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Short Communication

Ascogregarina taiwanensis infection in *Aedes aegypti* and *Aedes albopictus* in Santa Catarina, South Brazil

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Abstract

Introduction: This study registers *Ascogregarina* spp. infection in field populations of *Aedes aegypti* and *Aedes albopictus* in a subtropical region of Brazil. **Methods**: Mosquito larvae collected in tires placed in four municipalities of Santa Catarina were identified morphologically and assessed for *Ascogregarina* sp. infection using morphological and molecular methods. **Results**: Both mosquito species harbored *Ascogregarina taiwanensis*, whose genomic DNA was confirmed in both the *Aedes* species by PCR. DNA sequences were deposited in GenBank. **Conclusion**: Both *Ae. albopictus* e *Ae. aegypti* harbor *Ascogregarina* sp.

Keywords: *Ascogregarina taiwanensis*. DNA sequence. South Brazil.

Aedes aegypti and *Aedes albopictus* (Diptera: Culicidae) are important mosquito species responsible for transmitting the etiological agent that causes dengue and yellow fever, mainly in the tropical and subtropical regions of the world. Marcondes and Ximenes[1] suggested that the presence of these mosquito species in Latin America is associated with additional risk because they are potential vectors of Chikungunya and Zika viruses. In Brazil, both the species have been reported to have developed resistance to chemical insecticides[2]. For this reason, some species of microorganisms have been investigated for their potential activity against these mosquito vectors. About 10 species of mosquitoes that can harbor protozoan parasites belonging to the genus *Ascogregarina* (Apicomplexa: Ascogregarinidae) have been identified[3]. These parasites have been suggested for biological control, especially of *Ae. aegypti* and *Ae. albopictus*, which are the natural hosts of *Ascogregarina culicis* and *Ascogregarina taiwanensis*, respectively[4].

According to Tseng[5], the infection of mosquitoes by *Ascogregarina* species occurs when insects in the first larval stages ingest their oocysts containing sporozoites, present in mosquito breeding sites. Inside the mid gut of the larvae, these sporozoites are released and penetrate the epithelial cells of the host and develop into trophozoites. Prior to the metamorphosis of larvae to pupae, the trophozoites migrate to the Malpighian tubules, where they transform into macro or microgamete. The residual trophozoites in the midgut of pupae are apoptosed. During the pupal stage, two gametes fuse to form a gametocyte, in which hundreds of oocysts are formed. These oocysts are then released into the breeding grounds during the metamorphosis of pupae to adults, and also when the females lay their eggs. They can also be released from the adult through defecation or after death.

Studies on the distribution, morphology, pathogenicity, and biology of *Ascogregarina* spp. are very important as they can be useful for biological control of insects[3]. However, these parasites have been poorly studied in South America. As of date, *Ae. aegypti* was found to be infected with *A. culicis* in Argentina[6,7]. In Brazil, Passos and Tadei[8] described the only encounter of *Ae. aegypti* and *Ae. albopictus* infected with *A. culicis* and *A. taiwanensis*, respectively, in Manaus.

In a mosquito survey in Santa Catarina, South Brazil, we collected *Ae. aegypti* and *Ae. albopictus* infected with a protozoan belonging to *Ascogregarina*. This is the first report on *Ae. aegypti* and *Ae. albopictus* infected with *Ascogregarina* species from a subtropical region of Brazil.

The surveys were conducted in four municipalities, Tubarão (28°28′00″S–49°00′25″W), Gravatal (28°19′52″S–49°02'07"W), Laguna (28°28′57″S–48°46′51″W), and Capivari de Baixo (28°26′41″S–48°57′28″W), with subtropical climate and average annual temperature ranging from 15.5 to 23.6°C[9].

From 2011 to 2013, during the summer, automobile tires were placed in the above-mentioned localities to collect

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immature larvae of *Ae. albopictus* and *Ae. aegypti*. The larvae were identified based on the comb-scales of the eighth abdominal segment and were brought to the laboratory to assess the ascogregarine infection.

The aim of this study was to verify whether the *Aedes* species could be infected with the parasite. For this purpose, 20% of third and/or fourth instars of larvae (100 larvae per species) from each municipality were dissected in the laboratory under a stereoscopic microscope (OLYMPUS CX31-P). To confirm the presence of *Ascogregarina* sp., the entire digestive tracts of the larvae were removed. The most effective method involved the application of pressure between the cephalic and thoracic parts of the body and of a counterforce on the respiratory siphon. Once the intact intestine of larva was removed, an accurate search was made to confirm the presence of trophozoites in the lumen, epithelium, and peritrophic membrane. When the presence of these parasites was confirmed in the larvae, they were photographed and/or filmed using an optical microscope (ZEISS STEMI 200 C) with VMS3.5 program.

After confirming *Ascogregarina* infection in *Ae. aegypti* and *Ae. albopictus* larvae, genomic DNA was extracted from these protozoans using a slightly modified *salting-out* protocol of Morales[10]. Briefly, a pool of 10 dissected intestines from each *Ae. aegypti* and/or *Ae. albopictus* larvae was placed separately in 1.5ml tubes. The digestive tracts were then macerated in 60µl buffer (0.16M NaCl, 0.06M sucrose, 0.5% Ethylenediaminetetraacetic acid (EDTA), 0.1M Sodium dodecyl sulfate (SDS), Tris-HCl, pH 8.6) and incubated at 65°C, for 30 min. Subsequently, 40µl of 8 M potassium acetate was added, and the homogenate was incubated at 4°C for 30 min. The solution was then centrifuged at 12,000rpm for 10 min. The supernatant was transferred to a new 1.5ml tube containing 100µl of absolute ethanol and centrifuged for 10 min at 12,000rpm. The DNA was washed with 70% ethanol and resuspended in 50µl of sterile distilled water.

The PCR amplification was performed as described by Morales[10], who developed primers, namely AT and AC, to specifically distinguish *A. taiwanensis* from *A. culicis*, respectively. A universal primer (AU) was also used. The primer AT (5′-GAG AAG CCG TCG TCA ATA CAG C-3′) binds to the ITS2 region of rDNA and the primer AC (5′-CAC TTA GTG TTT TGT TTG ATG TC-3′) binds to the ITS1 region of rDNA. The primer AU (5′-ACC GCC CGT CCG TTC AAT CG-3′) binds to the 18S DNA of both the species of *Ascogregarina*. Each PCR was performed in a total volume of 25µl containing 2µl of genomic DNA from the dissected intestine of *Ae. albopictus* or *Ae. aegypti*, 8.5µl of nuclease-free water (Promega), 125µl of GoTaq® Green Master mix (Promega), and 1µl each of 2mM primers AC or AT and AU. The PCR included an initial denaturation at 94°C for 1 min, followed by 30 amplification cycles (94°C for 1 min, 50°C for 1 min, 72°C for 2 min) and a final extension step at 72°C for 10 min. To avoid any false positive or negative results, the PCR was performed thrice on different days.

The amplified fragments were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under UV light and photographed. After the confirmation of *Ascogregarina* sp., the DNA products were purified using QIAquick® extraction kit (QIAGEN GmbH, Hilden, Germany), according to the recommendations of the manufacturer. The genomic fragments were sent to Ludwig Biotec (Alvorada – Rio Grande do Sul) for sequencing on a MegaBace 1000 automated sequencer.

In the surveys of 2011 and 2012, only the *Ae. albopictus* larvae were found in the four cities that were included in the study. The immature larvae of *Ae. aegypti* were found only in Tubarão in 2013, coexisting with *Ae. albopictus*. The larvae of *Ae. albopictus* were found to be infected with *Ascogregarina taiwanensis* (**Figure 1**), in the four municipalities. The larvae of *Ae. aegypti* (**Figure 2**) collected from Tubarão were also infected with this parasite species.

All the trophozoites of *A. taiwanensis* detected in *Ae. albopictus* and *Ae. aegypti* larvae were, in general, morphologically similar to a *comma* and had a brown pigment when observed under a phase contrast microscope. The trophozoites differed in size, ranging from 50 to 170µm. Additionally, giant trophozoites (223, 337, and 621-µm in length) were sometimes found in the midgut of *Ae. albopictus* larvae. The trophozoites, matured extracellularly, were found migrating through or in the Malpighian tubules, in some larvae.

We used molecular techniques to avoid ambiguity or error in the identification based only on the morphology of gregarine. Using PCR, we confirmed *Ae. albopictus* harboring *A. taiwanensis* in the four cities, as well as *Ae. aegypti* in Tubarão. No amplification was obtained from the DNA of *A. culicis* (**Figure 3**). The 275- and 212-bp sequences of the amplified products from two *A. taiwanensis* samples obtained from *Ae. aegypti* and *Ae. albopictus* were aligned with the sequences present in NCBI using BLAST, revealing an identity of 97% for *Ae. aegypti* and 98% for *Ae. albopictus*.

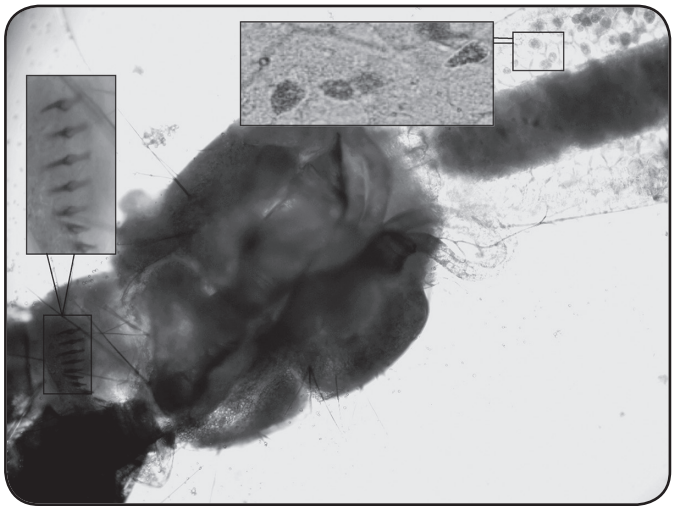

FIGURE 1 - Larvae of *Aedes albopictus* infected with *Ascogregarina taiwanensis*. The comb-scales on the eighth abdominal segment and the trophozoites inside the mid gut are evident. The larvae were dissected under a stereomicroscope.

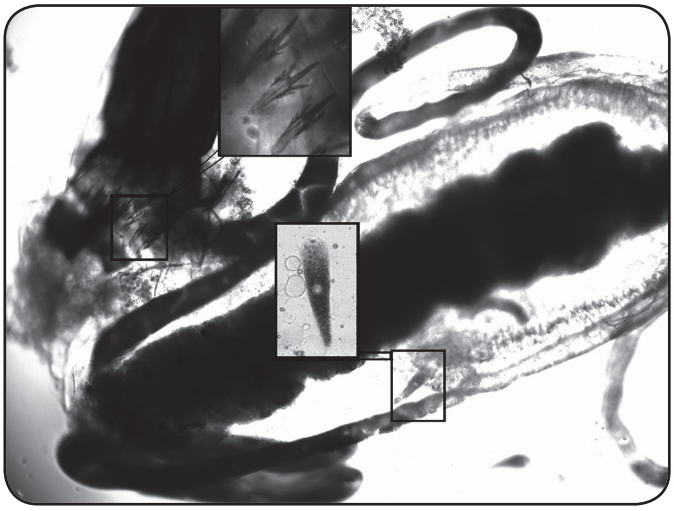

FIGURE 2 - Larvae of *Aedes aegypti* infected with *Ascogregarina taiwanensis.* The comb-scales on the eighth abdominal segment and trophozoites inside the mid gut are evident. The larvae were dissected under a stereomicroscope.

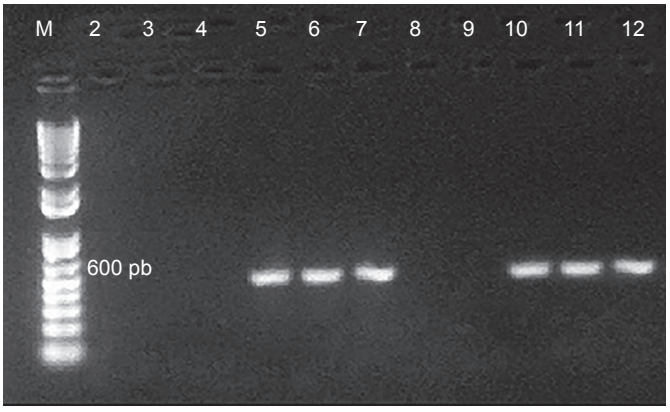

FIGURE 3 - Visualization of the PCR amplified products on 2% agarose gel. The primers AC and AT were used for the identification of *Ascogregarina culicis* and *Ascogregarina taiwanensis*, respectively. Lane M: 100-bp ladder; **Lane 2:** negative control (water); **Lane 3:** *Aedes albopictus* negative (AC); **Lane 4:** *Aedes aegypti* negative (AC); **Lanes 5-7:** *Aedes albopictus* positive for *Ascogregarina taiwanensis* (AT)**; Lane 8:** *Aedes aegypti* negative (AT); **Lane 9:** *Aedes albopictus* negative (AT); **Lanes 10-12:** *Aedes aegypti* positive for *Ascogregarina taiwanensis* (AT). **PCR**: Polymerase chain reaction; **AC:** (5′-CAC TTA GTG TTT TGT TTG ATG TC-3′); **AT:** (5′-GAG AAG CCG TCG TCA ATA CAG C-3′).

Our observations regarding the morphological characterization of *Ascogregarina* were similar to those of Reyes-Villanueva et al.[11] who also used these methods for identification of *A. culicis* and *A. taiwanensis* obtained from *Ae. aegypti* and *Ae. albopictus*, respectively, collected from Tampa, Florida, USA. However, Morales et al.[10] reported that morphological differentiation may be involved in the process of desiccation of insects. The larvae collected in the field could have ingested oocysts of both the gregarine species at different times during the larval development, resulting in variations in the ages of gamonts and trophozoites in the samples.

Based on the morphological characters, Passos and Tadei[8] found that *Ae. aegypti* were infected with *A. culicis* and *Ae. albopictus* were infected with *A. taiwanensis* in the same breeding place, in the Amazon region of Brazil. However, according to Blackmore[4], in studies related to competition between *Ae. aegypti* and *Ae. albopictus* for breeding places, an accurate identification of the gregarines is important for better estimation of the prevalence of infection among these mosquito species.

We found some bigger trophozoites than those reported by Albicocco and Vezzani[7] (148.35-217.58µm) in the midgut and Malpighian tubules of *Ae. aegypti*, infected with *A. culicis*. Chen and Yang[12] demonstrated that the size of *A. taiwanensis* trophozoites depends on water temperature. In the mosquito larvae collected in this study, we expected to find bigger trophozoites than those reported by these authors.

We also observed trophozoites, matured extracellularly, migrating through, or in the Malpighian tubules. According to Chen and Fan-Chiang[13], migration toward these tubules is normally unidirectional and usually occurs among trophozoites that are liberated in the midgut of early pupae.

Desportes[3] described that the gregarines, *A. culicis* and *A. taiwanensis*, are host-specific parasites of *Ae. aegypti* and *Ae. albopictus*, respectively. Further, Albicocco and Vezzani[7] presumed that only *A. culicis* is found infecting *Ae. aegypti*, worldwide. However, we found a possible cross-infection between *Ascogregarina* and *Aedes*. We confirmed *Ae. albopictus*, as well as *Ae. aegypti*, harboring *A. taiwanensis*, using PCR and DNA sequencing. This species of *Ascogregarina* has been also found in other mosquito species, such as *Aedes epactius* and *Culex restuans*[14]. Vezzani and Wisnivesky[14] described that gregarine infection in a non-natural mosquito host could be harmful. However, some studies have reported that *A. taiwanensis* can infect *Ae. aegypti* and in some circumstances can cause high mortality, whereas *A. culicis* is not pathogenic to *Ae. albopictus*[4,11,14]. Therefore, additional studies must be conducted on *Ae. aegypti* harboring this parasite, once it is considered a non-natural host.

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Conflict of interests

The authors declare that there is no conflict of interest.

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