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Original Article

Isolation and Identification of Pathogenic *Acanthamoeba* Species from Air Conditioning Systems, Egypt

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SUMMARY: *Acanthamoeba* are free-living amoebae that cause granulomatous amoebic encephalitis and keratitis. In this study, we aimed to isolate and identify *Acanthamoeba* from air conditioning systems using *in vitro* cell culture and polymerase chain reaction assays. We also estimated the pathogenicity of the isolates by measuring their thermotolerance and studying mice models inoculated with these isolates. Of the 80 dust samples acquired, 41 (51.25%) were found to be positive for *Acanthamoeba* spp. using *in vitro* cell culture and the results were validated using PCR. Out of these 41 samples, 27 (65.9%) were thermotolerant and 16 (39%) samples could infect mice and cause histopathological effects. Highly pathogenic *Acanthamoeba* isolates were characterized by their thermotolerance and the ability to disseminate in all organs after infection, causing early death of infected animals. Our study thus validated the presence of pathogenic isolates of *Acanthamoeba* in air conditioners that may be potentially infectious to humans.

INTRODUCTION

Free-living amoebae (FLA) are protozoans that are prevalent both in the natural environment as well as in artificial samples. *Acanthamoeba* is the most abundant genus in FLA (1) and is known to cause granulomatous amoebic encephalitis (GAE) and *Acanthamoeba* keratitis (AK) (2). GAE involves an infection of the central nervous system, with approximately 204 cases estimated worldwide (3). GAE mainly affects immunocompromised individuals and is usually fatal (2). On the other hand, AK is a corneal infection occurring in individuals with a normally functioning immune system (4).

Approximately 24 species of *Acanthamoeba* have been distinguished based on morphology. They have been largely categorized according to cyst characteristics into three different groups, namely I, II, and III. Group I is nonpathogenic, whereas group II contains many pathogenic species, including *A. polyphaga*, *A. castellanii*, *A. hatchetti*, and *A. rhysodes*. On the other hand, group III contains only one pathogenic species, *A. culbertsoni* (5). Most cases of GAE are mainly due to infection with *A. castellanii* and *A. culbertsoni*, whereas AK is due to infection with different species, such as *A. castellanii* and *A. polyphaga* (5).

Although morphology is still used to identify isolates, the molecular identification technique of sequencing genes that code for the small subunit nuclear 18S rRNA is currently the main tool for taxonomic characterization of *Acanthamoeba*. However, genotyping alone does not provide sufficient evidence for the pathogenicity of an isolate, and physiological properties, such as thermotolerance and infectivity in mice, are 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. In addition, they are icons of development. However, air conditioning systems are a source of airborne diseases caused by bacteria and viruses (6). *Acanthamoeba* spp. have been isolated from air conditioning units in many countries such as Malaysia (9), Iran (14,15), Australia (16), and Egypt (17). In this study, we aimed to examine the presence and pathogenicity of *Acanthamoeba* in dust samples obtained from different air conditioning systems placed in different buildings in Egypt.

MATERIALS AND METHODS

Sample collection and study design: Our cross-sectional study was performed during the period between January and December 2018, wherein 80 dust samples were collected from air conditioners placed in buildings situated in four different places in Minia Governorate, Egypt, which are as follows: (a) households in Bani Mazar, Maghagha, Matay, and

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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 using sterile cotton swabs from the framework and filters of the air conditioner. Samples were labeled with sampling details, namely date, time, and location of collection. All samples were examined on the same day in the Parasitology Department, Faculty of Medicine, Minia University, Egypt.

Ethical considerations: The study was approved by the Ethical Committee of Scientific Research, Faculty of Medicine, Minia University, Egypt.

In vitro cell culture: One gram of each dust sample was inoculated on non-nutrient agar (NNA) seeded with *Escherichia coli*. NNA was prepared with 1.5% bacteriological agar in Page's amoeba saline (PAS), which consists of 120 mg NaCl, 136 mg KH_2PO_4, 142 mg Na_2HPO_4, 4 mg $CaCl_2.2H_2O$, and 4 mg $MgSO_4.7H_2O$ in 1 L distilled water. All cultures were maintained at 28°C. Plates were monitored daily for up to two weeks for *Acanthamoeba* growth (7).

Samples were considered positive for *Acanthamoeba* growth when upon microscopic examination (100× objective) of the agar surface, feeding tracks on the NNA were visible. If no tracks were visible, plates were monitored daily for another 14 days for *Acanthamoeba* growth before the sample was reported as negative for the same.

Morphological identification: Morphological identification, based on the shape and size of the cysts, features of the endocyst and ectocyst, and morphotyping keys by Page (8), was performed using a light microscope with a 100× objective. Since polymorphisms in cyst shape, size, number of arms, and shape of the wall were observed within an *Acanthamoeba* isolate, all isolates were characterized based on the average cyst morphology within a isolate. Isolates that had cyst sizes ≤18 µm were identified as either Group II or Group III *Acanthamoeba* (9).

DNA extraction and PCR amplification: Cysts from individual *Acanthamoeba* isolates were harvested in sterile PAS from the surface of amoeba culture plates. After washing in PAS, the suspension of PAS and cysts was centrifuged at 3,000 rpm for 10 min. The pellet was then resuspended in PAS and was further centrifuged at 15,000 rpm for 15 min (10). The resultant pellet was stored at −20°C, and DNA extraction was performed using a QIAamp DNA Mini Kit (Cat. no. 40724; Qiagen, Hilden, Germany). To amplify the DF3 region of 18S rRNA (rDNA), specific primers JDP1 (forward: 5′ GGC CCA GAT CGT TTA CCG TGA 3′) and JDP2 (reverse: 5′ TCT CAC AAG CTG CTA GGG AGT CA 3′) were used (11). Reaction volume was set at 25 µL, and contained 12.5 µL of Ampliqon (Taq DNA Polymerase Master Mix RED, Amplicon, Copenhagen, Denmark), 1 µL of forward and reverse primers (10 pmol), 3 µL of DNA template, and 8.5 µL of double-distilled water. PCR amplification was performed with a primary denaturing step at 94°C for 1 min, 35 cycles at 94°C for 35 s, annealing at 56°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

Gel electrophoresis: PCR products were electrophoresed on a 1.5% agarose gel (Biolab, London, UK, Cat no. Bio-41026, Lot. no. ES520-B025720) and visualized with ethidium bromide (10 mg/mL in deionized H_2O). Positive samples yielded bands of approximately 500 base pairs on the agarose gel. Negative samples demonstrated no bands.

Thermotolerance test: *Acanthamoeba* isolates were incubated at 30°C, 40°C, and 50°C on *E. coli*-seeded NNA. The area occupied by the trophozoites and cysts, starting from the inoculum area, was recorded every 24 h for 2 weeks (7).

Animal inoculation: Eighty male Balb/c laboratory-bred mice (6-weeks old) were divided into four groups for the animal inoculation, and these are as follows: 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; and Group D (N = 13), immunosuppressed non-infected mice.

Animals were housed in groups of 2–5 mice per cage, and were maintained under controlled conventional conditions. Mice in groups B and D were immunosuppressed by the administration of 10 mg/kg of methylprednisolone sodium succinate, prepared in 0.1 mL of 0.9% saline (amounting to 0.22 mg of methylprednisolone), intraperitoneally for 5 days before amoeba inoculation.

The animals (groups A and B) were inoculated intranasally with 3 µL of suspension containing 10,000–20,000 amoebae, whereas control mice (groups C and D) were administered the same volume of sterile physiological solution (3 mL of 0.9% NaCl solution). Fourteen days post *Acanthamoeba* spp. infection, mice in all groups were anesthetized with an overdose of pentobarbital sodium (200 mg/kg administered intraperitoneally), sacrificed, and the brain, lung, heart, liver, and kidney were collected for histopathology and re-culturing on bacteria-seeded NNA at room temperature to recover *Acanthamoeba* species from mouse 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 formalin solution, embedded in paraffin, sectioned into 8-µm thick slices, and stained with hematoxylin and eosin, which is a technique employed universally for routine tissue examination.

Statistical analysis: The data obtained in this study were analyzed using SPSS (version 22). The chi-square test was used to evaluate qualitative data. P value <0.05 was considered statistically significant.

RESULTS

Out of a total of 80 air conditioner dust samples, 41 samples (51.25%) were found to be positive for *Acanthamoeba* spp. based on *in vitro* cultures. Dust samples collected from university (17 [85%]) and hospital buildings (14 [70%]) had a statistically higher proportion of *Acanthamoeba* contamination than those from factory (6 [30%]) and household buildings (4 [20%]), (P value = 0.0001; Table 1). Positive culture samples were examined microscopically upon iodine staining, and *Acanthamoeba* amoeba were identified

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[1)]
NNA culture N (%)	4 (20)	17 (85)	6 (30)	14 (70)	41 (51.25)	0.0001[2)]
PCR N (%)	4 (20)	17 (85)	6 (30)	14 (70)	41 (51.25)	0.0001[2)]

[1)]: P value was calculated by using Chi-square test.
[2)]: Significant difference in between groups (P value < 0.05).

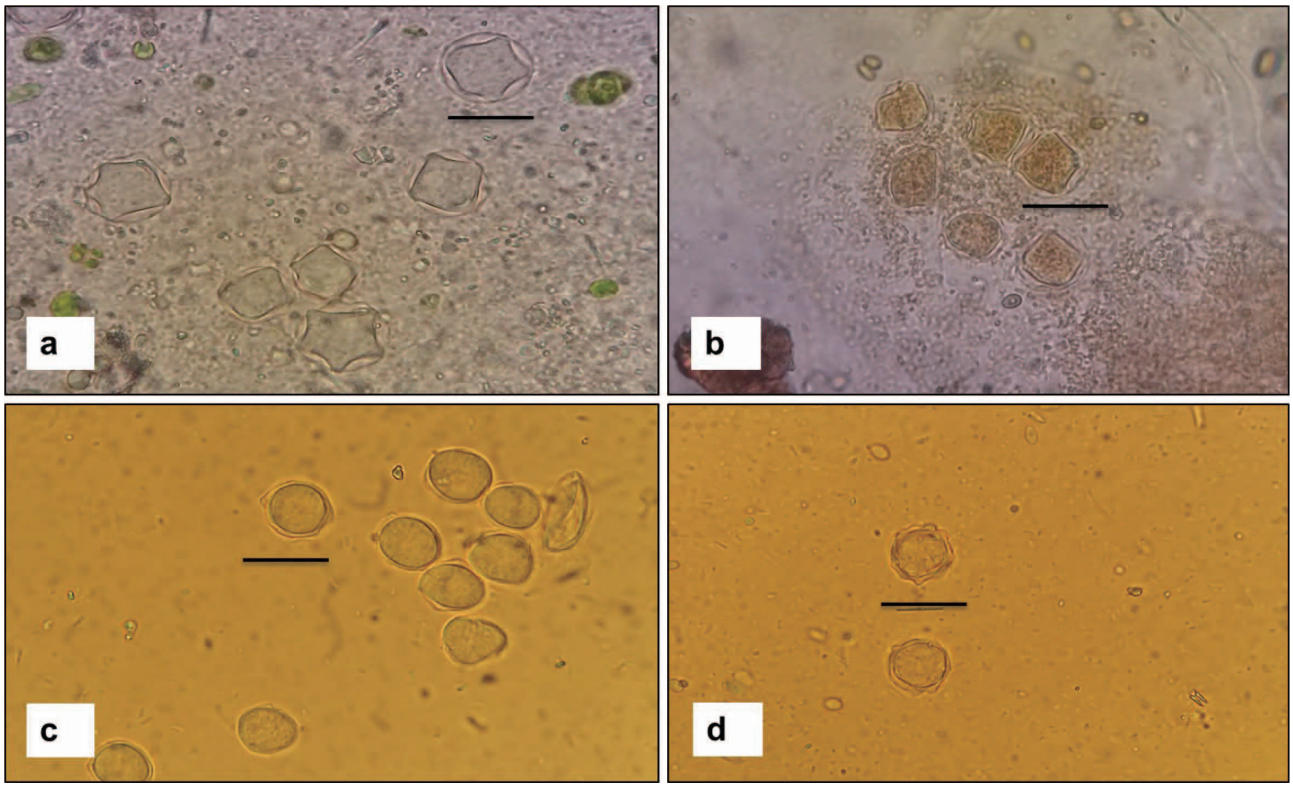

Fig. 1. (Color online) Light microscopy showing different morphological groups of *Acanthamoeba* cysts (I, II, III) by iodine wet mount stain (Objective, 100×). Bars represent 20 µm. Large sized cyst (>18 µm) and wrinkled endocyst forming 4 or 5 arms belonged to group I (a). Small sized cysts (<18 µm) with wrinked encocyst (b, c, d), and ectocyst (b, c) or wrinked ectocyst only (d) belonged to group II and III.

in all the positive samples, as shown in Fig. 1. All 41 (51.25%) air-conditioner samples identified positive using *in vitro* culture were PCR-validated using specific primers for *Acanthamoeba*, as shown in Table 1. Fig. 2 shows the representative PCR product of the 18S rRNA gene of *Acanthamoeba* spp. upon agarose gel electrophoresis.

Among the 41 *Acanthamoeba* isolates, 27 (65.9%) were found to be thermotolerant when grown at 40°C, and 16 (39%) samples were found to infect mice; however, these were not statistically significant findings (P = 0.9 and 0.3 respectively). The pathogenicity of *Acanthamoeba* species was higher in university and hospital samples compared to that of factory and household samples. Among the university and hospital samples, 12 (70.6%) and 9 (64.3%) species were thermotolerant, respectively, and 8 (47.1%) and 5 (35.7%) species could infect mice, respectively. The distribution of pathogenicity at different locations is shown in Table 2.

Mouse organs, including the brain, lungs, heart, liver, and kidneys, were examined for the presence of *Acanthamoeba* and any histopathological effects as a result of the infection. All the isolates of the pathogenic *Acanthamoeba* could be re-isolated from the brain. Lung tissues and other organs, including the liver and kidneys, were also affected. The most virulent isolate had invaded all the studied organs of infected animals. In addition, the time of death of the infected mice was also recorded. Some *Acanthamoeba* isolates caused early death within the first week of infection, indicating their higher virulence. Organs that were affected and time of death after infection of the different *Acanthamoeba* isolates from the four different locations are illustrated in Table 3. The histopathological effects in different organs are illustrated in Fig. 3.

DISCUSSION

Acanthamoeba are widely distributed in the environment and can be isolated from air, soil, water, contact lenses, and air conditioning units. Air conditioning units are considered essential in hot climates. However, their use could increase the risk

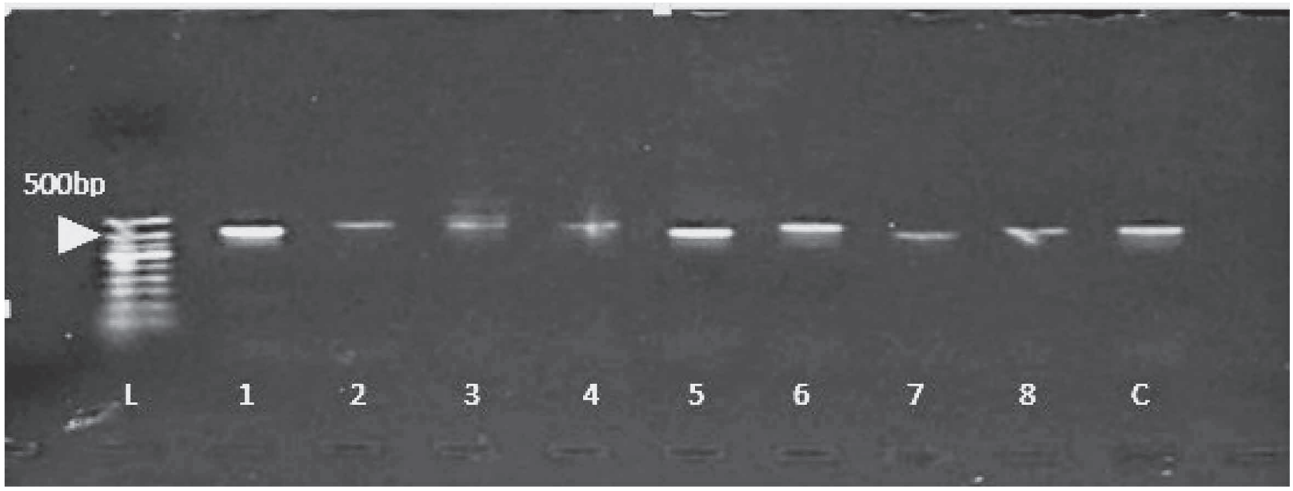

Fig. 2. Agarose gel electrophoresis showing representative PCR product of the 18S rRNA gene of *Acanthamoeba* spp. DNA molecular weight marker (100 bp) (lane L). Positive genomic DNA (lanes 1 to 8). Genomic DNA from *Acanthamoeba castellanii* as a positive control (lane C).

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[1)]
Thermo-assay + ve *N* (%)	2 (50)	12 (70.6)	4 (66.7)	9 (64.3)	27 (65.9)	0.9[2)]
Animal inoculation + ve *N* (%)	1 (25)	8 (47.1)	2 (33.3)	5 (35.7)	16 (39)	0.3[2)]

[1)]: *P* value was calculated by using Chi-square test.
[2)]: Significant difference in between groups (*P* value < 0.05).

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 (*N* = 2)	2	2	1	A	2	−	−	−	−	0	2
				B	2	1	−	−	−	1	1
University (*N* = 12)	12	12	8	A	12	3	2	1	1	1	11
				B	12	5	2	1	1	1	11
Industry (*N* = 4)	4	4	1	A	4	−	−	−	−	0	4
				B	4	2	1	1	1	1	3
Hospital (*N* = 9)	9	9	3	A	9	2	1	−	−	1	8
				B	9	3	3	2	1	2	7

A, group A of immunocompetent infected mouse group; B, group B of immunosuppressed infected mouse group; N, number of infected mice; −, absence of infection in mice.

of exposure to indoor air pollutants, such as cysts and trophozoites of *Acanthamoeba* spp. Therefore, in this present study, we sought to detect *Acanthamoeba* spp. in air conditioners and investigate their pathogenic potential using thermotolerance tests and mice inoculation models.

In this study, the prevalence rate of *Acanthamoeba* spp. in air conditioners was found to be 51.3%, and our finding was complementary to that of Astorga et al. (18), wherein 41 (56.9%) of the 72 samples collected from air-conditioner samples in Santiago, Chile revealed *Acanthamoeba* spp. However, Chan et al. (9) detected *Acanthamoeba* spp. in air conditioners with a prevalence rate of 23%. Furthermore, Özpinar et al. (6) recovered *Acanthamoeba* spp. from 4 (16.7%) out of 24 swab samples from air conditioners. In contrast, FLA could not be isolated from air conditioning systems in a previous study conducted in Egypt (19).

In the present study, *Acanthamoeba* spp. from all three morphological groups were detected. Spherical cysts with ectocysts separated from the star-shaped endocyst, four to six conic branches, and a cyst diameter >18 μm belonging to group I were identified. In addition, cysts with furrowed ectocysts, predominantly smooth endocysts, and a diameter <18 μm belonging to groups II and III were also identified. *Acanthamoeba* from group I is known to be non-pathogenic, while those from groups II and III are usually associated with

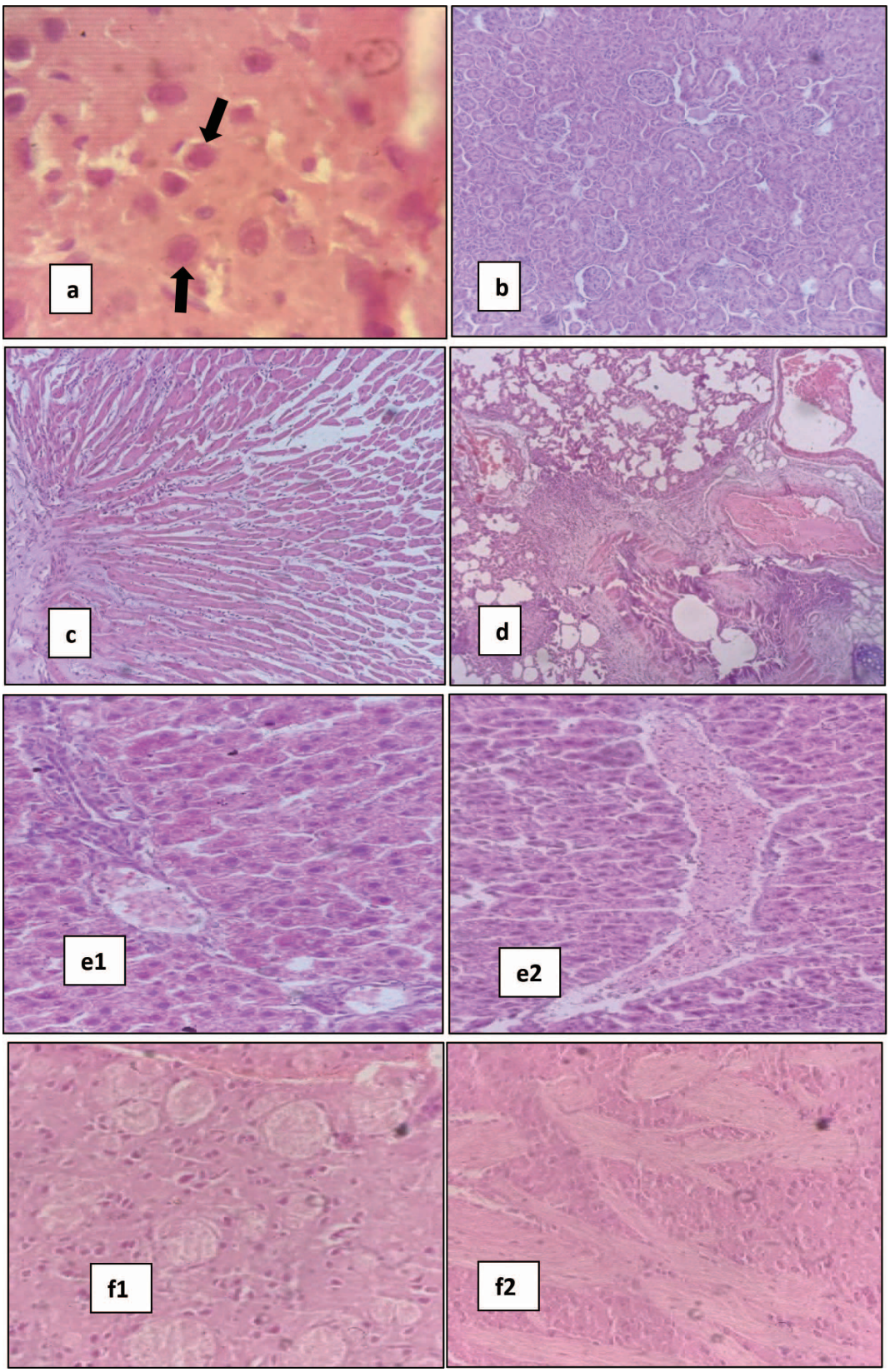

Fig. 3. (Color online) Histopathological effect of *Acanthamoeba* infection on different organs of mice by light microscopy of hematoxylin and eosin stained sections (Objective, 40×). Brain section illustrated *Acanthamoeba* cysts marked by black arrows (a). Kidney section illustrated the degeneration 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 (b). Section of the heart showed partial loss of muscle fibers with focal inflammation formed of infiltration by neutrophils, histocytes and plasma cells (c). Lung tissues showed inflammation, area of emphysema in the alveoli, hyperplasia of bronchial walls and thickening of some alveoli with an area of hemorrhage (d). Liver tissues demonstrated minimal focal portal tract inflammation (e1), area of hemorrhage and congestion of central veins (e2). Brain pathology ranged from mild inflammation and area of hemorrhage to an area of necrosis (f1 and f2). Numerous neutrophils, macrophages, plasma cells, and giant cells could be detected (f1) to gliosis (f2).

clinical conditions (25).

In this study, 41 (51.3%) dust samples were found to be positive for the growth of *Acanthamoeba* spp; further, PCR analysis confirmed that the bacteria present in the samples are indeed related to the genus *Acanthamoeba*. Similar results have been reported in studies conducted in Egypt (20,21,23) and Spain (22). Additionally, in a study by Gabriel et al. (24) in Malaysia, 64% of isolated FLA that were positive for the growth of *Acanthamoeba* spp. were validated by PCR. This indicated that the culture method is accurate in the detection of *Acanthamoeba* spp., and although being simple and inexpensive, it is time consuming.

Thermotolerance tests have been performed in many studies to test for pathogenicity (26,27). The advantages of the thermotolerance test are its feasibility and specificity for pathogenic effects. In addition, the thermotolerance test is one of the simplest tests to assess the pathogenic potential of *Acanthamoeba*, since isolates from clinical cases have been found to be thermotolerant (28,29). In this study, all thermotolerant *Acanthamoeba* species that were able to grow at 40°C, could also infect mice, indicating that thermotolerance is a strong indicator of pathogenicity. However, some non-pathogenic isolates can also tolerate temperatures of 37°C according to Schuster and Visvesvara (2), making the criterion of thermotolerance alone inconclusive to demonstrate the pathogenicity of *Acanthamoeba*. Costa et al. (27) reported that the thermotolerance could be used for an initial screening of pathogenic potential, although it must be used in combination with other tests, such as animal inoculation, which was performed in this study.

Animal inoculation of *Acanthamoeba* spp. was used as a means to test virulence, and the day of death was used as an indicator for the virulence of the *Acanthamoeba* species. The earlier the day of death of the infected mice, the more virulent was the *Acanthamoeba* species. In this study, certain isolates were tolerant to temperatures up to 50°C, and upon inoculation in mice, they infected all organs and caused an early death of the mice. In this study, we detected highly virulent *Acanthamoeba* isolates, which we speculate may be a real threat to humans based on the limited data concerning the distribution of AK cases in Egypt (30).

The animal model of nasal infection of *Acanthamoeba* spp. used in this study showed an early dissemination of the infection in mice with low mortality. The most virulent isolate invaded all the studied organs of the infected animals. All the isolates of the pathogenic amoebae studied could be re-isolated from the brain, which was the most common site of isolation, followed by the lungs. These results match those of Górnik K and 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 follow the olfactory nerves to reach the brain (32). The difference in *Acanthamoeba* tissue invasion may be due to the variability in virulence between isolates or even within the same isolate and the expression of different virulence factors following the first infection.

In conclusion, our findings provide additional evidence for the presence of pathogenic *Acanthamoeba* species in air conditioning systems, and this could pose a risk to human health. Therefore, awareness among clinicians should be raised. It also highlights the importance of surveying air conditioner systems in Egypt. Thermotolerance at higher temperatures is a reliable test for pathogenicity in animal inoculation, although further pathogenic testing and genotyping studies are required.

Conflict of interest None to declare.

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