiae larvae* (3rd and 4th instar) were exposed to five different concentrations of *L. giganteum* and *L. ajelloi* zoospores; 1000, 2000, 3000, 4000, and 5000 zoospores/mL, respectively. The larval mortality was recorded after 24, 48, 72, and 96 hours post-exposure, until all larvae were dead. The results obtained showed that *L. giganteum* was not pathogenic to *A. gambiae* larvae after 24 and 48 hours post-exposure to all concentrations. Larval mortality was recorded at 72 and 96 hours. There were no significant differences observed in the mortalities ($p > 0.05$) from all treatments. No mortalities were observed in deionized water (negative control) whereas 100% mortality was recorded in larvae exposed to *Bti* (positive control). Probit analysis showed that LC_{50} after 72 hours and 96 hours was 2.32×10^4 and 3.51×10^3 zoospores/ml, respectively. *L. ajelloi* caused larval mortalities at all the 5 test concentrations after 24-, 48-, 72- and 96-hours post-exposure with LC_{50} values of 1.18×10^5, 1.43×10^4, and 6.05×10^2, and 27.08 zoospores/ml, respectively. This study isolated and tested two species of *Lagenidium* from field collected larvae. *Lagenidium ajelloi* recorded greater pathogenicity than that of *L. giganteum* against *A. gambiae* larvae, making them potential candidates for use in the development of bio-larvicide for the control of *Anopheles* larvae.

1. Introduction

Malaria remains the leading cause of mortality and morbidity in sub-Saharan Africa, where *Plasmodium falciparum* is the main malaria parasite. The World Malaria Report indicates that approximately, 241 million cases of malaria were reported in 2020 in 85 malaria endemic countries. The WHO African region accounts for 95% (228 million) of all reported malaria cases. An increase in malaria cases was recorded compared with 227 million cases reported in 2019, which is attributed to the disruption of services due to the COVID-19 pandemic [1]. In addition, there is increasing evidence of the emergence of partial resistance towards artemisinin-based combination therapies (ACTs), the main chemotherapeutic intervention for malaria in the African Region [2]. Consequently, WHO encourages an integrated approach towards vector-borne disease control, calling on countries to prioritize research on the exploration and

development of novel mosquito control strategies [3]. The current study was aimed at exploring alternative biological interventions for malaria vector control using fungi.

Sub-Saharan Africa continues to record high malaria infections in the world mainly due to the presence of efficient vectors of the *Anopheles gambiae* and *Anopheles funestus* complexes, favorable weather (hot and humid) that allows the transmission to occur all year round, scarce resources and social-economic instability that have hindered efficient malaria control [4]. In western Kenya, malaria is hyperendemic, with the perennial transmission of parasites by *Anopheles* mosquitoes. Both *Anopheles gambiae* and *A. funestus* complexes are present at Ahero, with *A. arabiensis* being the predominant species [5, 6]. Although considerable gains have been made towards malaria elimination in Africa, the development of the recent transformative technologies such as vaccines and gene-drive mosquitoes may soon quicken malaria control [7]; however, total eradication of the disease faces challenges [4]. For a long time, the control of malaria in most parts of the world and indeed in Kenya has been based on case management and vector control using long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [7, 8]. However, the use of chemical pesticides has led to the increasing development of insecticide resistance of the malaria vectors to pyrethroid-based insecticides, hence compromising the effectiveness of insecticide-based vector control programs [9–11]. Besides, the persistent use of chemical pesticides contaminates the environment and also poses a health risk to nontarget organisms [12], and the need for ecologically safe alternatives is clear [13]. To counteract these challenges, integrated approach to malaria vector control by use of both chemical and nonchemical methods including biological control and environmental management has been proposed [14]. Integrated approach strategies work by combining different control methods that target either adult mosquitoes or/and the immature aquatic stages of mosquitoes.

Immature stages of mosquito control mainly follow the WHO recommendation on larval source management, which includes habitat modification, habitat manipulation, larviciding, and biological control [15]. Controlling mosquitoes by targeting their immature stages is convenient since they are confined in their aquatic habitat, where they have a relatively limited mobility range, and therefore controlling them is far easier than controlling the adult stage [16]. Biological control agents that can be used to control mosquitoes and other insect pests include plants, fish, nematodes, viral, bacterial, protozoan, and fungal pathogens [16, 17]. Of these, entomopathogenic fungi are the most well-suited for development as biopesticides against vector mosquitoes [18, 19]. Fungal biopesticides are easy to deliver, and they provide flexibility in improving their formulations and hence being able to target and control a wide range of insects with different feeding habits [20]. Entomopathogenic fungi are pathogens of insects or they cause some level of harm within insects [21, 22]. Eighty-five genera and over 750 species of entomopathogenic fungi are known. Amongst these, two genera, *Beauveria* and *Metarhizium,* which belong to the class Hyphomycetes in Deuteromycota are being used as an ingredient for "myco-insecticides" or "myco-acaricides" [23]. Control of mosquito larvae supplements adult mosquito control strategies, and it is advantageous since larvae are confined in their habitats, unlike adult mosquitoes that fly, making their control difficult [24, 25].

Entomopathogenic fungi have shown natural variations in their prevalence and virulence against mosquitoes. It is, therefore, advisable to carry out pathogenicity tests of different isolates of the same fungus from different geographical locations to ascertain their susceptibility to mosquito larvae obtained from the habitat of interest.

The genus *Lagenidium* is a group of aquatic fungi that belong to the class Oomycota and whose members are either saprophytes or parasites [26]. Only one species of this group, *Lagenidium giganteum* has been shown to be parasitic to mosquito larvae [27]. It was first described by Couch [28] from a combined collection of copepods and mosquito larvae (*Culex* and *Anopheles*) in North Carolina, USA. The infective stage is the motile spore (zoospores) which attacks and destroys mosquito larvae in water. Depending upon temperature and zoospore density, the larvae die of starvation within 1–4 days [29]. *Lagenidium ajelloi* is a water mold, and although it has previously been isolated in mammals in the past, little is known about its entomopathogenic potential against mosquito larvae and its interaction with other organisms, except for its morphology [30]. The current study therefore aimed to isolate *L. giganteum* and *L. ajelloi* from wild *Anopheles* larvae from Ahero rice fields in western Kenya and to test their potency against laboratory-reared *Anopheles gambiae* larvae.

2. Materials and Methods

2.1. Study Area. The study was carried out at the Ahero rice irrigation scheme (34.9°E, −0.17°S), located in Kisumu County along the shores of Lake Victoria in western Kenya. The region receives long and short rains between the months of March to May and September to December, respectively, with annual variability. River Nyando passes through Ahero and provides water to the local population for irrigation, with the main cash crops being rice and sugar cane [10]. Due to the presence of water, Ahero provides favorable breeding grounds for mosquitoes throughout the year. The main malaria vectors in Ahero belong to *A. gambiae* and *A. funestus* complexes. Both *A. arabiensis* and *A. gambiae sensu stricto* occur sympatrically in the Ahero region [31].

2.2. Mosquito Larvae Sampling. A sampling of wild *Anopheles* mosquito larvae was carried out in July, 2018, from 10 randomly selected irrigation canals within a radius of 1 km. *Anopheles* larvae were distinguished from *Culex* by observing their resting positions on the water surface. *Anopheles* larvae were sampled and put in plastic containers and then transported to the laboratory at the Technical University of Kenya in Nairobi, where fungal isolation and characterization were carried out.

2.3. Fungal Isolation. In the laboratory, the dead larvae were surface sterilized by dipping them in a 2% sodium hypochlorite solution for two minutes to kill any external microbial contamination. The larvae were then introduced into potato dextrose agar (PDA) media for fungal isolation. Gentamycin was added as a bacteriostatic agent in the media to prevent bacterial contamination. The larvae were inoculated into the media with a sterile pair of forceps. To ensure complete fungal isolation, twenty replicates were carried out with each plate containing 10 larvae. A negative control was set with 3 plates in which no larvae were inoculated. The plates were placed in the incubator set at $24 \pm 2^{\circ}$C for fungal cultivation for 14 days, with observations carried out, starting from the 3rd day for fungal growth and colony characteristics. On the eighth day, a fungal colony survey was carried out and subculturing carried out on Sabouraud dextrose agar (SDA) plates supplemented with gentamycin to obtain pure cultures.

2.4. Fungus Identification. Fungal species were then identified using both macroscopic characteristics of colonies and microscopic observation of morphological features of mycelia, hyphae, and spores. Microscopic identification was performed under objectives X40 and X100 using an OPTIKA B-380 compound microscope manufactured by OPTIKA Srl in Italy at Ponteranica, and images were taken using an OPTIKA 4083.B2 digital camera attached to the B-380 microscope. Microscopic slide preparations were performed using a modified Riddell's slide culture technique to enable viewing of the specimen in its live state with minimal distortion to morphological features [32]. Existing published work and taxonomic keys [28, 30, 33] were used in the identification of the fungi. Only *Lagenidium* species were selected for further tests after carrying out molecular analysis to ascertain their identities.

2.5. DNA Extraction and Sequencing. Sabouraud dextrose broth was used to inoculate the 2 fungal strains in a 250 mL flask. Incubation was then performed for 72 h at 37°C on a rotating shaker at 150 rpm. Merthiolate (0.02%, wt/vol) was used to kill the cultures after incubation before filtration to obtain the hyphal cell mass for DNA extraction. The hyphal cell mass was grounded using a pestle and mortar in the presence of the CTAB buffer. The mixture was subjected to sodium dodecyl and proteinase K treatment and incubated for 1 h at 60°C before adding CTAB/NaCl (heated at 65°C) and incubating at 10 min. The total DNA was extracted from the solution with phenol: isoamyl alcohol (25 : 24 : 1). ITS gene was amplified by hot start PCR protocol using primers for *Lagenidium* strains as described by Vilela et al. [34]. ITS is recognized as a fungal barcode because it is the most sequenced region of fungi and is routinely used for systematics, phylogenetics, and identification [35]. The PCR products were analyzed in 2% agarose gel and purified using Exosap-it® (Affymetrix, Santa Clara, CA), as per the manufacturer's instructions. Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit reaction mix (Applied Biosystems, USA) using a 3500xL sequencer.

2.6. Phylogenetic Analysis. The raw sequence data in the form of chromatograms from both forward and reverse primers were edited manually to remove poor quality reads. The edited reads were then exported to BioEdit software version 7.0.5.3 [36], aligned using MAFFT alignment software [37] and assembled into contigs after resolving potential ambiguities by eye. Multiple sequence alignment is important in the identification of gaps, matches, and mismatched nucleotides in the three genes, which is a prerequisite for the construction of phylogenetic trees. The two ITS genes from *L. giganteum* and *L. ajelloi* were analyzed independently against twenty-three *lagenidium* species' sequences obtained from the National Centre for Biotechnology Information (NCBI), of which four sequences were from *L. giganteum* and the other four were from *L. ajelloi*. This led to the construction of a phylogenetic tree showing the evolutionary relationship of both *L. giganteum* and *L. ajelloi* with other *Lagenidium* species obtained from NCBI. Phylogeny construction was carried out in PhyloSuite [38]; the models assumed the rates and patterns of substitution were uniform among the four nucleotide sites. The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model [39]. The test for phylogeny was bootstrap resampling [40], where the number of bootstrap replications was set at 5000 for the ML tree constructed.

2.7. Anopheles gambiae Larvae Colony. *Anopheles gambiae* colony was established at the University of Nairobi insectary with eggs obtained from the International Centre of Insect Physiology and Ecology (ICIPE), Mbita point, western Kenya. As per the standard operating procedures (SOPS) of the University of Nairobi insectary, environmental conditions in the insectary were set at temperatures of 32°C and relative humidity of 70–80%. The adults were kept separately at room temperatures of 26°C–28°C. From this colony, 3rd and 4th instars of *A. gambiae* larvae were picked and used for the bioassay according to WHO recommendations [15]. Because both *A. gambiae* and *A. arabiensis* occur in the study area, *A. gambiae sensu stricto* was selected for laboratory bioassays as it was readily available.

2.8. Pathogenicity Tests on Mosquito Larvae. Pure cultures of the two *Lagenidium* species were removed from the Petri dishes in storage at 4°C and each blended together with 100 mL of deionized water, then kept for 12 hours to initiate zoospore production. For each fungus, the number of zoospores in an aliquot of 10 microliters of this stock solution was counted by using a hemocytometer, and the number of spores per mL was determined. Then, concentrations of 1000, 2000, 3000, 4000, and 5000 zoospores/mL were calculated using the formula $C_1V_1 = C_2V_2$. These were then used as the concentrations upon which the late instars of *Anopheles* mosquitos were tested. This was informed by Shathele [41], who found that a concentration of 2000 *L. giganteum* zoospores/ml caused a 100% kill of 1st instar of *Culex quinquefasciatus*. Each sample was added to a 500 mL plastic container filled with 100 mL deionized

water in which 25 late instar *A. gambiae* larvae were introduced, with each concentration having 4 replicates [15]. For each concentration, deionized water was used as a negative control where no fungus was added. *Bacillus thuringiensis* var *israelensis* (Bti) at a concentration of 0.21 mg/L was used as a positive control [42]. The larval mortality was recorded after 24, 48, and 72 hours, and any dead larvae were recorded [43].

2.9. Data Analysis. Data were subjected to Finney Probit analysis [44] using Biostat 2009 to determine the concentration of the fungal zoospores required to kill 50% of the larvae (LC_{50}) and the time taken by a specific concentration of zoospores to kill 50% of larvae (LT_{50}). The data were also subjected to ANOVA to determine any significant differences between the treatments and the positive control. Comparisons of mortalities caused by the two fungal species were performed by ANOVA using the Prism software package.

3. Results

Based on the observation of macroscopic and microscopic morphological features obtained by DNA analysis, 2 species of *Lagenidium* (*L. ajelloi* and *L. giganteum*) were identified.

3.1. Morphological Characteristics of Lagenidium giganteum. *L. giganteum* colonies were circular, velvety, and orange-white in colour on an agar plate and were about 1.5 cm in diameter on the 3rd day of growth (Figure 1(a)). The hyphae were aseptate at first but later became septate, hyaline, and constricted at the septum and had almost parallel branching to form mycelium with a whitish gleam evident in the protoplasm (Figure 1(b)) at ×40 magnification. Zoosporangiophores appeared and formed zoospores that were subglobose, colorless, thick-walled, and formed inside sporangia (Figure 1(c)) as observed under a microscope at ×100 magnification. Zoospores were pale black, laterally biciliate, and swum sluggishly. Homothallic sexual reproduction was observed (Figure 1(d)) to form a spherical zygote with a subcentral sphere of fat surrounded by cytoplasm (Figure 1(e)). Both structures were observed under a compound microscope at ×100 magnification. Finally, the hyphae cellulose changed to purple on staining with chloro-zinc iodide (Figure 1(f)). This observation was in agreement with the findings reported by Couch [28].

3.2. Morphological Characteristics of Lagenidium ajelloi. Macroscopically, the colony was irregular, 4.5 cm in diameter on the 7th day, and yellow in colour (Figure 2(a)). Microscopically, the thallus was filamentous with strong restrictions at the septa forming long detached segments. Hyphae were septate and hyaline, with protoplasm with pale whitish gleam and with conspicuous shiny globules (Figure 2(b)). Exit tubes were present and were short and extended through hyphal wall superficially. Zoosporangiophores were club-shaped with sporangia at the tip, and a conspicuous shiny globule was present at the foot, while some were constricted at the center with pale black coaxial fabric running in the middle. Zoosporangiophores contained two sporangia in most cases, which were lobed and irregular and sometimes ovoid in shape. Zoospores were pale black to golden yellow, laterally biflagellate, swum aggressively in circular paths and rolled in gyrates, and after sometime, formed cysts that were pointed at the anterior and rounded at the posterior end (Figure 2(c)). Homothallic sexual reproduction was characterized by antheridia moving horizontally along the hyphae to meet archegonia and hence formed pyriform zygote with subcentral male and female gametes. These observations were in agreement with the findings reported by Mendoza et al. [30].

3.3. Phylogenetic Analysis. Molecular phylogeny was performed to confirm the identity of the two species already identified from morphological characteristics. Initial trees for the ML heuristic search were attained through the application of Neighbor-Join and BioNeighbor-Join algorithms to pairwise distances matrix generated from *Lagenidium* sequences. The final tree selected was the tree topology with a superior log likelihood value. Bootstrap resampling was selected as a test of phylogeny, and it measured the reliability of the tree generated [40]. In addition, NCBI Blast search was also conducted using *L. ajelloi* as query sequence (13a) and yielded 100% identity of organisms with GenBank accession numbers KJ506135, KJ506135, and KJ506135 that correspond to *L. ajelloi.* This is in line with the ML phylogeny obtained, where all the five sequences from *L. ajelloi* species are grouped together, forming a clade that is strongly supported by a bootstrap value of 100, implying the query sequences (13a) was indeed *L. ajelloi* (Figure 3). Similarly, 100% sequence identity was observed in KT257472, AY151183, KY965927, and KT257343 which correspond to *L. giganteum* when blast search was carried out using *L. giganteum* (11) as the query sequence. Identity of *L. giganteum* has also confirmed from the ML phylogeny obtained where all the five *L. giganteum* species have been grouped together in a clade which is supported by a strong bootstrap support value of 97 (Figure 3). Hence, the ITS genes from the two species strongly indicated that the species were indeed *L. ajelloi* and *L. giganteum* as previously identified from the morphological characteristics.

3.4. Pathogenicity Tests. It was observed that *L. ajelloi* was more toxic against *Anopheles gambiae* larvae than *L. giganteum*. No larval mortalities were recorded due to *L. giganteum* zoospores at 24 and 48 hours post-exposure to all 5 zoospores concentrations tested. However, larval mortalities were recorded for *L. ajelloi* after 24, 48, 72, and 96 hours post-exposure in all the 5 test concentrations. For both fungi, it was observed that the larval mortalities increased with exposure time (Figure 4). The LC_{50} of *L. giganteum* zoospores against *A. gambiae* larvae after 72 and 96 hours was 2.32×10^4 and 3.51×10^3 zoospores/mL, respectively, while that of *L. ajelloi*

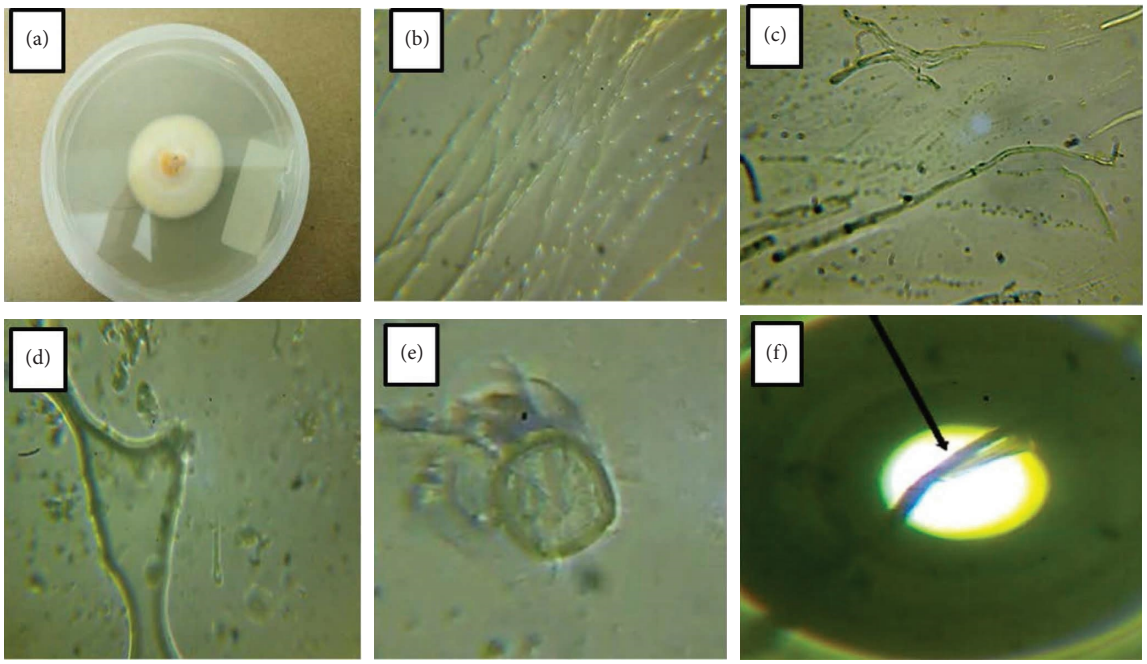
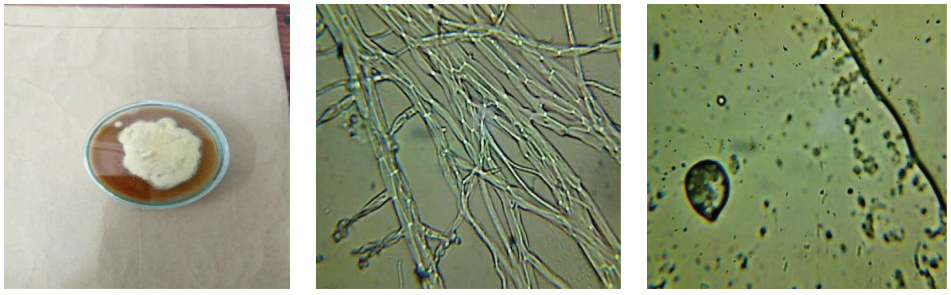


Figure 1: Macroscopic and microscopic characteristics of *Lagenidium giganteum*. (a) Colony on agar; (b) mycelium; (c) zoospores; (d) homothallic reproduction; (e) zygote; (f) stained hyphae.



(a) (b) (c)

Figure 2: Macroscopic and microscopic characteristics of *Lagenidium ajelloi*. (a) Irregular colony; (b) hyphae; (c) zoospore cysts.

zoospores after 24, 48, 72, and 96 hours was 1.18×10^5, 1.43×10^4, 6.05×10^2, and 27.08 zoospores/ml, respectively (Table 1). No mortalities were recorded in deionized water (negative control), while 100% mortality was recorded in larvae exposed to *Bti* (positive control) within 24 hours. It was observed that the longest LT_{50} was in larvae exposed to 1000 zoospores/mL concentration for both oomycetes, which was 105 hours for *L. giganteum* and 62.58 hours for *L. ajelloi*. On the other hand, the shortest LT_{50} was observed under 5000 zoospores/mL, which was 89 hours for *L. giganteum* and 58.19 hours for *L. ajelloi,* respectively (Table 2). The results indicate that the increase in zoospore concentrations was directly proportional to larval mortality for both fungi, with *L. ajelloi* causing significantly more larval mortalities than *L. giganteum* (Figure 4). However, the larvicidal activity of both *L. giganteum* and *L. ajelloi* was significantly slower than the positive control (*Bti*) in all test zoospore concentrations (Figure 5).

4. Discussion and Conclusion

The results of this study indicate that *Anopheles* mosquito larvae from the Ahero rice irrigation scheme in Western Kenya are naturally parasitized by *Lagenidium giganteum* and *L. ajelloi*. It was evident that both species are infective to the larvae of *A. gambiae* and the pathogenicity was observed to increase proportionately with an increase in the concentration of zoospores. *L. ajelloi* was found to be more pathogenic than *L. giganteum* to *A. gambiae* larvae under all concentrations of zoospores tested. Pathogenicity due to *L. ajelloi* and that of *Bti* were found to be similar, whereas significant differences were recorded between pathogenicity of *L. ajelloi* and that of *L. giganteum*.

As a facultative parasite, *L. giganteum* can grow vegetatively both as a pathogen on mosquito larvae or as a saprophyte in aquatic environments [45–47]. Isolation of *L. giganteum* from *Anopheles* larvae has previously been reported by studies performed in the USA [26, 28, 33].

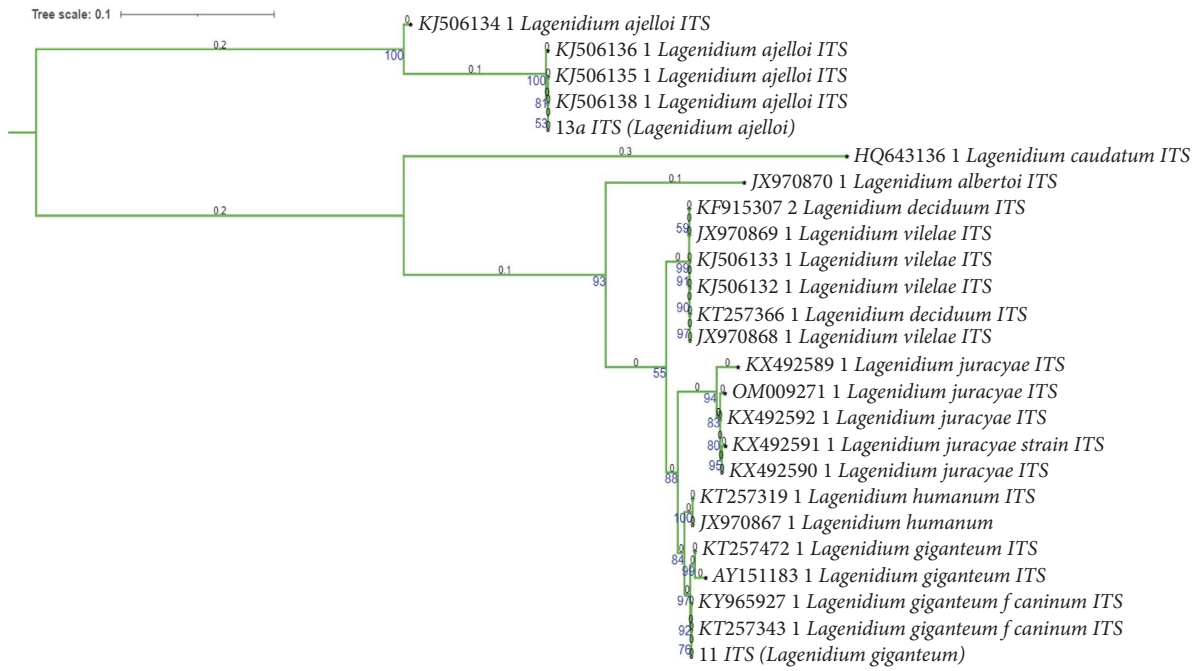


Figure 3: Phylogenetic tree of *Lagenidium* species.

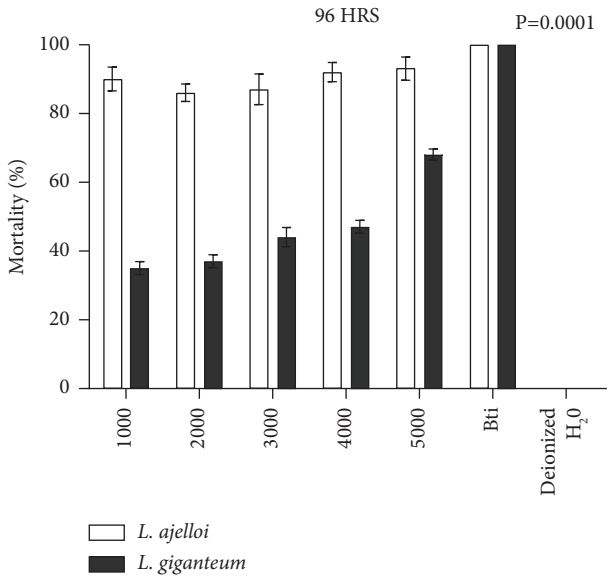


Figure 4: Comparison of percent mortality of *Lagenidium giganteum* and *L ajelloi* against *A. gambiae* larvae at different zoospores concentrations and controls. *L. ajelloi* caused significantly more larval mortalities than *L. giganteum* in all zoospore concentrations under investigations.

Couch [28] was the first scientist to discover the fungus in copepods (*Daphnia* sp.) and a mixture of *Culex* and *Anopheles* larvae in North Carolina, USA. Thereafter, Umphlett [48] and Kerwin et al. [49], both in the USA, isolated and tested the pathogenicity of the fungus on several species of *Culex* mosquitoes. Umphlett found *L. giganteum* to be toxic against larvae of *Culex restuans* and *Culex quadrimaculatus* [48], while Kerwin et al. found *L. giganteum* toxic against larvae of *Culex quinquefasciatus* [49].

The highest concentration (5000 zoospores/mL) of *L. giganteum* zoospores tested against *A. gambiae* larvae killed 68% of the exposed larvae in 96 hours. However, these results differed slightly from those of Golker et al. [50], who found that 56% of *A. gambiae* larvae exposed to *L. giganteum* zoospores were protected from death by the larval immune defense. Both findings support the fact that the pathogenicity of *L. giganteum* to *A. gambiae* increases with zoospore concentration and that different strains of the fungus may produce different virulence and pathogenicity [51]. The weak pathogenicity of *L. giganteum* zoospores observed in the study can also be attributed to their inability to sometimes recognize late instars of otherwise susceptible mosquito larvae [52]. However, the findings of this study may not authentically suggest that *L. giganteum* zoospores have weak pathogenicity against *A. gambiae* larvae since laboratory findings may not necessarily always reflect the ideal field situations due to a wide range of differences in environmental factors [53]. Although little previous entomopathogenic research work has been carried out on *L. ajelloi,* it was found to be more pathogenic with the highest tested concentration (5000 zoospores/mL) killing 94% larvae in 96 hours. It was observed that *L. ajelloi* zoospores swim faster than those of *L. giganteum,* which agreed with the findings by Mendoza et al. [30]. Zoospores that swim faster could imply many come into contact with larvae, consequently resulting in more infections and pathogenicity as compared to slow swimming zoospores.

Previous works report that Anopheline mosquito larvae have been found to produce immunity characterized by melanization response, resulting in the encapsulation of foreign particles and parasites with a dark layer of melanin

Table 1: Lower and upper limits for LC_{50} of *Lagenidium giganteum* and *L. ajelloi* zoospores of the *A. gambiae* larvae at various time intervals.

Fungal species	Time in hours	LC_{50} lower limit (zoospores/ml)	LC_{50} (zoospores/ml)	LC_{50} upper limit (zoospores/ml)	SE
L. ajelloi	24	2.14×10^4	1.18×10^5	2.15×10^5	±97148.20
	48	1.27×10^4	1.43×10^4	1.58×10^4	±803.61
	72	1.67×10^2	6.05×10^2	9.98×10^2	±285.609
	96	3.261	27.084	51.842	±22.72
L. giganteum	24		Nil		
	48		Nil		
	72	2.06×10^4	2.32×10^4	2.58×10^4	±1326.12
	96	2.87×10^3	3.51×10^3	4.14×10^3	±325.45

Larval mortalities increased with exposure time and lower LC_{50} observed with *L. ajelloi* zoospores indicated that *L. ajelloi* is more toxic to *A. gambiae* larvae compared to *L. giganteum*.

Table 2: Time taken by a specific concentration of *Lagenidium ajelloi* and *L. giganteum* zoospores to kill 50% larvae (LT_{50}).

Fungal species	Concentration (zoospore/ml)	LT_{50} lower limit (hours)	LT_{50} (hours)	LT_{50} upper limit (hours)	SE
L.ajelloi	1000	45.43	62.58	86.19	±4.66
	2000	35.59	63.58	113.58	±8.89
	3000	55.71	58.95	62.18	±1.64
	4000	55.68	58.87	62.01	±1.62
	5000	54.51	58.19	61.88	±1.87
L. giganteum	1000	101.24	105.55	109.87	±2.19
	2000	99.96	104.17	108.38	±2.14
	3000	96.62	100.59	104.56	±2.01
	4000	93.74	97.52	101.30	±1.92
	5000	86.15	89.31	92.48	±1.60

As expected, an increase in the concentration of zoospores resulted in a shorter LT_{50}. The shortest LT_{50} was observed in *L. ajelloi* zoospores indicating that *L. ajelloi* is more potent against *A. gambiae* larvae compared to *L. giganteum*.

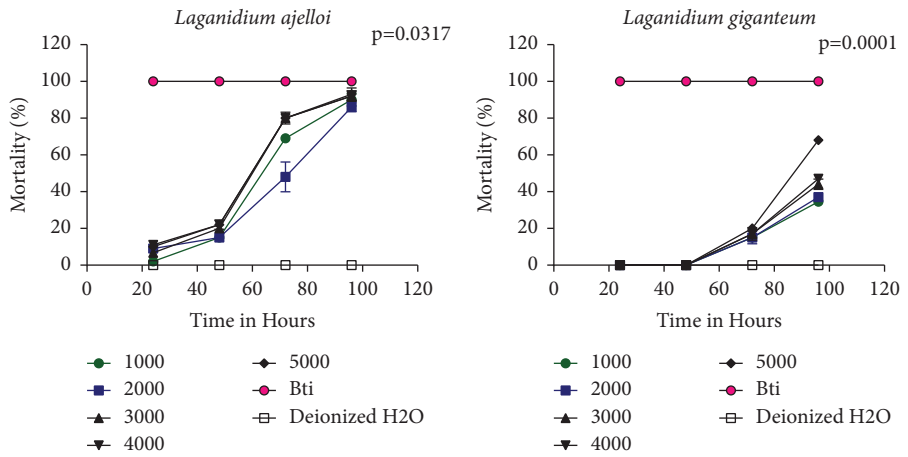


Figure 5: Percent mortality of *Lagenidium giganteum* and *L. ajelloi* zoospores against *A. gambiae* larvae at different time intervals. The positive control (*Bti*) caused larval mortalities significantly faster than all the test zoospore concentrations, but there were no significant differences in killing time between the various zoospore concentrations for both fungi.

[54]. The larval immune melanization starts at the cuticle before moving on to the hemocoel; hence, some larvae that mount intense cuticle defense escape infection. Further studies by Golker et al. [50] revealed that some *A. gambiae* larvae that do not mount enough cuticle defense get infected but may also escape death. This is because after the fungi enter the hemocoel, there is complete melanin encapsulation of the fungal hyphae rendering them unable to exchange both gases and metabolites leading to death of the invading oomycete due to asphyxia or starvation. However, the present study has shown *L. ajelloi* to be more potent against the larvae of *A. gambiae* causing significant larval mortalities

that are closely comparable to *Bti* with the only difference in exposure time. These results may suggest that melanization by *L. ajelloi* was more intense as compared to that caused by *L. giganteum,* indicating a difference in the number of infecting zoospores and hence pathogenicity. Faster swimming *L. ajelloi* zoospores could result in more infections than the sluggish swimming *L. giganteum* zoospores. Infection by many zoospores would then lead to overwhelming of the immune defense regardless of melanin development.

The delayed larval mortalities due to *L. giganteum* and *L. ajelloi* infections, as compared to mortalities due to *Bti* could be attributed to the mode of infection, and action between the two treatments. Infection is faster with *Bti* since the larvae directly ingest the bacteria in water as opposed to *Lagenidium* infection where the zoospores take time to recognize (by chemotaxis) and penetrate the larval cuticle [26]. Secondly, the larvae die within hours after ingestion of *Bti* due to protein toxins produced by the bacteria inside the gut of the larvae [25]. On the other hand, larval death due to *Lagenidium* infection is slower since the fungi have to penetrate and proliferate within the larvae [26], which takes longer than pathogenicity due to *Bti* infection.

Unlike viruses and bacteria parasites that need to be ingested by the mosquito larvae in order to cause pathogenicity [55], infection due to *Lagenidium* is advantageous since after killing the mosquito larvae; more zoospores are produced in the cadavers resulting in the recycling of the fungus in the water and hence being able to infect current and subsequent generations of mosquito larvae [46]. Besides, due to the specificity of *Lagenidium* zoospores to mosquito larvae, it is expected to have minimal to no risk to natural enemies of mosquito larvae [28].

In general, the findings of this study are in tandem with earlier findings that *L. giganteum* is a facultative entomopathogenic oomycete of mosquito larvae [28, 43, 49]. The findings of this study support the findings of earlier studies that susceptibility of mosquito larvae to *L. giganteum* is variable and depends upon the mosquito species [50] and also upon different isolates of the fungus from different geographical locations [56]. The successful discovery and isolation of *L. ajelloi* as a mosquito larval pathogen is encouraging and brings on board a new candidate for further evaluation. There is a need for further research on molecular characterization to ascertain the possibility of having several strains of *L. giganteum* and *L. ajelloi* in mosquito-infested water from different parts of the country. Perhaps, different strains of the oomycete would present different toxicity levels against *A. gambiae* larvae [51]. Despite the promising opportunities and advantages associated with entomopathogenic fungi, there has been little work on the use of fungal biopesticides against malaria vectors. There is also a need to explore possibilities of producing fungus-based biocontrol agents that will complement existing adult-based strategies and also slow mosquito resistance development [18, 19].

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

SSI and VWW conceived the study. SSI provided expertise with field work and sample collection. MMS did sample collection and laboratory experiments and prepared the first manuscript draft. VWW provided expertise on laboratory trials. FMM did fungal characterization and statistical analysis. SSI, VWW, and FMM reviewed and contributed to the final manuscript. All authors have read and approved the final manuscript.

Acknowledgments

The authors are grateful to the TWAS Basics Sciences Research (grant no. RG 16-164) for providing equipment and consumables for this work through a grant accorded to Dr. Imbahale. The authors appreciate Professor Richard Mukabana of the School of Biological Sciences, University of Nairobi, for his support in mosquito rearing. The authors thank the Technical University of Kenya for providing laboratory space for this work. Dr. S. Imbahale (grant no. RG 16-164) received funding from TWAS for the purchase of equipment and consumables.

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