Culex (Culex) Pipiens Mosquitoes Carry and Harbor Pathogenic Fungi during Their Developmental Stages

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Objective: Fungi are the main source of aflatoxin contamination in nature. The present study aimed to assess the role of the cosmopolitan mosquito species, *Culex pipiens*, in the circulation and dissemination of pathogenic fungi in nature, and to evaluate its capability to harbor these fungi.

Materials and Methods: Fungi were isolated and identified from both, the external surface and the internal organs of the developmental stages and from the breeding environment of *Cx. pipiens*.

Results: A total of 35 fungal isolates were isolated from both, the internal organs and the external surface of the developmental stages and from the breeding environment of *Cx. pipiens*. These isolates were identified as eleven *Penicillium notatum* isolates, eleven *Aspergillus flavus* isolates, six *Rhizopus stolonifer* isolates, four *Candida albicans* isolates, two *Fusarium solani* isolates, and one *Aspergillus niger* isolate. Antagonistic activity showed that the *P. notatum* growth inhibited the growth of the bacteria, *Staphylococcus aureus*.

Conclusions: This study revealed that the different developmental stages of *Cx. pipiens* mosquito were capable of harboring many fungal species. Subsequently, this mosquito will be incriminated in the mechanical dissemination, circulation, and transmission of these fungi in nature, during its life cycle.

Keywords: *Cx. pipiens*, mosquitoes, pathogenic fungi, developmental stages, breeding environment

INTRODUCTION

Mosquitoes are unquestionably the most important arthropod vectors of diseases. The maintenance and transmission of pathogens that cause malaria, lymphatic filariasis, and numerous viral infections are absolutely dependent on the availability of competent mosquito vectors (1, 2).

Among the culicine mosquitoes in Egypt, *Culex pipiens* stands first and foremost as a widely distributed species that is frequently encountered in prodigious numbers in many regions of the Egyptian territory. It has also been incriminated in the transmission of the nocturnally periodic bancroftian filariasis in the Nile Delta of Egypt and the Rift Valley fever (1, 2).

Fungi can occasionally attack insects or develop symbiotic relationships (3). Many interactions between fungi and mosquitoes have been reported worldwide (4-10). Other interactions between bees and fungi have been documented, as well (11-13). Termite-fungi relationships have also been observed (14). Other insect-fungi interactions have also been recorded, including lepidopteran-fungi (15), beetle-fungi (5, 16), and triatomine-fungi interactions (17-20).

The main objectives of this study were to assess the role of the cosmopolitan mosquito species, *Cx. pipiens*, in the circulation and dissemination of pathogenic fungi in nature, and to evaluate its capability to harbor these fungi. Antagonistic activity between the isolated fungi and associated bacteria was also investigated.

MATERIALS and METHODS

Colonization of *Cx. pipiens* A colony of the mosquito, *Cx. pipiens*, was maintained under controlled laboratory conditions (27±2°C, 60-70% Relative humidity (RH) and 10/14 light/dark photoperiod cycle) in the insectary of the Department of Entomology, Faculty of Science, Cairo University, Giza, Egypt, up till now. This colony was used for supplying the immature stages (eggs, larvae, and pupae) of *Cx. pipiens* during this study. Briefly, the eggs were set up to hatch, and the 1st instar larvae were seeded into plastic cups (25·35·7 cm³) containing water at a constant density of 300 individuals per cup. The
larvae were provided with activated yeast or tetramin, every 2 days, until pupation. Water was changed on feeding days to avoid bacterial growth on the water surface. On pupation, cups were placed inside an emergence cage (27x40x35 cm³) and provided with a source of 10% sugar solution for the emerged adults (21). All necessary permits for this study were obtained from the local ethics committee of Cairo University. This study did not involve endangered or protected species. The informed consent rules are not applicable for this study.

**External fungal isolation**

Twenty individuals from Cx. pipiens egg rafts, 4th larval instars, pupae, and adults (males and females) were anesthetized using cold 70% ethanol, and then, the insect samples were transferred to centrifugal filters and washed five times with 70% ethanol in order to sterilize the insect surface. The surface-sterilized insects were spun for 5-10 seconds in a table-top microcentrifuge at a low speed setting (around 1,000 g) to dispose most of the ethanol. Samples were washed thrice with sterile distilled water. The surface-sterilized samples were homogenized in sterile Ringer’s solution using a glass tissue grinder. A suspension of 0.1 mL of the washing saline was inoculated onto Czapek-Dox’s agar medium using a sterilized scalpel, inside the laminar air flow hood. The plates were incubated at 28°C for 5-7 days (22).

**Internal fungal isolation**

Twenty individuals from Cx. pipiens egg rafts, 4th larval instars, pupae, and adults (males and females) were anesthetized using cold 70% ethanol, and then, the insect samples were isolated from the different developmental stages of Cx. pipiens. Out of these 11 isolates, 2 (18%), 3 (27%), 1 (9%), 3 (27%), and 2 (18%) isolates were isolated from the egg, larval, pupal, adult male, and adult female stages, respectively.

Results of the fungal identification revealed that we had isolated four different fungal strains (Table 1). Among these 4 fungal strains, Aspergillus flavus and Penicillium notatum were the most persistent fungi and appeared in four mosquito developmental stages, followed by Rhizopus stolonifer, which appeared in two developmental stages (larva and adult male), and Candida albicans, which was isolated from the egg rafts and was lost in the other stages. It was observed that A. flavus, P. notatum, and R. stolonifer were isolated from the larvae and the adult male mosquitoes (Table 1).

**RESULTS**

**Fungal isolates**

A total of 35 fungal isolates were identified during this study. These isolates were isolated from the different developmental stages of the mosquito and from its breeding environment.

**External fungal isolation from the insect**

Out of the 35 isolates, 11 fungal isolates (31%) were isolated from the external surface of the eggs, 4th larval instar, pupae, the adult male, and the adult female of Cx. pipiens. Out of these 11 isolates, 2 (18%), 3 (27%), 1 (9%), 3 (27%), and 2 (18%) isolates were isolated from the egg, larval, pupal, adult male, and adult female stages, respectively.

**Table 1. External fungal isolates from eggs, larvae, pupae, and the adult male and adult female**

<table>
<thead>
<tr>
<th>Stages</th>
<th>Eggs</th>
<th>Larvae</th>
<th>Pupae</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Penicillium notatum</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Rhizopus stolonifer</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
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<td>✓: appearance</td>
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</tbody>
</table>

**Internal fungal isolation from the insect**

Out of the 35 isolates, 13 fungal isolates (37%) were isolated from the internal organs of the different developmental stages of Cx. pipiens. Out of these 13 isolates, 1 (7.5%), 3 (23%), 1 (7.5%), 4 (30.5%), and 4 (30.5%) isolates were isolated from the egg, larval, pupal, adult male, and adult female stages, respectively.

**Table 2. Internal fungal isolates from eggs, larvae, pupae, and adults (males & females)**

<table>
<thead>
<tr>
<th>Stages</th>
<th>Eggs</th>
<th>Larvae</th>
<th>Pupae</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Penicillium notatum</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Rhizopus stolonifer</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<td>✓: appearance</td>
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</tbody>
</table>

**Characterization of fungal isolates**

The isolated fungal colonies were identified according to their macroscopic morphology, and the selected colonies were mounted and stained with lactophenol for light microscopic examination. This was presented according to the method of Arx (25).

**Antagonistic activity between isolated fungi and other associated bacteria**

Dox medium was prepared and poured into plates. One milliliter of each isolated fungal suspension was inoculated onto the DOX media. On cooling, a disk of filter paper immersed in bacterial suspension was placed on the DOX plate. The tested bacterial strains were previously isolated from the different developmental stages of the mosquito, Cx. Pipiens (21). Measurements of growth inhibition zones were recorded and compared to a reference chart after incubation at 30°C for 5-7 days. Each experiment was repeated thrice.
peared in four mosquito developmental stages, followed by *R. stolonifer*, which appeared in three developmental stages (larva, adult male, and adult female), and *C. albicans*, which was isolated from the adult stages (male and female) and lost in the immature stages. The four fungal strains, *A. flavus*, *C. albicans*, *P. notatum*, and *R. stolonifer*, were isolated from both, the male and female adult mosquitoes (Table 2).

### Isolation from the mosquito environment

Out of the 35 isolates, 11 fungal isolates (31%) were isolated from the environmental sources of the immature and mature stages of *Cx. pipiens*. Out of these 11 isolates, 3 (27%), 3 (27%), and 5 (45%) isolates were isolated from air, sucrose water, and breeding water, respectively.

#### Table 3. Fungal isolates from environmental sources (air, sucrose water, and breeding water)

<table>
<thead>
<tr>
<th>Environmental sources</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>From air</td>
<td><em>Penicillium notatum</em></td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus flavus</em></td>
</tr>
<tr>
<td></td>
<td><em>Fusarium solani</em></td>
</tr>
<tr>
<td>From sucrose water</td>
<td><em>Aspergillus flavus</em></td>
</tr>
<tr>
<td></td>
<td><em>Penicillium notatum</em></td>
</tr>
<tr>
<td></td>
<td><em>Rhizopus stolonifer</em></td>
</tr>
<tr>
<td>From breeding water</td>
<td><em>Aspergillus flavus</em></td>
</tr>
<tr>
<td></td>
<td><em>Penicillium notatum</em></td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td></td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td></td>
<td><em>Fusarium solani</em></td>
</tr>
</tbody>
</table>

Results of the fungal identification revealed that one additional fungal strain (*Fusarium solani*) was identified from the air and breeding water samples (Table 3). Among the five identified fungal strains, *A. flavus* and *P. notatum* were the most persistent fungi and appeared in all the breeding environments (air, sucrose, and water), followed by *F. solani*, which appeared in the air and water environment. *C. albicans* and *Aspergillus niger* were isolated from only the breeding water environment, whereas *R. stolonifer* was isolated from only the sucrose water environment (Table 3).

#### Common fungal isolates between external and internal isolates

It was observed that all the fungal strains shared the external and internal isolations, except *C. albicans*. It did not share the internal isolation in the egg stage and the external isolation in the adult stages (male and female) with the other fungal strains. In addition, *R. stolonifer* did not share the external isolation in the adult female stage (Table 4). It was clear that *A. flavus* was the most prevalent strain that appeared in all isolations (external and internal). However, *A. flavus* was not isolated from the pupal stage at all (Table 4). *P. notatum* was not isolated from the egg stage. Meanwhile, *P. notatum* was the most prevalent strain that appeared in all other stages (larva, pupa, male, and female) in both, external and internal isolations (Table 4). *R. stolonifer* was the third strain that shared external and internal isolations in the larval and adult male stages (Table 4).

### Characterization of Fungal Isolates

#### Macromorphosopic characterization

A total of 35 fungal isolates were identified from both, the internal organs and the external surface of the different developmental stages, and from the breeding environment of the mosquito, *Cx. pipiens*. The fungal isolates were identified macroscopically as six...
different strains: *P. notatum* (11 isolates), *A. flavus* (11 isolates), *R. stolonifer* (6 isolates), *C. albicans* (4 isolates), *A. niger* (one isolate), and *F. solani* (2 isolates).

On an agar plate, *P. notatum* appeared granular, flat, often with radial grooves, yellow at first, but quickly becoming bright to dark yellow-green with age. *A. flavus* was observed as powdery masses of yellow-green spores on the upper surface and reddish-gold on the lower surface. *R. stolonifer* was observed to fill the culture plate with dense, cottony, aerial mycelium, first white, which then became gray. *C. albicans* appeared white in color. *A. niger* was observed as powdery masses of numerous black dots, and *F. solani* was observed to be white to creamish in color, becoming bluish-brown when sporodochia were present (Table 5).

**Microscopic characterization**

Microscopic examination of the fungi revealed that mycelia were branched in all the tested fungi, except in *C. albicans*. The hyphae of all the examined fungi were septate, except in *R. stolonifer* and *C. albicans*. Conidiophores were observed to be branched, fusiform, long smooth, and coarse roughened in *P. notatum, F. solani, A. niger*, and *A. flavus*, respectively. Sporangioles were observed in *R. stolonifer*. Spores were observed in different colors in all the tested fungi, except in *C. albicans* (Table 5).

**Antagonistic activity**

A combination of two or more microorganisms in a single culture medium may indicate synergism if all of them can grow on the medium and antagonism when only a single species can grow on the medium.

Our results revealed that *P. notatum* was antagonistic to the multidrug resistant bacteria, *Staphylococcus aureus*. The fungus inhibited the bacterial growth and tolerance towards fungi. The mean diameter of the inhibition zone was 0.9 cm (Figure 1). No antagonistic activity was observed in other fungi-bacteria combinations (Table 6).
A total of 35 fungal isolates were isolated from both, the internal organs and the external surface of Cx. pipiens developmental stages and from its breeding environment. These isolates were definitively identified as six fungal strains, namely A. flavus, A. niger, P. notatum, R. stolonifer, F. solani, and C. albicans. These fungi are known to be widely spread in nature, and the most frequent genera were Aspergillus and Penicillium.

Many authors have isolated fungi from insects (11, 12, 22, 26-33). Other authors have studied the association of fungi with mosquitoes. (4, 10, 24, 34, 35).

Agreeable results were presented by Burnside (11) who isolated A. flavus and A. niger from bees; Gillian and Prest (12) who isolated various fungi from bees and identified the species, A. niger and A. flavus; Costa and Oliveira (4) who isolated various species of Penicillium from the mosquitoes of tropical regions as well as from the mosquitoes of Brazil; Kaaya and Okech (27) who reported various species that were isolated from the pupal and adult Glossina palpalipes, including A. flavus, A. niger, and Penicillium spp.; and Zarrin et al. (23) who identified Aspergillus, Penicillium, and yeast fungi from the external surface of house flies in Ahwaz, Iran.

Other fungal strains were isolated from insects. Penicillium cyclopium, Cladosporium ladosporoides, and Alternaria spp. were isolated from bees (11,12); Fusarium species were isolated from larvae and adult insects (26); Spiroplasma sabaudiense sp. was isolated from mosquitoes (36); the new Spiroplasma taiwanense sp. was isolated from the Culex tritaeniorhynchus mosquitoes in Taiwan (34); Metarhizium anisopliae and Beauveria bassiana were commonly found on terrestrial insects (37); Cladosporium and Fusarium were isolated from the external surface of house flies (23); Alternaria spp., Penicillium oxalicum, and Aspergillus tamari were isolated from the house fly (Musca domestica L.) larvae, and other species, such as Neurospora sp., Alternaria spp., and Fusarium oxysporum were isolated from the larvae of the sheep blow fly, Lucilia cuprina (29,33).

Six fungal strains (A. flavus, P. notatum, R. stolonifer, A. niger, C. albicans, and F. solani) were isolated and re-characterized using macroscopic and microscopic properties. These results indicated that mosquito eggs, larvae, pupae, and adults were exposed to fungal infection, either through oral or dermal contamination. However, among these fungi, F. solani and A. niger showed negative contamination in all the mosquito stages (isolated from the environment, but not in the mosquito developmental stages). It was not clear if transstadial transmission from larvae and pupae to the adult stage had occurred or not; hence, it needs further confirmatory study.

It is well-known that microbes may compete with each other for nutrients, and this competition often results in the inhibition of growth among one of them. This phenomenon is called antagonism (38). In our study, the growth of P. notatum inhibited the growth of S. aureus. This is due to the secretion of penicillin, which is a well-known antibacterial agent (39). Contrary to our results, Harriott and Noverr (40) reported the antagonism between S. aureus and C. albicans. However, bacteria or fungi may also rely on other species for nutrient supply.

CONCLUSION

In conclusion, the present study described the isolation of six fungal species from the external surface and the internal organs of the Cx. pipiens mosquito developmental stages, and from its breeding environment, as well. Our study demonstrated that the mosquito, Cx. pipiens, could harbor many fungal spores, either internally or externally, during its developmental stages. Subsequently, this mosquito will be incriminated in the mechanical dissemination, circulation, and transmission of these fungi in nature, during its life cycle.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethical committee of Cairo University.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Authors’ Contributions: Conceived and designed the experiments or case: FHG, AMS. Performed the experiments or case: AMS, FHG, OZ, AA. Analyzed the data: FHG, AMS, AA, IA. Wrote the paper: AMS, FHG, IA, AA. All authors have read and approved the final manuscript.
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Conflict of Interest: No conflict of interest was declared by the authors.

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