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Isolation and identification of pathogenic *Acanthamoeba* species from air conditioning systems, Egypt

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**Running title:** Pathogenic *Acanthamoeba* in air conditioners.

**Key words:** *Acanthamoeba*; Dust; Culture; PCR; Histopathology
**Summary:** *Acanthamoeba* species are free-living amoebae causing granulomatous amoebic encephalitis and keratitis. This study aimed to identify *Acanthamoeba* isolated from air conditioning systems by culture method and Polymerase Chain Reaction (PCR) and testing its pathogenicity by thermo-tolerance and animal inoculation. Out of the 80 dust samples, 41 (51.25%) were found to be positive as *Acanthamoeba* spp. by culture method. These samples were confirmed positive by PCR. Regarding pathogenicity, out of 41 samples, 27 (65.9%) were thermo-tolerant and 16 (39%) samples could infect mice and caused histopathological effects. Highly pathogenic *Acanthamoeba* isolates were characterized by a thermo-tolerance ability and disseminated in all mice organs after infection causing early mice death. This work confirmed that the presence of pathogenic isolates of *Acanthamoeba* potentially infectious to humans in air-conditioners.

**Introduction**

Free-living amoebae (FLA) are very frequent protozoa in the environment as well as artificial samples. *Acanthamoeba* is the most abundant genera of FLA (1). *Acanthamoeba* causes granulomatous amoebic encephalitis (GAE) and *Acanthamoeba* keratitis (AK) (2). GAE is a central nervous system infection with about 204 cases estimated worldwide (3).
GAE affect mainly immune-compromised individuals and are frequently fatal (2). On the other hand, AK is a corneal infection occurring in normal immunological individuals (4).

There are about 24 species of *Acanthamoeba* morphologically distinguishable. They are divided according to cyst characteristics into three different groups (I, II, III). Group (I) are nonpathogenic, while group (II) contain many pathogenic species including *A. polyphaga*, *A. castellanii*, *A. hatchetti* and *A. rhysodes*. On the other hand, group (III) contain only one pathogenic species which is *A. culbertsoni* (5). Most GAE cases are mainly due to infection with *A. castellani* and *A. colbertessoni* while AK resulted from different spp., particularly *A. castellani* and *A. palyphaga* (5).

Although morphology is still used in the identification of isolates, a molecular technique focusing on the sequences of small subunit nuclear 18S rRNA genes are currently the main tool for taxonomic characterization of *Acanthamoeba*. As genotyping alone does not provide sufficient indication of the pathogenicity of an isolate. physiological properties, such as thermo-tolerance and infectivity to mice is also tested.

Nowadays, air-conditioners are considered the most important devices as a modern lifestyle level in hot weather countries. They are the principle
for relaxation. Also, they are icons of development. Moreover, air conditioning systems are the source of air-borne diseases caused by bacterial, viral, etc. (6). *Acanthamoeba* spp. have been isolated from air-conditioning units in many regions such as Malaysia (9), Iran (14,15), Australia (16) and Egypt (17). Therefore, the present study aimed to examine *Acanthamoeba* in dust samples obtained from different air-conditioning systems of many buildings in Egypt with respect to their pathogenicity.

**Materials and Methods**

**Samples collection and study type:**

A cross-sectional study was performed during the period from January to December 2018, where 80 air-conditioners dust samples were collected from buildings of four different places in Minia Governorate, Egypt as follow: (a) household in Bani Mazar, Maghagha, Matay and Samalot, districts of Minia City, (b) Parasitology, Microbiology and Public Health Departments, Faculty of Medicine, Minia University, (c) Mills Company in New Minia city and (d) Internal Medicine and Surgery Departments, Minia university hospital. Samples were collected by using sterile cotton swabs from frameworks and filters of different unit air-conditioners. Samples were labeled with sampling details of date, time and place of collection. All
collected samples were examined on the same day in the Parasitology Department, Faculty of Medicine, Minia University, Egypt.

**Ethical consideration:**

The study was started after being approved by the Ethical Committee of Scientific Research, Faculty of Medicine, Minia University, Egypt.

**Culture method:**

One gram of dust sample was inoculated on the center of non-nutrient agar (NNA) seeded with *Escherichia coli* (*E. coli*). The NNA media was prepared with 1.5% bacteriological agar in Page’s amoeba saline (PAS) (120 mg NaCl, 136 mg KH₂PO₄, 142 mg Na₂HPO₄, 4 mg CaCl₂.2H₂O, 4 mg MgSO₄.7H₂O and 1 L distilled water). All cultures were maintained at 28 °C. Plates were observed daily up to two weeks for *Acanthamoeba* growth (7).

Samples were considered positive when feeding tracks on NNA media were visible and the surface of the agar was examined using traditional microscopy with a 100x objective. If no tracks are visible, plates were examined daily for up to another 14 days before reporting the sample as a negative one.

**Morphological identification:**
Morphological identification under light microscopy with a 100x objective, was based on the shape and size of the cysts and features of the endocyst and ectocyst according to morphotyping keys by Page (8). Since polymorphism of cyst shapes, sizes, number of arms and shape of the wall could be observed within an *Acanthamoeba* isolate, all isolates were characterized based on the average cyst morphology within the respective isolate. It was observed that isolates had cyst sizes \( \leq 18 \text{ mm} \) were identified as either Group II or Group III *Acanthamoeba* (9).

**PCR:**

**DNA extraction and PCR amplification:**

Cysts from individual *Acanthamoeba* isolates were harvested by sterile PAS, from the surface of amoeba culture plates. After washing in PAS, centrifugation at 3000 rpm for 10 minutes was done. The pellet was resuspended in PAS and the suspension was centrifuged at 15,000 rpm for 15 min (10). The pellet was stored at \(-20\) °C for DNA extraction using the QIAamp DNA Mini Kit (Cat. no. 40724, Qiagen, Hilden, Germany, 50 reactions). To amplify the DF3 region of 18S rRNA (rDNA), specific primers JDP1 (forward: 5’ GGC CCA GAT CGT TTA CCG TGA A 3’) and JDP2 (reverse: 5’ TCT CAC AAG CTG CTA GGG AGT CA 3’) were used (11). Amplification reactions were set for a total volume of 25 μl, containing
12.5 μl Ampliqon (Taq DNA Polymerase Master Mix RED, Denmark), 1 μl forward and reverse primers (10 pmol), 3 μl DNA templates, and 8.5 μl double-distilled water. PCR amplification was performed with a primary denaturing step at 94 °C for 1 min, after 35 cycles at 94 °C for 35 s, the annealing step was 56 °C for 45 s, and 72 °C for 1 min. After this process, the final extension was performed at 72 °C for 5 min.

**Gel electrophoresis:**

Electrophoresis of PCR products was visualized using agarose gel electrophoresis on the 1/5% agarose gel (Biolab, UK, Cat no. Bio-41026, Lot. no. ES520-B025720) stained with ethidium bromide (10 mg/ml in deionized H2O). Positive samples gave bands of about 500 base pairs on the agarose gel. Negative samples had no bands.

**Thermo-tolerance test:**

*Acanthamoeba* isolates were incubated at 30 °C, 40 °C, and 50 °C on bacteria-coated NNA. The area occupied by the trophozoites and cysts, starting from the inoculum area, was recorded every 24 h until 2 weeks (7).

**Animal inoculation:**

This study was conducted on 80 male Balb/c laboratory bred mice (6-weeks old). The mice (n=60) were divided into 4 groups:
- Group A (n=27): immunocompetent mice infected with *Acanthamoeba* spp.

- Group B (n=27): immunosuppressed mice infected with *Acanthamoeba* spp.

- Group C (n=13): immunocompetent non-infected mice.

- Group D (n=13): immunosuppressed non-infected mice.

Animals were housed in groups of 2-5 mice per cage. The mice were kept in controlled conventional conditions. Mice groups B and D were immunosuppressed by administering 0.22 mg (10 mg/kg) of methylprednisolone as methylprednisolone sodium succinate (MPS) in 0.1 ml of 0.9% saline intraperitoneally for 5 days before amoeba inoculation.

The animals (groups A and B) were inoculated intra-nasally with 3 µl of suspension containing 10-20 thousand amoebae. Control mice (groups C and D) were given the same volume of sterile physiological solution (3 ml of 0.9% NaCl solution). The mice in all groups were anesthetized by an overdose of pentobarbital sodium (200 mg/kg intraperitoneally). The mice were sacrificed at 14 days post *Acanthamoeba* spp. infection. During necropsy, the brains, lungs, hearts, livers and kidneys were collected for: (i) histopathology and (ii) reculturing on bacteria-coated NNA media at room
temperature to recover *Acanthamoeba* species from mice tissues as evidence of its presence (12,13).

**Histopathology:**

The tissues of different organs (brain, lung, heart, liver and kidney) were fixed in a 4% phosphate-buffered saline (PBS) buffered formalin solution, embedded in paraffin, sectioned to 8 µm thick slices, and stained with hematoxylin and eosin, a technique employed universally for routine tissue examination.

**Statistical analysis:**

The data obtained from this study were analyzed using SPSS (Version: 22). Chi-square test was used to evaluate the qualitative data. P value <0.05 was considered significant.

**Results**

Out of 80 air conditioners dust samples, 41 samples (51.25%) were found positive as *Acanthamoeba* spp. based on the culture method. Dust samples collected from university and hospital buildings had a higher level of *Acanthamoeba* contamination than those from factory and households buildings; 17 (85%) and 14 (70%) versus 6 (30%) and 4 (20%) respectively with statistical significance (P value = 0.0001) as shown in Table 1. Positive culture samples were examined microscopically with iodine stain. All
Acanthamoeba groups were found as shown in Fig. 1. All 41 (51.25%) air-conditioner samples diagnosed positive by culture method were confirmed positive by PCR using specific primers for Acanthamoeba species as shown in Table 1. Agarose gel electrophoresis showed a representative PCR product of the 18s rRNA gene of Acanthamoeba spp. as in Fig. 2.

About pathogenicity, among 41 Acanthamoeba isolates, 27 (65.9%) were thermo-tolerant, grown at 40 °C, and 16 (39%) samples could infect mice. These data were not statistically significant (P= 0.9 and 0.3 respectively). Pathogenicity of Acanthamoeba species was higher in university and hospital samples 12 (70.6%) and 9 (64.3%) respectively by thermo-tolerance tests and 8 (47.1%) and 5 (35.7%) respectively by animal inoculation. The distribution of pathogenicity according to different places is showed in Table 2.

Regarding animal inoculation, mice organs including the brain, lungs, heart, liver and kidneys were examined for the presence of Acanthamoeba and its histopathological effect. All the isolates of the pathogenic Acanthamoeba studied were re-isolated from the brain. Lung tissues were the second target to be affected then other organs including liver and kidneys were affected. The most virulent isolate had invaded all the studied organs of the infected animals. Also, the time of mice's death after the infection was
detected. Some *Acanthamoeba* isolates caused early mice death within the first week indicating higher virulence ability. Organ affections and time of death after infection of different *Acanthamoeba* isolates from the four different places were illustrated in Table 3. Histopathological effects in different organs were illustrated in Figure 3.

**Discussion**

*Acanthamoeba* species are widely distributed in the environment and have been isolated from the air, soil, water, contact lenses, and air conditioning units. Air conditioning units are very important and essential in hot climates. This could increase the risk of exposure of indoor air-pollutants such as cysts and trophozoites of *Acanthamoeba* spp. Therefore, the present study sought to detect *Acanthamoeba* spp. in air-conditioners and investigate for their pathogenic potential using thermo-tolerance tests and animal inoculation method.

In this work, the prevalence rate of *Acanthamoeba* spp. in air-conditioners was 51.3%. Our finding was complementary to the findings by Astorga et al. (18) who found that 41 (56.9%) of the 72 samples revealed *Acanthamoeba* spp. from air-conditioner samples in Santiago, Chile. On the other hand, Chan et al. (9) in Malaysia detected *Acanthamoeba* spp. in air-conditioners with a prevalence rate of 23%. Furthermore, ÖZPINAR et al.
could recover *Acanthamoeba* spp. from 4 (16.7%) out of 24 swab air-conditioner samples. Contrary, FLA could not be isolated from air-conditioning systems in a study done in Egypt (19).

In the present study, all morphologically groups of *Acanthamoeba* spp. were detected. Spherical cysts with ectocyst separated from the star-shaped endocyst with 4 to 6 conic branches with a cyst diameter of more than 18 um belong to group I were identified. Also, cysts with furrowed ectocyst and predominantly smooth endocyst with a diameter less than 18 um belong to group II and III were identified. *Acanthamoeba* group I is known to be non-pathogenic, while group II and III include several species more usually associated with clinical conditions (25).

This work showed that all 41 (51.3%) positive culture samples for *Acanthamoeba* spp., proved to be related to genus *Acanthamoeba* when they tested by PCR technique. Similar results have been reported in Egypt (20,21,23) and Spain (22). Additionally, in Malaysia, Gabriel et al. (24) showed that 64% of isolated FLA by culture method were positive as *Acanthamoeba* spp. in PCR. This indicated that the culture method is very accurate in the detection of *Acanthamoeba* spp. besides its simplicity and cheapness still, it is time-consuming.
Thermo-tolerance test was done in many studies to test pathogenicity (26,27). The advantages of the thermo-tolerance test are feasibility and specificity to pathogenic effect. Also, the thermo-tolerance test is one of the simplest tests to assess the pathogenic potential of *Acanthamoeba*, since the isolates from clinical cases are thermo-tolerance (28,29). In this study, all thermo-tolerant *Acanthamoeba* species showed growth on cultures at 40 °C temperature which could infect mice indicating that thermo-tolerance is a strong indicator for pathogenicity. However, some non-pathogenic isolates can also tolerate temperatures 37°C according to Schuster and Visvesvara (2), making the criteria of thermo-tolerance alone inconclusive to demonstrate *Acanthamoeba* pathogenicity. Costa et al. (27) reported that the thermo-tolerance could be important to provide an initial screening for the pathogenic potential and it must be associated with other parameters as animal inoculation which was done in this study.

Regarding animal inoculation as a test for virulence of *Acanthamoeba* spp., the day of mice's death gave us an indicator for the virulence of the *Acanthamoeba* species. As, the earlier of the death day of the mice, the more of the virulence of *Acanthamoeba* species. In this study, there were some isolates with thermo-tolerance to 50°C, infecting mice organs and cause the early death of the mice. This research detected highly virulent
*Acanthamoeba* isolates which might be a real threat to humans in spite of limited data concerning the distribution of AK cases in Egypt recently done by Taher et al. (30).

The animal model of nasal infection of *Acanthamoeba* spp. in this study showed an early disseminated infection of mouse with low mortality. The most virulent isolate had invaded all the studied organs of the infected animals. All the isolates of the pathogenic amoebae studied could be re-isolated from the brain as the commonest site followed by the lungs. These data matched with research done by Górnik K, Kuźna-Grygiel, who found that the brain was the most frequent site of the primary infection following intranasal inoculation (13). On the other hand, Veríssimo et al. (31) reported that the most affected site was the lung tissue. The principal invasion route of *Acanthamoeba* is the nose, where the amoebae cross the cribriform plate and following the olfactory nerves to reach the brain (32). The difference in *Acanthamoeba* tissue invasion may be due to the change of virulence between isolates or even the same isolate and the expression of different virulence factors after the first infection.

**Conclusions:**
The current findings serve as additional proof for the presence of pathogenic *Acanthamoeba* species in the air conditioners system which could make a risk for human health. Thus, awareness in clinicians should be raised. Also, it highlights the importance of surveying air conditioner systems in Egypt. Thermo-tolerance at higher temperatures is a reliable test for pathogenicity with animal inoculation. Further pathogenic testing and genotyping studies may be needed.

**Conflict of interest**

None to declare

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Figure legends

**Fig. 1:** Light microscopy showing different morphological groups of *Acanthamoeba* cysts (I, II, III) by iodine wet mount stain (x100)

Bars represent 20 µm

a. Large sized cyst (>18 um) and wrinkled endocyst forming 4 or 5 arms belonged to group I.

b, c, d, Small sized cysts (<18 um) with wrinkled encocyst and ectocyst (b,c) or wrinked ectocyst only (d) belonged to group II and III.

**Fig. 2:** Agarose gel electrophoresis showing representative PCR product of the 18s rRNA gene of *Acanthamoeba* spp.

L: DNA molecular weight marker (100 bp)

1,2,3,4,5,6,7,8: positive genomic DNA

C: Genomic DNA from *Acanthamoeba castellanii* as a positive control

**Fig. 3** Histopathological effect of *Acanthamoeba* infection on different organs of mice by light microscopy of hematoxylin and eosin stained sections (x 400)

a. Brain section illustrated *Acanthamoeba* cysts marked by black arrows.
b. kidney section illustrated the degeneration of renal tubules in the form of cloudy swelling with interstitial mild chronic inflammation with inflammatory cellular infiltrate formed mainly of lymphocytes and plasma cells.

c. Section of the heart showed partial loss of muscle fibers with focal inflammation formed of infiltration by neutrophils, histocytes and plasma cell.

d. Lung tissues showed inflammation, area of emphysema in the alveoli, hyperplasia of bronchial walls and thickening of some alveoli with an area of hemorrhage.

e. Liver tissues demonstrated minimal focal portal tract inflammation (e1), area of hemorrhage and congestion of central veins (e2).

f. Brain pathology ranged from mild inflammation and area of hemorrhage to an area of necrosis. Numerous neutrophils, macrophages, plasma cells, and giant cells could be detected (f1) to gliosis (f2).
### Table 1: Prevalence of *Acanthamoeba* spp. in different air-conditioners samples by NNA culture

|                | Household (N=20) | University (N=20) | Factory (N=20) | Hospital (N=20) | Total (N=80) | P value |
|----------------|------------------|-------------------|----------------|-----------------|--------------|---------|
| NNA culture    | 4 (20%)          | 17 (85%)          | 6 (30%)        | 14 (70%)        | 41 (51.25%)  | 0.0001* |
| PCR            | 4 (20%)          | 17 (85%)          | 6 (30%)        | 14 (70%)        | 41 (51.25%)  | 0.0001* |

* P value was calculated by using Chi-square test.

* Significant difference in between groups (P value < 0.05).
Pathogenic *Acanthamoeba* in air conditioners

Table 2: Results of thermo-assay test and animal inoculation of *Acanthamoeba* isolates from air-conditioners samples

|                     | Household (N= 4) | University (N= 17) | Factory (N= 6) | Hospital (N= 14) | Total (N= 41) | P value* |
|---------------------|------------------|--------------------|---------------|-----------------|---------------|----------|
| Thermo-assay +ve N (%) | 2 (50%)          | 12 (70.6%)         | 4 (66.7%)     | 9 (64.3%)       | 27 (65.9%)    | 0.9      |
| Animal inoculation +ve N (%) | 1 (25%)     | 8 (47.1%)         | 2 (33.3%)     | 5 (35.7%)       | 16 (39%)      | 0.3      |

* P value was calculated by using Chi-square test.

* Significant difference in between groups (P value < 0.05).
Pathogenic *Acanthamoeba* in air conditioners

Table 3: Results of thermo-tolerance and animal inoculation of all *Acanthamoeba* isolates

| Sample source | Thermo-assay +ve | Animal group | N | Animal inoculation +ve | Time of mice death after infection |
|---------------|------------------|--------------|---|------------------------|----------------------------------|
|               | 30°C 40°C 50°C   |              |   | Brain Lungs Liver Kidneys | Early death No death              |
| Household     | 2 2 1            | A            | 2 | - - - -                | 0 2                              |
| (N= 2)        |                  | B            | 2 | 1 - - -                | 1 1                              |
| University    | 12 12 8          | A            | 12| 3 2 1 1               | 1 11                             |
| (N= 12)       |                  | B            | 12| 5 2 1 1               | 1 11                             |
| Industry      | 4 4 1            | A            | 4 | - - - -                | 0 4                              |
| (N= 4)        |                  | B            | 4 | 2 1 1 1               | 1 3                              |
| Hospital      | 9 9 3            | A            | 9 | 2 1 - -                | 1 8                              |
| (N= 9)        |                  | B            | 9 | 3 3 2 1               | 2 7                              |

(A) group A of immunocompetent infected mice group

(B) group B of immunosuppressed infected mice group

(N) number of infected mice

(−) absence of infection in mice
Figures

Fig. 1: Light microscopy showing different morphological groups of *Acanthamoeba* cysts (I, II and III) by iodine wet mount stain (1000 x).
Fig. 2: Agarose gel electrophoresis showing representative PCR product of the 18s rRNA gene of *Acanthamoeba* spp.
Pathogenic Acanthamoeba in air conditioners
Fig. 3: Histopathological effects of *Acanthamoeba* infection on different organs of mice (hematoxylin and eosin staining sections under light microscopy 400x)