Research article

A preliminary study on *Naegleria* species in water bodies of Kurunegala district, Sri Lanka

JANS Gunarathna[1], D Iddawela[1], S Wickramasinghe[1]

Sri Lankan Journal of Infectious Diseases 2018 Vol.8(2)74-83

DOI: http://dx.doi.org/10.4038/sljid.v8i2.8216

Abstract

Introduction and Objective: Species belonging to the genus *Naegleria* are free-living ubiquitous protozoa. They have been isolated from most regions of the world. *N. fowleri* causes an acute, fulminant and rapidly fatal infection involving the central nervous system (CNS) in humans. It is known as primary amoebic meningoencephalitis (PAM). Infection is generally acquired while swimming, diving and total submersion for bathing in freshwater-lakes and ponds. Many inland fresh water bodies are present in Sri Lanka. These water bodies are frequently used by people for their daily needs. However, studies have not yet been conducted to determine the prevalence of *Naegleria* species occurring in local water bodies. The present study was therefore, carried out to isolate *Naegleria* species from selected water bodies located in four Divisional Secretariat (DS) divisions in the Kurunegala district, Sri Lanka.

Methods: Two different sites (clear and turbid water) of each tank were selected for sampling. Two water samples (surface water and deep water) were collected from each site (4 samples from one tank). Altogether, eighty water samples were collected from 20 tanks. Culture, enflagellation test and staining were done to detect *Naegleria* species. ArcGIS 10.3 and MINITAB (14) software were used for the data analysis.

Results: Flagella transformation was observed in 19 (47.5%) surface water samples and 11 (27.5%) deep water samples. Of 20 tanks, 10 were positive for *Naegleria* species.

Conclusions: Findings of the present study suggest that more specific genotyping studies are needed to confirm the presence of pathogenic *N. fowleri* in the study area.

Keywords: Primary amoebic meningoencephalitis, Naegleria fowleri, Tanks, Sri Lanka

Introduction

[1]*Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka*

Address for correspondence: Dr S Wickramasinghe, Department of Parasitology, Faculty of Medicine, University of Peradeniya Telephone: +94770074544 Email: susijijp@yahoo.co.jp

https://orcid.org/0000-0003-2046-1093

Received 2 May 2018 and revised version accepted 18 August 2018

Naegleria species are free-living amoebae which inhabit warm fresh water bodies (rivers, lakes and hot springs) and soil.[1] There are several species in the Genus *Naegleria*. However, *Naegleria fowleri* is the only species known to cause infection in humans. It causes fulminant and rapidly fatal primary amoebic meningoencephalitis (PAM).[2] While PAM is a rare disease, it could be acquired while swimming, diving and total submersion for bathing in freshwater-lakes and ponds. Other *Naegleria* species such as *N. australiensis* and *N. italic* are known to cause PAM in experimentally infected animals only.[3] In addition to PAM, humidifier fever can be caused by *Naegleria* spp.[4] It is a nonlethal hypersensitivity reaction caused due to antigenic material of *Naegleria* spp. present in humidifier systems.

Three distinct stages (amoeba, flagellate and cyst) of this organism can be identified in the life cycle.[5] Trophozoites (10μm-25 μm)[6] multiply by binary fission[7] and encyst in response to unfavourable conditions.[8] The cyst is round in shape with a single wall and size is varied (8μm-12 μm).[7]

Studies have shown 92.9%, 35.3% and 15.0% prevalence of *Naegleria* species in environmental water samples, natural hot springs and recreational water in China, Thailand and Iran respectively.[9,10,11]

There are many fresh water bodies (locally known as "tanks") in the dry zone of Sri Lanka. These tanks are primarily built for agricultural purposes. However, these water bodies are frequently used by people for their daily needs (mainly for washing and bathing). Conditions of these water bodies are ideal for the growth of *Naegleria* species. Except for one report[12], the occurrence of *Naegleria* spp. in local water bodies has not yet been investigated.

This preliminary study was therefore carried out to isolate *Naegleria* species from 20 water bodies of four Divisional Secretariat divisions in the Kurunegala district, Sri Lanka using culture techniques and the enflagellation test.

Methods

Study area

The Kurunegala district is located in the North-Western province of Sri Lanka. The total population of the study area was approximately 142,078 in a land area of 719 square kilometres[13] with a mean elevation of approximately 76 m from sea level. The annual rainfall is around 2,316.1 mm with the highest rainfall occurring in October and November during the North-East monsoon. The mean annual temperature is approximately 27.4 ˚C and the annual relative humidity varies from 71-87%.[14]

The Kurunegala District consists of 30 Divisional Secretariat (DS) divisions from which the Maho, Nikaweratiya, Kotawehera and Abanpola DS divisions were selected for the study, based on the density of water-bodies. There are approximately 654 water bodies in the study area. A standard random number table was used to select the tanks. Of the 654 water bodies, 50 were randomly selected from the four DS Divisions.

As some of the randomly selected tanks were located in rural localities with no proper road access, 20 of the 50 tanks were selected for the study based on the convenience of collection and transportation of samples.

Collection of water sample

There were several entryways to a tank in the study area. Two different entryways were selected for sampling. The bathing area frequently used by people was selected as the first sampling site. A turbid area (frequented by cattle and buffaloes) was selected as the second sampling site. Two water samples were collected from each site (4 samples from one tank). One sample was collected from the surface water approximately 0.5 m away from the edge of the dam. The second water sample was collected by opening the lid of the container at a depth of 1m. The purpose of sampling from surface and deep water was to determine the presence of *Naegleria* species in either deep or surface water in the study area.

Samples were collected during day time (12.00 noon to 14.00 pm) in sterile universal glass containers (30 ml). Sample collection was carried out during a warm and dry month (September 2016) of the year with a minimum rainfall. Containers were capped and labelled immediately after collection. The samples were transported at room temperature to the Department of Parasitology, Faculty of Medicine, University of Peradeniya for further investigations.

Global positioning system (GPS) coordinates

Geographical coordinates (Latitude and Longitudes) of sampling sites were recorded using an android GPS receiver.

Culture

The water samples were mixed well, and 15 ml of water transferred into new conical tubes. The tubes were centrifuged at 2000 rpm for 2 minutes (International centrifuge; GEC A4378x1). The supernatant was discarded, and the sediment was used for inoculation. Cultures were carried out on non-nutrient agar plates with *Escherichia coli* (NCTC10418) as described by Ash and Orihel (1987).[15] Plates were sealed with parafilm to prevent contamination. Culture plates were incubated at 37˚C overnight. The plates were observed for five consecutive days using an inverted microscope (Leitz Diavert). Positive growth was identified by increased localised amoebic count in the culture plate. Localised areas were marked on the plate.

Examination for flagellates (enflagellation test)

Scrapings from culture plates with growth were inoculated into 1 ml of distilled water and incubated at 37 ˚C for 30 minutes. Wet smears were prepared and observed for transformation of trophozoites into pear shaped bi-flagellates or multi-flagellates.[16] Preservation of flagellates was done using polyvinyl alcohol (PVA) fixative for trichrome staining. Trichrome stained trophozoites and flagellate forms were examined with a light microscope separately. Dimensions of trophozoites and flagellate forms were measured using a calibrated micrometer at (x100) magnification.

GIS analysis

GIS analysis was done using ArcGIS 10.3 software which works with maps to compile geographic information.

Statistical analysis

The results were analysed using MINITAB (14) statistical software. Two proportions and correlation tests were performed. Positivity in surface water vs deep water and positivity in first site vs second site were considered as variables.

Results

Eighty water samples were collected from 20 tanks. From each tank, sampling was done from two sites (clear and turbid water). From each site, two samples were collected (surface and deep).

Flagella transformation was observed in 19 surface water samples (47.5%) and 11 (27.5%) deep water samples. Of 20 tanks, 10 (50%) were positive for *Naegleria* spp. (Table 1, 2 and Figure 1).

Table 1: Results of enflagellation test

Name of the Tank	GPS N	GPS E	1st site positivity	2nd site positivity	Overall remark
Hulugallewewa	7.785488	80.14305	S	S	S
Magollewewa	7.740626	80.12363	0	0	0
Diwullawewa	7.765771	80.13234	S,D	S	S,D
Mahagirilllawewa	7.829951	80.11597	S	S,D	S,D
Udagirillawewa	7.834991	80.13112	0	0	0
Thabarambuwamahawewa	7.822655	80.11482	0	0	0
Mahakirindewewa	7.814126	80.11163	S,D	S,D	S,D
Olupeliyawawewa	7.805042	80.11689	0	0	0
Tubullawewa	7.794186	80.09382	0	0	0
Malabediyawawewa	7.812652	80.11006	S,D	S,D	S,D
Ipalogamamahawewa	7.8193	80.209	S	S,D	S,D
Thammitagamawewa	7.825202	80.22933	S,D	S,D	S,D
Uduweriyawewa	7.849772	80.24091	0	0	0
Kaburupitiyawewa	7.794035	80.20798	S	S,D	S,D
Ithewwewa	7.805821	80.18694	0	0	0
PahalaManingamuwawewa	7.832664	80.21649	0	S,D	S,D
Ehetuwewa	7.78442	80.15906	0	0	0
Hithkadawalawewa	7.856582	80.26146	S	S	S
Dalupotawewa	7.819508	80.21903	0	0	0
Mahowewa	7.830137	80.2798	0	0	0

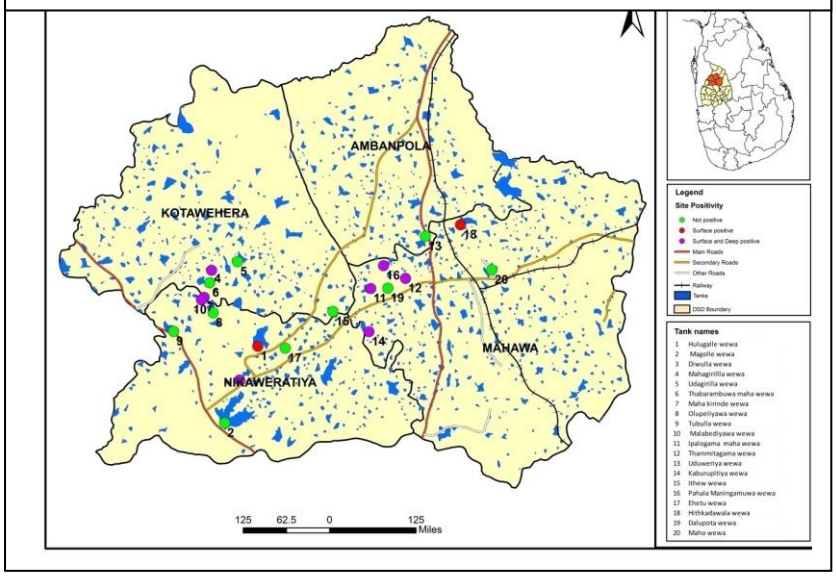
S: Enflagellation test positive-surface water D: Enflagellation test positive-deep water 0: Enflagellation test negative

Table 2: Sample positivity in DS Divisions

DS Divisions							
					Total		
Sample type	**Mahawa**	**Abanpola**	**Nikeweratiya**	**Kotawehera**	**No of samples**	**No positive**	**%**
Surface water	9	0	6	4	40	19	47
Deep water	5	0	3	3	40	11	27.5
Total tanks (surface or deep)	5	0	3	2	20	10	50
1st site (clear water)	5	0	7	1	40	13	32.5
2nd site (turbid water)	9	0	6	2	40	17	42.5

There was no significant positive correlation ($p>0.05$) between clear and turbid water. However, we found a significant positive correlation between surface water and deep water ($p<0.05$).

Figure 1: GIS map indicating sample sites and results of enflagellation test.



The shape and size of the flagellate, trophozoite, and cyst were evaluated on trichrome stained slides for confirmation.

The trophozoites were active and constantly changing their size and shape. The length varied from 18-22 µm. The cytoplasm was finely granular and contained a conspicuous clear nuclear halo and a dense central nucleolus. Few vacuoles were visible in the cytoplasm.

The trophozoites were motile and moved by extending blunt rounded lobopodia (Figure 2). Bi-flagellates were pear-shaped (Figure 3). They were 10-12 µm in length and 5-7 µm in width. Flagella was fixed into the conical edge of the organism and showed sluggish circular movements. Multi-flagellates were not observed in cultured samples. Cysts were rounded and spherical in shape with smooth outer coverings. The length of the cyst varied between 6-9 µm. In most cases, the cytoplasm was granular (Figure 3).

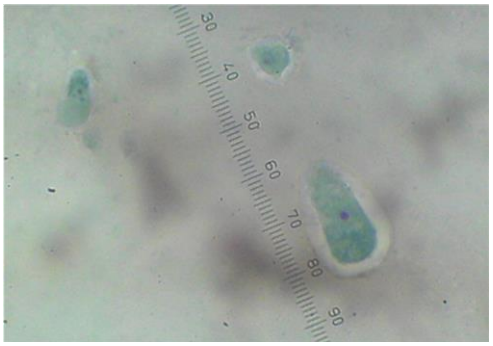


Figure 2: N*aegleria* trophozoites (Trichrome staining, X100)

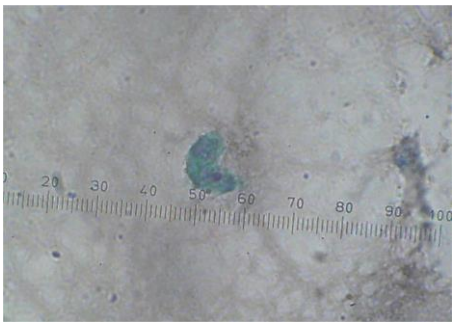


Figure 3: *Naegleria* bi-flagellate form (Trichrome staining, X100)

Discussion

Epidemiologically, *Naegleria* has a worldwide distribution. Primary amoebic meningoencephalitis was first described by Malcolm Fowler in Australia.[17] Thereafter, cases of PAM were identified in many parts of the world. Over 440 cases of PAM were reported worldwide. Among them, nearly half of the cases were reported from the United States.[18] In Asia, the highest number of cases has been reported in India[19,20] followed by Pakistan.[21] A single case of PAM has been reported due to freshwater pearl diving in Vietnam.[22]

The main method of contracting PAM is by swimming in *N. fowleri* contaminated fresh water collections. However, recent reports suggest that the use of "neti pots" for nasal irrigation can also predispose to PAM.[23] In addition; *N. fowleri* has been isolated from the nares of apparently healthy children.[24,25,26]

Neither PAM nor *N. fowleri* has been reported in Sri Lanka. However, the climatic conditions prevailing in Sri Lanka is favourable for growth of *Naegleria* species in local water bodies. Thus, isolation and identification of *N. fowleri* in local water bodies is a necessity. However, identification of *N. fowleri* based on morphological characteristics is not easy due to the existence of several genera of amoebae with similar morphological features in the same ecological habitat.[27,28] Furthermore, pathogenic *N. fowleri* and non-pathogenic *Naegleria lovaniensis* are antigenically related species.[28]

Enflagellation test (Amoeba-to-Flagellate Transformation) was performed to detect *Naegleria* species in the present study. However, the enflagellation test is not considered the gold standard test for detecting *Naegleria* species, because few non-flagellating *Naegleria* strains have been isolated from France.[29] In addition, trophozoites isolated in one location of Australia have failed consistently to transform into flagellates. However, non-flagellating *Naegleria* species have not been reported so far in Asia or the Indian subcontinent.

Current methods for detection and enumeration of *Naegleria* species are based on culture techniques[30] followed by various other methods (species-specific monoclonal antibodies[31], PCR[32], enzyme electrophoresis[27], isoenzyme electrophoretic profiles[33,34] and DNA restriction fragment length polymorphisms (RFLPs)[32,35,36] to identify the isolate up to the species level.

Randomly amplified polymorphic DNA typing has been used to differentiate *Naegleria* spp. and could be used to detect minor variations in *N. fowleri* strains.[37] In addition, *N. fowleri* positive cultures could be tested for pathogenicity by mice inoculation.

Records on the isolation of *Naegleria* from water bodies in tropical countries are very limited. Prevalence of 29% and 10% were reported in aquatic habitats in a human environment[38] and in stagnant water in an industrial area in Thailand.[39] A recent study carried out in natural hot springs in 13 provinces of Thailand showed 35% positivity for *Naegleria*[10] indicating a high health risk to those exposed to such waters. Findings of our study also suggest a high prevalence (50%) of these free-living amoebae in aquatic habitats in the study area. These water-bodies form a network of reservoirs where water cascades and spills over to the tank situated below and finally drains into a common canal or stream. Hence, *Naegleria* can easily migrate from this network to other tanks. This could be one reason for the high prevalence of the organism in these lakes.

Usually in the dry season water is clear as no muddy rain water is drained to the tank. The most plausible reason for the turbid water in these tanks was the wallowing of agricultural animals (such as buffalos) for long hours in the water. These animals enter the tanks through specific entry points. These entry points are also used by the local people for bathing and various other purposes. The role of animals in *Naegleria* ecology and epidemiology is yet unclear. A study in Tennessee, USA, showed that 13 wild mammalian species had serum antibodies against *Naegleria* indicating past exposure[40]. This was the reason we compared sites in the tanks having both clear and turbid water. However, we could not find a significant difference ($p>0.05$) between the two sites, clear and turbid water. A study done in India showed 34.5% prevalence of *Naegleria* spp in surface water.[41] It was 7.6% in deep well water in Arizona.[42]

The significant finding of this study was that the organism thrives more in surface water than in deep water ($p<0.05$). A similar result has been reported in some DS divisions in the same district previously.[43]

Limitations: Small sample size and not performing specific tests (such as FAT or PCR) to detect *Naegleria* species were the limiting factors of this study. However, the findings of the present study would encourage more epidemiological investigations on *Naegleria* species occurring in local water bodies.

Conclusions

Our results show a high prevalence of *Naegleria* species in the water bodies of the study area. Consequently, there is a possibility of the existence of pathogenic *Naegleria fowleri* in these water bodies. More specific and accurate genotyping is required to confirm the presence of pathogenic *Naegleria* in these water bodies, as it may pose a health risk to people who use such water bodies for domestic and recreational activities. The findings of this study will be used in designing a long-term study for genotyping of isolates from different sites.

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