Short Communication

Microsporidium Infecting *Anopheles superpictus* (Diptera: Culicidae) Larvae

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Abstract

Background: Microsporidia are known to infect a wide variety of animals including mosquitoes (Diptera: Culicidae). In a recent study on the mosquito fauna of Chahar Mahal and Bakhtiari Province, at the central western part of Iran, a few larvae of *Anopheles superpictus* were infected with a microsporidium-resembled microorganism. Current investigation deals with the identification of the responsible microorganism at the genus level.
Methods: Fresh infected larvae were collected from the field. After determining the species identity they were dissected to extract their infective contents. Wet preparations were checked for general appearance and the size of the pathogenic microorganism. Fixed preparations were stained with Geimsa and Ryan-Blue modified Trichrome techniques to visualize further morphological characters. The obtained light microscopy data were used in the identification process.
Results: The infected larvae were bulged by a whitish material filling the involved segments corresponding to a microsporidium infection. Bottle-shaped semioval spores ranged 4.33±0.19×2.67±0.12 and 4.18±0.43×2.45±0.33 micron in wet and fixed preparations, respectively. They were mostly arranged in globular structures comprised of 8 spores. These data was in favor of a species from the genus *Parathelohania* in the family Ambliosporidae.
Conclusion: This is the first report of a microsporidium infection in *An. superpictus*. The causative agent is diagnosed as a member of the genus *Parathelohania*. Further identification down to the species level needs to determine its ultrastructural characteristics and the comparative analysis of ss rRNA sequence data. It is also necessary to understand the detail of the components of the transmission cycle.

Keywords: Microsporidia, Mosquito, *Parathelohania*, *Anopheles superpictus*, Iran

Introduction

Mosquitoes are of major health concern not only because of their irrititating bites but also because of the capability to transmit life-threatening diseases to human being and his livestock (Tolle 2009). While this is so, the effective control of mosquitoes is still a far-reaching dream (Ranson et al. 2010). Apart from the resistance problem, one of the main obstacles is the scarcity of control measures, which are specific and also safe to use (Kamareddine 2012). In this context, the discovery of a new event on the natural enemies of mosquitoes may be a promising occurrence in the field of finding environmental-friendly control measures to overcome these troublesome insects.

Microsporidia are single celled intracellular eukaryotic fungi parasitizing all groups of animals (Corradi et al. 2009, Wittner et al. 1999). To date more than 1300 species of these microorganisms have been described in the literature (Vávra et al. 2013). While 14 species are known to infect humans, at least 150 more species have been recognized to parasitize 14 genera of mosquitoes (Canning 2001, Andreadis 2007). Based on these evi-

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dences, it has been stated that all of the mosquito species could be possibly the host of at least one microsporidium agent (Andreadis 2007).

Microsporidia are transmitted horizontally and vertically between and within hosts by means of a specific form called spore. The spores are very small and characterized by their unique invading behavior and the exclusive ultrastructure (Wittner et al. 1999). While the monomorphic forms (eg *Anncaliia* and *Vavraia*) produce only one spore type, the polymorphic ones (eg *Amblyospora* and *Parathelohania*) have a complex life cycle and produce different types of spores. The latter are generally more host specific and commonly there is an obligatory intermediate copepod host in their life cycle (Becnel et al. 2005).

Microsporidia can be recognized by light microscopy techniques such as Geimsa and different versions of Trichrome staining (Garcia 2002). Further verification can be achieved by transmission electron microscopy (Wittner et al. 1999). The molecular information on the ss rRNA sequence is an asset and facilitates phylogenetic analysis (Andreadis 2007).

Anopheles superpictus Grassi (Diptera: Culicidae) is a major malaria vector in Asia (Zahar 1974). It has been incriminated in the stable malaria as its longevity frequently fits for the completion of the parasite sporogonic cycle (Macdonald 1957). This mosquito is also known to transmit malaria in Iran with a widespread distribution (Saebi 1987, Edrissian 2006).

Recently, in a study on the mosquito fauna of Chahar Mahal and Bakhtiari Province, at the central western part of Iran, a few lactophenol-preserved larvae of *An. superpictus* were found disfigured by the swelling of their thorax and/or first abdominal segments (Fig. 1). Close observation showed that the multiple packets of a tiny microorganism have been infiltrated in the affected areas.

This picture was suggestive of a microsporidium infection (Andreadis 2007). Thus, a preliminary study was conducted to identify the responsible microorganism at the genus level.

Materials and Methods

A few trips were arranged to collect fresh specimens from the field. The locality was the same area in Kiar district of Chahar Mahal and Bakhtiari Province where the infected larvae of *An. superpictus* were previously discovered in 2012. Anopheline larvae were collected with the aid of a dipper and an eyedropper based on their surface swimming behavior. Close observation of the larvae in a small black pan was used to detect the whitish discoloration of body segments as the primary sign of microsporidium infection.

In laboratory, larval identities were checked with the aid of a valid local taxonomic key (Azari-Hamidian and Harbach 2009). The infected larvae were observed under a low power stereomicroscope and dissected to remove their infective contents. Wet preparations were prepared by adding a small sample from the extracted tissues to a drop of tap water. Simple pressure with the tip of a pencil was applied on the coverslip to test if it is possible to extrude polar tube. Other preparations were prepared by the gentle spreading of the infective material on microscope slides. The air-dried specimens were fixed in absolute methanol for 10 minutes. For Geimsa staining 1:14 v/v concentrate solution was buffered at 7.41 by distilled water and used for 20 minutes. For Trichrome staining the modified procedure previously introduced by Ryan et al. 1993) was followed. All of the preparations were viewed under the 1000-x power of a conventional compound microscope. Spore measurements were performed at random for 50 spores in both wet and fixed preparations.

The morphological characters proposed by Andreadis (2007) and Hazard and Anthony (1974) were used to identify the microsporidium genus.

Results

The infected larvae were recollected from the same locality previously discovered in Chahar Mahal and Bakhtiari in 2012 (Fig. 2). In a typical larva the first 3 abdominal segments was the primary site of infection. At times, the thorax was also affected. The involvement of the whole abdomen was relatively rare. The infected larvae were sluggish and tended to remain more silent at the bottom of the pan once they were physically stimulated by an eyedropper.

Close observation of the involved segments under a stereomicroscope showed that they were filled with the globular masses of a fine opaque material indicative of a microsporidium infection (Fig. 3). In wet preparations, the *in situ* stirring spores ranged 4.33±0.19×2.67±0.12 micron. They were seemed semioval and dually refractive (Fig. 4). The posterior, transparent and larger half had a rounder border. The anterior, smaller and opaque half was seemed to be a bit blunt and composed of heterogenous components with a small projection into the aforementioned section. This was conferring a bottle shape appearance to the spores. The refractive surrounding border was in favor of the presence of a tick wall around the body.

In the stained specimens, spores were ranged 4.18±0.43×2.45±0.33 micron. The blurred internal structures were a little apparent (Fig. 5). The posterior and anterior light spots could be attributed to the posterior vacuole and anterior sporoplast, respectively. The spores were frequently arranged in spherical aggregates comprised of 8 spores (Fig. 6). Immature spheres were blue tint containing multiple purple nuclei.

Simple pressure applied on wet preparations was not able to extrude the polar filaments of spores.

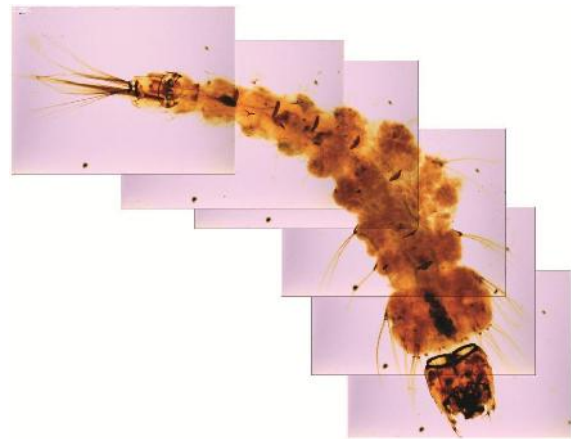Overall, these features suggested the presence of a microsporidium infection from the genus *Parathelohania*.

Fig. 1. Apparent infection the thorax and first 3 abdominal segments in a lactophenol-preserved larva of *Anopheles superpictus*

Fig. 2. One of the typical breeding places in which the infected *Anopheles superpictus* larvae were collected
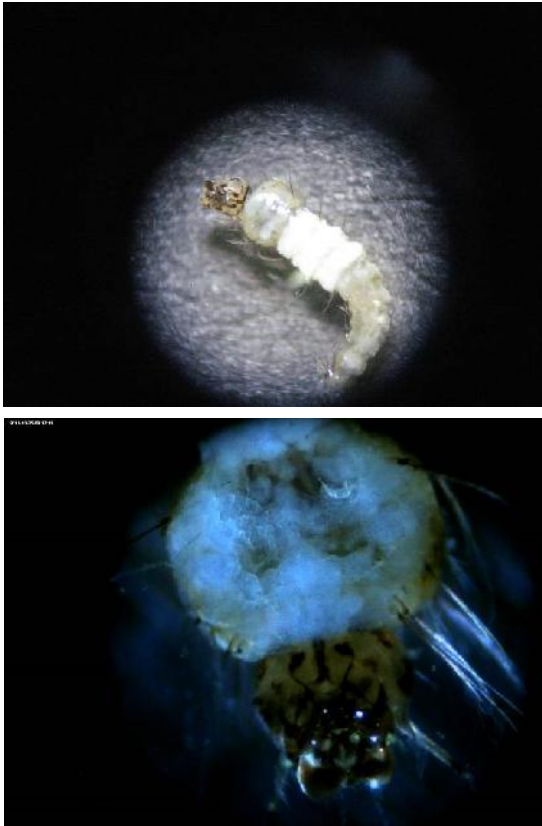
Fig. 3. Infected 4th instar larvae of *Anopheles super-pictus*. Heavy infection of first 3 abdominal segments (Above) Multiple globular masses of the infective material in thorax (Below)
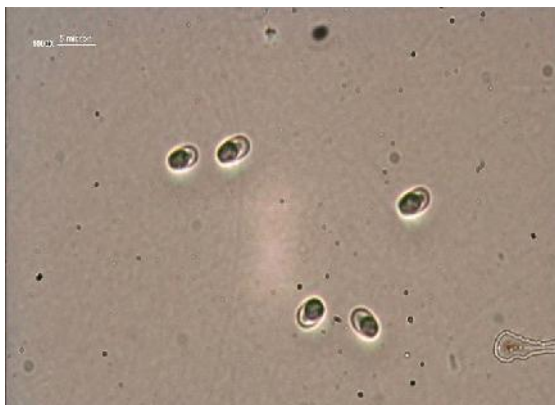

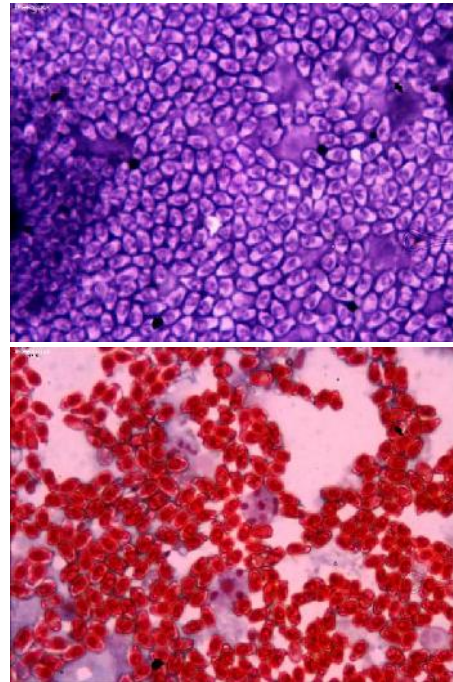**Fig. 4.** Microsporidia in wet preparations from infected *Anopheles superpictus* larva

Fig. 5. Geimsa (Above) and Ryan-Blue modified Trchrome staining (Below) of the microsporidia in *Anopheles superpictus* larva
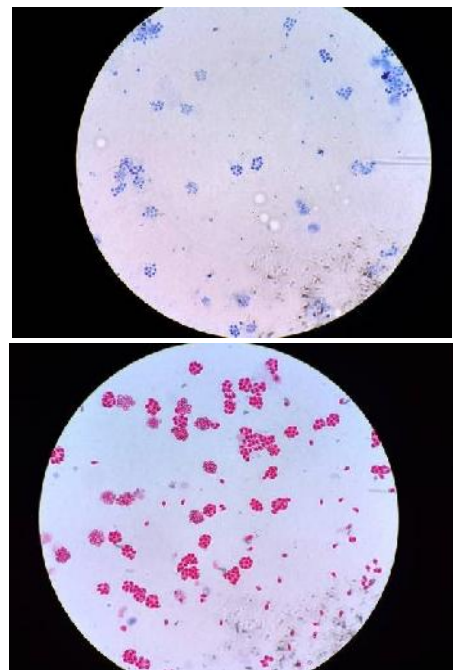

Fig. 6. Typical clusters of *Parathelohania* sp spores in *Anopheles superpictus* larva. (Above) Geimsa and (Below) Ryan-Blue modified Trichrome stained specimens

Discussion

This study indicates that the detected infection in the *An. superpictus* larva has been caused by a microsporidium species from the genus *Parathelohania*. To our knowledge, this is the first report of microsporidium infection in *An. superpictus*. It is also the first report of natural microsporidium infection in a mosquito species from of Iran.

Parathelohania, with at least 22 species, is the second largest polytypic genus next to *Amblyospora* in the family Amblyosporidae (Andreadis 2007). This genus which was firstly described by Codreanu in 1966, redescribed later by Anthony and Hazard in 1974 (Hazard and Anthony 1974). The type species is *Parathelohania legeri* (Synonym: *Thelohania legeri*) which was discovered in *Anopheles maculipennis* Meigen sl about 110 years ago (Andreadis 2007). The latest described species, *P. tomski*, *P. divulgata*, *P. sibirika*, *P. formosa* and *P. teguldeti* have been reported by Simakova and Pankova from Russia in 2004 (Simakova and Pankova 2004). All of these microsporidia were isolated from Anopheline mosquitoes. In reality, Anopheline mosquitoes constitute the principal hosts for the genus *Parathelohania* (Hazard and Anthony 1974). *Parathelohania chagrasensis* and *P. barra* are the only species described from *Aedomyia* and *Ochlerotatus* mosquitoes (Pillai 1968, Hazard and Oldacre 1976). Vertical and horizontal transmission is a common feature in *Parathelohania* and normally there is an intervening obligatory copepod host in the life cycle (Becnel et al. 2005).

In the infected larvae, the first 3 abdominal segments were primarily involved (Fig. 3). This region corresponds with the middle part of the midgut, which is highly alkaline. A series of digestive enzymes are maximally active in this portion of the gut (Clements 1992). Perhaps, from here the germinated spores access to their target cells in the fat tissues and progressively extend to invade other areas. While the severe overgrowth of spores kills male larvae, the infection in females is less pronounced. These larvae transmit their infection to the adulthood during which the vertical transmission will occur (Andreadis 2007).

The extrusion of the polar tube is a reliable sign of a microsporidium spore (Wittner and Weiss 1999). In this study, the application of simple mechanical pressure was not able to extrude the polar tube. This might be regarded as an exception to the general recommendation proposed by Andreadis (Andreadis 2007). Therefore, the chemical stimulation of the spores should be considered in future attempts.

Geimsa staining is a routine technique in the diagnosis of microsporidia infection in mosquitoes (Andreadis 2007). In the present study, with this method the central structures of the spores were deeply stained (Fig. 5). However, a better shadow of the internal structures was obtained by Ryan-Blue modified Trichrome technique (Fig. 5). At times, it was possible to detect the position of posterior vacuole and/or anterior poloroplast as one or two light spots in the stained spores. Since the introduction of the Weber-Green Trichrome staining technique, various modifications are proposed by different authors (Garcia 2002). While a few have addressed the quality of the visualization of the morphological characters, the others have tried to improve the practical items, or both. This study suggests that the Trichrome staining might be considered as a preferred technique in those situations in which more details of the spores are aimed at.

In the present study, the size of spores overlaps with the spores of *P. anophelis*, *P. evansae*, *P. periculosa*, *P. indica*, *P. tomski*, *P. divulgata* and *P. formosa* (Hazard and Anthony 1974, Garcia and Becnel 1994,

Simakova and Pankova 2004). While the first 3 species have been discovered in the Nearctic region, the remainder has been described from the Palearctic region. It is clear that the spore size cannot be used as a reliable method for species differentiation. Instead, with the aid of electron microscopy techniques, comparisons that are more accurate would be possible. In some occasions, the definitive identification of a microsporidium species needs a good knowledge on its ecological attributes and the ultrastructural detail of developmental stages in the hosts (Andreadis 2007). On the other hand, molecular information on the ss rRNA sequence is a valuable tool in the phylogenetic analysis of the microsporidium species (Vossbrinck et al. 2005).

The checklist of the Culicidae of Iran contains the name of 64 species (Azari-Hamidian 2007). A number of these mosquitoes might be considered as the potential host for microsporidia infection. This is because there is at least one report of natural microsporidium infection in these species from elsewhere (Castillo 1980). These mosquitoes include *An. hyrcanus* (Pallas), *An. maculipennis* Meigen s.l, *An. subpictus* Grassi s.l and *An. messeae* Falleroni, *Ochlerotatus caspius* (Pallas) s.l, *Aedes vexans* (Meigen), *Culex modestus* Ficalbi, *Cx. pipiens* Linnaeus, *Cx. quinquefasciatus* Say, *Cx. territans* Walker and *Cx. theileri* Theobald. The vital consequences of artificial infection with *Anncaliia* algerae (Synonym: *Nosema algerae*, Synonym: *Brachiola algerae*) on *An. stephensi* Liston has been explored in laboratory, as well (Haq et al. 1981). In the past, at least two extensive countrywide mosquito larval surveys have been performed in Iran (Saebi 1987, Zaim 1987). Although there is no report of microsporidium infection in these studies, it is still possible that new cases of microsporidium infection could be found with more intensive larval surveys.

There are few reports, which declare that some microsporidia species are connected with human infections (Cheney et al. 2000, Coyle et al. 2004). However, the research on the application of these microorganisms in the biological control of mosquitoes is going on (Solter and Becnel 2007). Mathematical modeling is also in favor of their effectiveness in the control of mosquitoes (Koella et al. 2009). Recent efforts have been focused on species with polymorphic development and maximum phylogenetic distance to human parasitizing microsporidia (Andreadis 2007). Although *Parathelohania* is a genus with average characters, the possibility of the utilization of these pathogens in the control of specific mosquitoes cannot be completely excluded.

Conclusion

The first report of *An. superpictus* larval infection with a microsporidium species from the genus *Parathelohania* is presented here. Diagnosis was made based on gross morphological characters in light microscopy. Although the definitive diagnosis of the species needs specific ultrastructural and molecular data, current ecological attributes predicts that it is likely a new microsporidium species.

Acknowledgements

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