Acta Ecologica Sinica xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Acta Ecologica Sinica

journal homepage: www.elsevier.com/locate/chnaes

Nested PCR assay for the rapid detection of *Naegleria fowleri* from swimming pools in Egypt

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ARTICLE INFO

Article history:
Received 30 April 2016
Received in revised form 13 June 2017
Accepted 15 June 2017
Available online xxxx

Keywords:
Naegleria fowleri
Swimming pools
PCR
Culture
Egypt

ABSTRACT

The free-living amoeboflagellate *Naegleria fowleri* is the only species infects humans world widely distributed. *N. fowleri* is the causative agent of very rare but severe brain infection called primary amoebic meningo-encephalitis (PAM), a rapidly fatal disease of the central nervous system mainly in immuno-compromised individuals. *N. fowleri* infects human through the entry of the nose, and it happens when human swimming or diving in warm freshwater, such as lakes, rivers and swimming pools. The disease is acute, and patients often die within 5–10 days and before the infectious agent can be diagnosed. Limited information is available about the existence of pathogenic *N. fowleri*, in Egypt, so the present of *N. fowleri* is an important public health. In the present study, we examined hundred water, dust and swap samples collected from 5 swimming pools in Cairo, Egypt. Based on morphological characteristics of trophozoite and cyst, flagellation test 56% of thermo-tolerant *Naegleria* like amoeba was detected. The incidence of thermo-tolerant free-living amoebae reached 84, 80and 70% from water, cotton swap and dust samples, respectively at cultivation temperature of 45 °C. The highest occurrence of thermo-tolerant amoebae were recorded in summer (100 & 87.5%) while the lowest one were recorded in winter (58 & 37.5%) in both water and dust samples, respectively. In swap samples, the highest occurrence of thermo-tolerant free-living amoeba was recorded in both summer and spring (100%), while the lowest one was recorded in winter (40%). *N. fowleri* was performed on 24 samples from a total of 56 (42.2%) samples which are positive by culture. Nested PCR using Mp2Cl5 gene primers that is unique to *N. fowleri* was carried out. The *N. fowleri* specific primer showed band at 166 bp against 24 of 56 (42.2%) samples. The majority of positive samples unique to *N. fowleri* was detected in water samples followed by swap samples and finally dust samples 14 of 24 (58%), 7 of 24 (29%), 3 of 24 (13%), respectively. In conclusion, swimming pools water may be the source of *Naegleria* invasion. The use of molecular methods to identify free-living amoebae *N. fowleri* could provide a more rapid means to diagnose infections caused by those *amoebae*.

1. Introduction

Free-living amoebae (FLA) are a very diverse and comprehensiveness organisms that have been isolated from soil and water environments [19,36,39]. *Naegleria* is a free-living amoeba that is distributed ubiquitously in the environment all over the world. *N. fowleri* is a thermo-tolerant, free-living amoebo-flagellate characterized by a life-cycle of trophozoite, flagellate and cyst stage [8,10,20,25,26,33]. Only *N. fowleri* was found to cause human disease, that result in primary amoebic meningo-encephalitis (PAM), which is quick and fatal infection of the central nervous system (CNS) [42]. The most frequently reported PAM in healthy young men with the recent history of swimming or other water activities in warm water [11,38,44]. Also, PAM was associated with pipe and storage ponds used by Muslims for routine ritual ablution, which involves the inhalation of water through the nostrils [23,34]. It was documented that, *N. fowleri* infects the CNS via inhalation through the nasal passages of the trophozoite, flagellate and cyst forms that pollute water or dust. Inside the nasal passage, amoebae travel up the olfactory mucosa, along olfactory nerve fibers, and through the cribriform plate into the brain. Inside the brain, *N. fowleri* amoebae feed on red blood cells, white blood cells, and brain tissue causing hemorrhagic necrosis and edema. Disease progresses vary rapidly and symptoms occur in a few days, followed by a dramatic clinical course and death within 3–7 days following the onset of symptoms. Most victims of PAM are healthy children and young adults with a relatively recent history of

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http://dx.doi.org/10.1016/j.chnaes.2017.06.013
1872-2032/

Table 1
Prevalence of thermo-tolerant free-living amoebae from different sources.

Sample sites	Thermo-tolerant free-living amoebae					
	Cotton swap		Water samples		Dust samples	
	No.	%	No.	%	No.	%
1	4\4	100	10\10	100	5\6	83.33
2	2\4	50	8\10	80	3\6	50
3	4\4	100	9\10	90	3/6	50
4	3\4	75	7/10	70	6\6	100
5	3\4	75	8\10	80	4\6	66.66
Total	16\20	80	42\50	84	21\30	70

Table 2
Incidence of thermo-tolerant free-living amoebae during different seasons of the year.

Seasons	Samples					
	Water		Swap		Dust	
	No	%	No	%	No	%
Summer	13/13	100	5/5	100	7/8	87.5
Spring	12/13	92.3	5/5	100	6/8	75
Autumn	10/12	83.3	4/5	80	5/8	62.5
Winter	7/12	58.3	2/5	40	3/8	37.5
Total	42/50	84	16/20	80	21/30	70

recreational exposure to warm and often shallow [29,37]. Therefore, risk prevention is essential require environmental monitoring using fast and accurate screening discrimination of *N. fowleri* diseases from other free-living amoeba in water samples. The usual habitat of *N. fowleri* is in freshwater lakes of natural or man-made, thermally polluted bodies of water, or not enough chlorinated domestic or swimming pool local supplies where the amoebae can feed on bacteria and reproduce [10,26,27,32]. High incubation temperatures, up to 45 °C, are used to screen for the presence of this human pathogen [10].

The identification of pathogenic free-living amoebae in swimming pools, which can be a risk for acquiring the disease, has taken great importance at present. Molecular methods are very sensitive and may allow the detection of microorganisms which are difficult to identify, and thus these methods are a useful alternative to microscopy and culture. PCR diagnostic methods are useful for the diagnosis of both clinical and environmental specimens [45]. Until now, we do not have enough studies to develop quantitative criteria for the number of pathogenic free-living amoebae in swimming pools in Egypt in order to develop procedures to reduce the risk of infections resulting from *N. fowleri* and/or other pathogenic species of diseases to humans.

The aim of this study was to monitor the population of thermo-tolerant *N. fowleri* in different swimming pools in Cairo, Egypt by microscopy and PCR using established primer sets designed from the ITS1-ITS2 regions.

2. Materials & methods

2.1. Sample collection

One hundred samples were collected from 5 swimming pools from Cairo, Egypt during the period from the beginning of January 2012 to the end of December 2012. Twenty sterile cotton swaps were used to scrape the debris from each wall of the swimming pool and placed onto non-nutrient agar (NNA) plates prepared from Page's amoebae saline (PAS) overlaid with thin layers of live *Escherichia coli* [14,16]. Thirty samples from dust near the swimming pools and fifty samples of water were collected (500 ml from each) in sterile bottles and transported to the Environmental Parasitology Lab, Water Pollution Research Department, National Research Centre, Egypt.

2.2. Cultivation & morphological characterization

Water samples were filtered using nitrocellulose membrane filters with 0.45 micron pores and a vacuum pump then, the filter was inverted and placed onto 1.5% non-nutrient agar (NNA) surface, covered by *E. coli*. The plates were sealed by Parafilm and incubated at 40 to 45 °C for 14 days to obtain thermo-tolerant amoebae [4,14,16]. The plates were examined by inverted microscope daily in order to identify positive amoeba samples. After growth, the agar was cut by a sterile scalpel and transferred to the new culture medium containing a lawn of *E. coli*. The plates were checked every day for amoeba growth and this step repeated until a pure plate from the desired ameba was obtained [3,4,14,15,21].

2.3. Flagellation test

A test for flagellates was performed on the select samples that had amoebae in the culture. Amoeba plaques that were seen to contain *Naegleria* trophozoites were transferred to sterile distilled water and placed in a shaker incubator. Samples were examined at 15-min intervals by light microscopy for transformation of amoebae to flagellates [4,6,14,16,22,27].

2.4. Polymerase chain reaction (PCR) analysis

The positive *Naegleria*-like samples were detected by amplifying a portion of a gene distinctive to *N. fowleri* using a nested polymerase chain reaction (PCR) as previously described [27,46]. This test with two primer sets for the detection of *N. fowleri* from other amoeba isolate

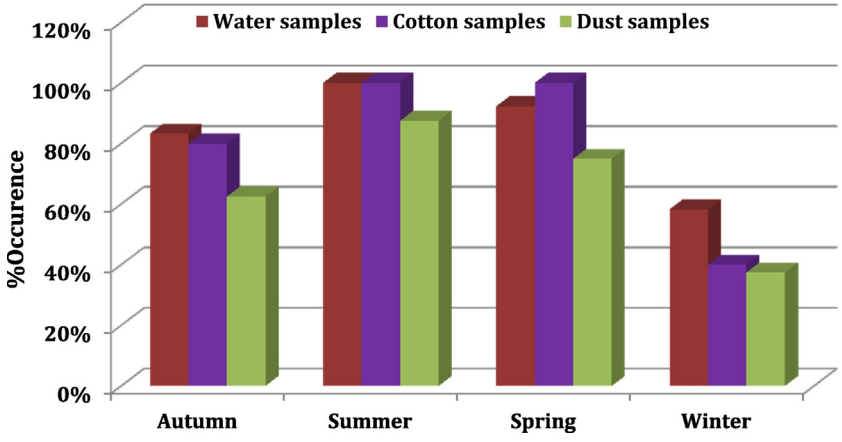

Fig. 1. Seasonal variation of thermo-tolerant free-living amoebae from different samples during different seasons at 45 °C.

Please cite this article as: W.M. Hikal, M.A. Dkhil, Nested PCR assay for the rapid detection of *Naegleria fowleri* from swimming pools in Egypt, Acta Ecologica Sinica (2017), http://dx.doi.org/10.1016/j.chnaes.2017.06.013