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Biochemical Criteria of *Naegleria* Species Isolated from the Egyptian Aquatic Environment

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Abstract

The free-living amoebae *Naegleria* species (spp.) have been recognized as etiologic agents of amoebic encephalitis, keratitis, otitis, lung lesions and other skin infections mainly in immuno-compromised individuals. In this study, morphological and biochemical characterization of *Naegleia* strains isolated from the Egyptian aquatic environment were surveyed. Some *Naegleria* species were cultivated on non-nutrient agar. Isolated strains of *Naegleria* were identified based on the morphology of trophic and cyst forms in addition to temperature and flagellation test. Biochemical characterization of the isolated amoeba strains using quantitative and qualitative (SDS-PAGE) assays as well as qualitative determination of proteolytic activity in zymograph analysis. Potentially pathogenic free-living amoebae were isolated from all of the examined water sources. Colorimetric assays showed protease activity in heat-tolerant isolates of *Naegleria*. All pathogenic isolates exhibited higher protease activity than non-pathogenic ones did. The zymographic protease assays showed various banding patterns for different strains of *Naegleria*.

Keywords

Free-Living Amoebae, *Naegleria*, Flagellation Test, Proteases, Drinking Water Sources

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1. Introduction

Infection of the central nervous system by free-living amoebae is an unusual event. Free-living amoebae are presented worldwide in fresh water as well as in marine water. Moreover, they have been recovered from various domestic water systems such as drinking tap water (Michel et al., 1998), cooling towers (Barbaree et al., 1986), swimming pools (Rivera et al., 1983), hydrotherapy baths (Scaglia et al., 1983) and hospital water networks (Thomas et al., 2006). Waterborne transmission, acquired through forceful inhalation of surface waters or poorly maintained swimming pools, is uncommon (Karanis et al., 2007). The genus *Naegleria* includes more than 30 species of freelivingamoebae that are extensively distributed in soil and freshwaterenvironments (CDC, 2010; Laseke et al, 2010). *Naegleria fowleri* is the only species of thisgenus known to be pathogenic to humans (Visvesvara et al, 2007). The disease most associated with *N. fowleri* infection is primary amoebic meningo-encephalitis (PAM), an acute, severe, necrotizing, and hemorrhagic form of meningo-encephalitis.PAM infection requires that *N. fowleri* trophozoites or cysts enter the central nervous system. Infection begins when contaminated water directs through

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the nose, usually through swimming or diving. Inside the nasal passage, travel amoebae reach the olfactory mucosa, along the olfactory nerve fibers, and through the perforated plate in the brain.Inside the brain, *N. fowleri* amoebae feed on red blood cells, white blood cells, and brain tissue. PAM is almost always fatal, usually killing its victims within 3–7 days after the onset of symptoms (CDC, 2010; Cabanes et al, 2001; John, 1982). Pathogenic FLA are not dependent upon a host for transmission and spread, nor does host-to-host transmission of these amoebic diseases occur. They feed by phagocytosis, mainly on bacteria, fungi and algae (Bass and Bischoff, 2001). They have the ability to multiply and grow well in tropical climate and in water body with high temperatures of 40-45ºC (De Jonckheere, 2006).There are few data on the occurrence of these pathogenic free-living amoebae in the aquatic environment of Egypt. So, the main objective of the present work is to illustrate the occurrence and identification of pathogenic free-living amoebae *Naegleria* spp in different types of water using morphological characteristics. A secondary objective is to characterize the potential pathogenicity of the isolated strains using biochemical assays.

Table 1. Samples and sampling sites.

Locality	Water type
Cairo	Nile and tap
Giza	Nile and tap
Qalubeya	Nile and tap
Behera	Nile and tap
Gharbeya	Nile and tap
Dakahleya	Nile and tap
Helwan	Nile and tap
Kafr-Elshikh	Tap
Sharkeya	Tap
Minofeya	Tap

Figure 1. Governorates of the Nile Delta, Egypt.

2. Materials and Methods

2.1. Samples and Sampling Sites

Water samples (2 liters each) were collected from different localities in Delta region, Egypt (Figure 1) for the detection and isolation of freshwater amoebae(Table 1). Samples were collected from the Nile Riverand tap water in clean, dry autoclavable polypropylene containers and sent to the laboratory in icebox and processed at the same day of collection.

2.2. Isolation and Morphologic Identification of *Naegleria* Spp. from Water Samples

Collected water samples (1 liter each) were concentrated by using the membrane filtration technique. One liter of each water sample was filtered through a nitrocellulose membrane filters (0.45 µm pore size and 47 mm in diameter) (Whatman, WCN type, Cat No. 7141-104) (Gradus et al., 1989). After filtration the membranes were separately inverted face to face on the surface of a non-nutrient (NN) agar plates previously seeded with 100 µl *Escherichia coli* suspension. All the inoculated plates were incubated at 40°C for one week with daily microscopic examination for the presence of any amoebic growth (Hikal, 2015). Identification of the obtained *Naegleria*spp. were achieved according to the morphological characteristics of both trophic and cyst stages (Pussard and Pons, 1977, Hikal, 2010, Al Herrawy et al., 2013).

2.3. Flagellation Test

The obtained amoebic trophozoites were gently scraped from the surface of agar plates with a bacteriological loop and suspended in a test tube containing 5 ml distilled water and incubated at 37°C for 30 minutes. Every 10 minutes one drop from the content of the tube was suspended in the concavity of a clean glass hanging drop slide and examined under the microscope for the formation of temporary flagella (Behets et al., 2003).

2.4. Biochemical Characterization of Isolated *Naegleria* spp.

Grown amoebae were harvested from cultured NN agar plates by scraping of the agar surface in eppendorf tubes containing 0.5 ml sterile Page's amoebae saline. The harvested amoebae were centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the final pellet was re-suspended in 100 µl Page's amoebae saline and homogenized for 5 minutes in a tissue grinder. After that, the homogenate was transferred to a fresh eppendorf tube and centrifuged at 14000 rpm for 10 min. The supernatant was aspirated, divided into aliquots and stored at -80ºC till being used.

These steps were repeated for each sample (Khan et al. 2000).

2.4.1. Quantitative Assays for Proteinase Activity Using Chromomeric Substrates

An aliquot was taken from samples prepared and stored at -80°C as mentioned above. The protease activities in different *Naegleria*were quantitatively measured using the trypsin-like proteases specific substrate (Boc-Val-Leu-Gly-Arg-PNA L-1195, Bachem Bioche-mica, Heidelberg, Germany) at λmax 405 nm using Sun Rise reader (TECAN, Austria) according to (Iwanaga, 1994; Bahgat and Ruppel, 2002; Bahgat et al., 2006). The intensity of the yellow colour was directly proportional to the enzyme activity.

2.4.2. Qualitative Determination of Proteolytic Activity inZymograph Analysis (Gelatin Sodium-Dodecyl Sulphate Polyacrylamide Gel Electrophoresis, SDS-PAGE Gels)

The proteolytic activity of *Naegleria*isolates were characterized by zymography on SDS-polyacrylamide gels copolymerized with gelatin (Bahgat et al., 2006).

2.5. Statistical Analysis

All obtained data were analyzed by the student's *t*-test using the Graph Pad InStat Soft ware.

3. Results

3.1. Morphological Characterization of Genus *Naegleria*

Genus *Naegleria* represented the amoebo-flagellates whose members could transform from amoebae to flagellate forms. Differentiation of *Naegleria* from other amoebae was based on their characteristic eruptive movement of the amoebic form, and their ability to transform to flagellates.

3.2. Prevalence of Genus *Naegleria* in Different Water Sources

Heat tolerance and morphological features were used in differentiating members of genus *Naegleria* from other amoebae. Heat-tolerant *Naegleria* species were isolated from only 24.6and 16.7% of the examined Nile water and Tap water, respectively (Figure 2).

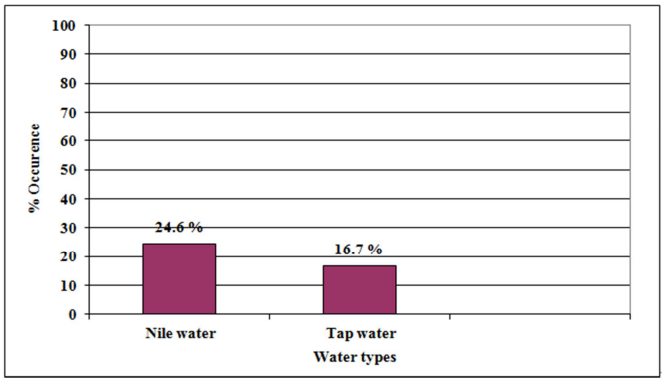

Figure 2. Occurrence of *Naegleria* at different water sources.

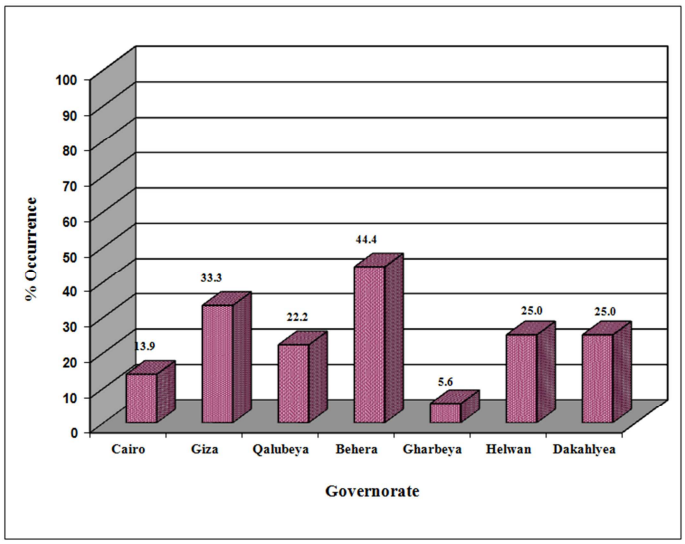

Figure 3. Occurrence of *Naegleria* in Nile water samples from different sites.

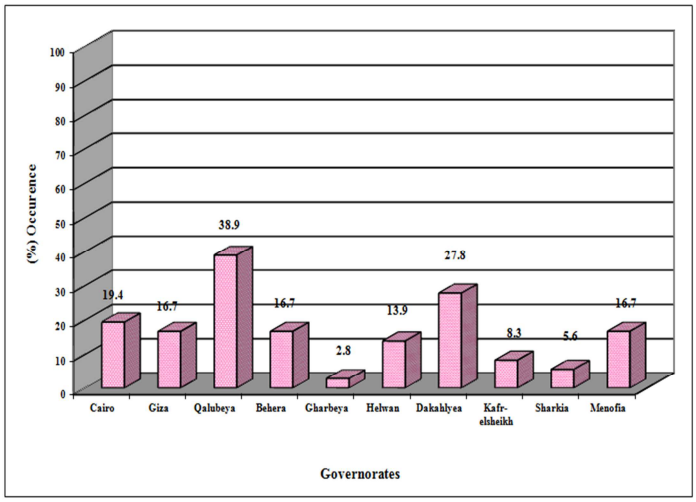

Figure 4. Occurrence of *Naegleria* in tap water samples from different sites.

Nile water samples collected from Behera Governorate showed the highest incidence of heat-tolerant Naegleria species (44.4%), followed by Giza in a percentage of 33.3%. The incidence of heat-tolerant Naegleria species was the same (25.0%) in raw Nile water collected from both Helwan and Dakahleya Governorates. The least incidence of Naegleria species in the Nile water samples occurred in Gharbeya (5.6%) and Cairo (13.9%) Governorates (Figure 3).

The highest incidence of heat-tolerant Naegleria species was recorded in tap water samples collected from Qalubeya Governorate (38.9%), followed by Helwan and Cairo in percentages of 27.8 and 19.4 %, respectively. The incidence of heat-tolerant Naegleria species was the same (16.7%) in tap water collected from Giza, Behera and Minofeya Governorates. The lowest incidence of heat-tolerant Naegleria species in tap water from Gharbeya, Sharkeya and Kafr-Elshikh Governorates in percentages of 2.8, 5.6 and 8.3%, respectively (Figure 4).

3.3. Quantitatively Absolute Enzyme Activity in *Naegleria*Isolates

In general, protease activities of the 6 examined *Naegleria* isolates at alkaline pH values seemed to be higher than those at the acidic pH values except in the sample number 2 (isolated from tap water) where the protease activities at alkaline pH values were lower than those at the acidic pH values. *Naegleria* isolates number 3 and 4 (isolated from tap water and Nile water, respectively) showed a great difference between protease activities at the acidic and alkaline pH values. At the alkaline pH *Naegleria* isolates number 3 and 4 showed the highest proteolytic activities (0.186 and 0.181, respectively) while the lowest ones appeared in *Naegleria* isolates number 5 and 2 (0.086 and 0.092, respectively. *Naegleria* isolate number 5 (isolated from Nile water) showed the lowest proteolytic activity at both the acidic and alkaline pH values. Concerning at the acidic pH, *Naegleria* isolates number 2 and 6 showed the highest proteolytic activity (0.11 in both), while the lowest ones appeared with *Naegleria* isolates number 3, 4 and 5 (0.057, 0.058 and 0.055, respectively (Figure 5). Statistically there is a significant difference between acidic and alkaline pH values in *Naegleria* isolates.

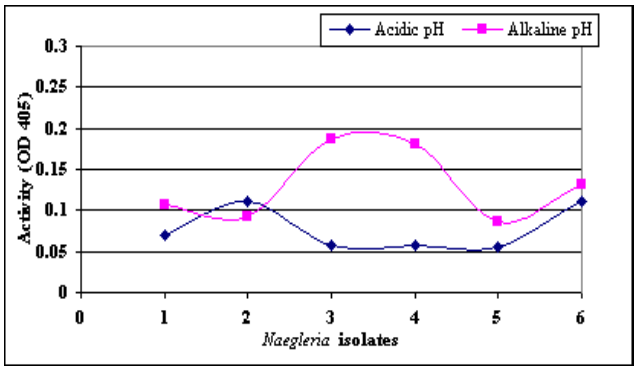

Figure 5. Tryptase activity in individual Naegleria isolates at both acidic and alkaline pH values.

3.4. QualitativelySilver Stain Profile of Resolved *Naegleria* Protein

The proteolytic profile of bacterial control sample without *Naegleria* was completely different from that of *Naegleria* isolates. The bacterial control sample without *Naegleria* had strong bands at molecular weights 124, 103, 90, 74, 66, 60, 53, 49, 45, 41, 35, 32, 27, 24, 21 and 15 kDa.The proteolytic profiles of prepared lysates from both *Naegleria* isolates number 1 and 3 (isolated from tap water) had 2 common bands at molecular weights 121 and 89 kDa. The lysate of isolate number 1 showed nine strong bands at 135, 121, 89, 69, 60, 50, 23, 19 and 15 KDa and three weak bands at 45, 35 and 27 kDa, while the lysate of isolate number 3 was resolved into seven strong bands at 143, 121, 109, 89, 75, 63, 48 kDa and two weak bands at 24 and 20 kDa. *Naegleria*

isolates number 2 and 4 (isolated from tap and Nile water) showed different proteolytic activities. The lysate of *Naegleria* isolate number 2 showed four strong bands at 141, 112, 60, and 50 kDa, while *Naegleria* isolate number 4 showed five strong bands at 139, 121, 91, 61, 52 KDa and one weak band at 44kDa. *Naegleria*isolates number 5 and 6 (isolated from Nile water) had a common band at 44 kDa. The lysate of *Naegleria* isolate number 5 showed bands at molecular weights 139, 119, 107, 61, 51, 44, 32 and 14 kDa, while in *Naegleria* isolate number 6 the lysate was resolved into five strong bands at 141, 121, 89, 73, 60 and 49 kDa and two weak bands at 105 and 44 kDa (Figure 6).

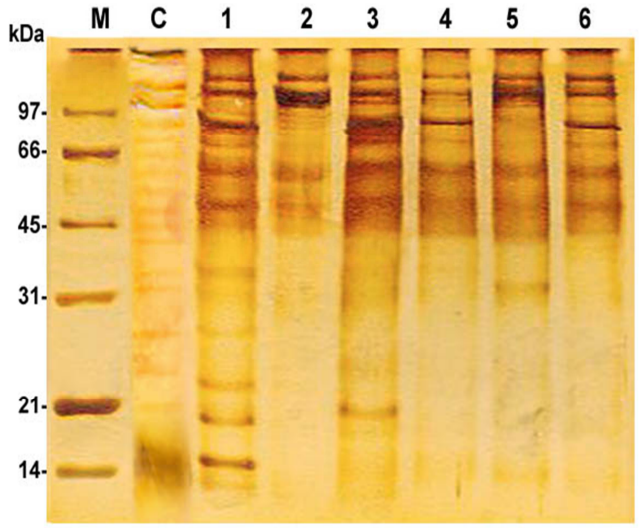

Figure 6. Protein profile of Naegleria isolates followed by silver stain, whereC: negative control bacteria; (1, 2, 3, 4, 5 and 6): N. spp.; M: marker.

3.5. Qualitatively Proteolytic Activity in Lysates of Different *Naegleria* Isolates Visualized by Gelatin SDS-PAGE

The proteolytic profile of prepared lysates from bacterial control samplecontaining no *Naegleria* was totally different from that of different *Naegleria* isolates. Lysates from bacterial control sample had molecular weights at 147, 110, 87, 70, 59, 53, 46, 31 and 23 kDa.The proteolytic activities of prepared lysates of *Naegleria* isolates showed activity at both alkaline and acidic pH values**.** Isolates number 1 and 3 (isolated from tap water) showed different profiles. *Naegleria* isolate number 1 visualized proteolytic activity bands at 143, 115, 96, 78, 59, 51, 40 and 31 kDa. In *Naegleria* isolate number 3, the proteolytic activity visualized bands at 164, 133, 112, 90, 73 and 63 kDa. *Naegleria* isolates number 2 and 4 (isolated from tap and Nile water) also showed different proteolytic profiles. In *Naegleria* isolate number 2 the gelatin digestion bands were evidenced at 137, 113, 92, 76, 64, 58, 42 and 30 kDa.*Naegleria* isolate number 4, the proteolytic activity visualized bands at 167, 141, 117, 89, 69, 57 and 52 kDa. *Naegleria* isolate number 5 (isolated from Nile water) showed three strong proteolytic bands at 139, 113 and 93 kDa and four weak bands at 74, 62, 54 and 48 kDa (Figure 7).

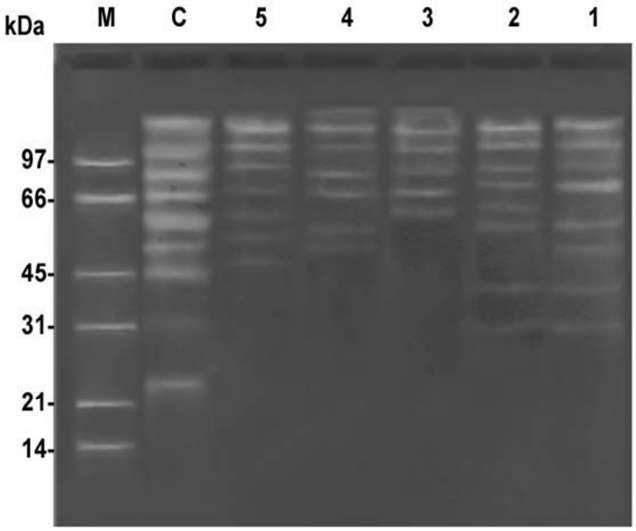

Figure 7. Serine-like protease activity in Naegleria isolates was visualized in gelatin SDS-PAGE,whereC: negative control bacteria; (1, 2, 3, 4, and 5): N. spp.; M: marker.

4. Discussion

The present study deals with the natural distribution of pathogenic *Naegleria* in the aquatic environment at different localities in the Nile Delta region, Egypt. Of special interest were the isolates capable of proliferating at temperatures of 37°C and above, as well as the biochemical characterization of these organisms. Previous studies on this subject in Egypt are non-existent. Therefore this study represents the first field investigation initiated in Egypt to estimate the presence of potentially pathogenic *Naegleria* in the Nileand tap waters by using culture and biochemical techniques.

4.1. Prevalence of Heat Tolerant Free-Living Amoebae in Different Types of Water

In the present study the incidence of *Naegleria* reached 24.6% in the examined Nile water samples. Other workers in Egypt also detected *Naegleria* in 66.7 and 60% of the examined freshwater samples by Hilali et al. (1994) and Hamadto et al. (1993), respectively. In USA, other workers detected *Naegleria* from pond water and James River in percentages of 56 and 46%, respectively (John and Howard, 1995; Ettinger et al., 2003). Also in Saudi Arabia, Al-Herrawy and Al-Rashied (1995) detected *Naegleria* in 47.22% of the examined freshwater samples. In Thailand, Nacapunchai et al. (2001) recorded an incidence (28.6%) lower than that recorded in our results.In the present work, the incidence of *Naegleria* reached 16.7% in the examined

tap-water samples. Other workers in Egypt detected *Naegleria* in 9.7% of the examined tap water (Hilali et al., 1994).

4.2. Biochemical Characterization of Isolated Free-Living Amoebae

Proteases are enzymes that catalyze the hydrolysis of peptide bonds in a broad spectrum of important biological reactions including the pathogenesis of parasitic disease (McKerrowet al., 1993). In the present work, *Naegleria* isolates showed different proteolytic activity bands ranging from 30 to 167 kDa. Other workers showed that five out of fifteen enzymes: aldehyde oxidase (ALDOX), aldolase (ALD), a-glycerophosphate dehydrogenase (a-GPDH), xanthine dehydrogenase (XDH), and glutamate oxaloacetate transaminase (GOT), were undetectable in the pathogenic strains, while the other enzymes: esterase (EST), fumerase (FUM), glucose-6-phosphate dehydrogenase (G-6-PDH), glucose phosphate isomerase (GPI), isocitate dehydrogenase (IDH), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malic enzyme (ME), glucose phosphomutase (GPM), and malate dehydrogenase (MDH), were detected (Tiewcharoen et al., 2004). Other workers in Malaysia (Amin, 2004) showed that *N. fowleri* possesses two high molecular weight proteases on gelatin gels at molecular weights 128 and 170 kDa.

5. Conclusion

The incidence and prevalence of the pathogenic free-living amoebae in different populations using morphological and biochemical diagnostic tools will provide baseline data against which the risk factors associated with waterborne transmission can be identified. The isolated species of *Naegleria*could provoke variable degrees of infections to the swimmers. The culture method is cheaper and easier than biochemical techniques that are faster for the detection of free-living amoebae.

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