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Full Length Research Paper

Distribution of free-living amoebae in springs in Morelos, Mexico

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Free-living amoebae (FLA) can accomplish their life cycle as free-living organisms; therefore it is important to know more about the distribution of FLA in the environment. The aim of the study was to determine the presence and distribution of free-living amoebae in recreational and drinking water springs. Eight springs were selected and the samples were taken on a bimonthly basis for one year. Filtration of water samples was performed and culture was done on non-nutrient agar medium with *Enterobacter aerogenes* bacteria. Identification of FLA was carried out taking in account the morphological characteristics of the trophozoite and cyst. Forty one of 48 (85.4%) samples tested positive for the presence of free-living amoebae and isolated amoebae belonging to 15 genera. Genera were grouping into 4 groups in according to spatial-seasonal distribution, having *Naegleria* and *Vermamoeba* the highest frequency and distribution. The study contributed to knowledge of the diversity of free-living amoebae in springs and provided interesting information about the spatial and seasonal distribution FLA in these water bodies.

Keywords: free-living amoebae, *Naegleria*, *Vermamoeba*, springs, recreational water.

INTRODUCTION

Free-living amoebae (FLA) comprise a large number of species, but not all have been studied to the same extent. Many studies have focused on potentially pathogenic amoebae because they can cause severe infections of the central nervous system that may lead to death, and serious eye and skin infections. Besides they can be reservoirs and dispersal of pathogens such bacteria, fungi, viruses and others protozoa (Visvesvara, 2014).

Free-living amoebae are found worldwide in soil and aquatic environments. They can be found in different types of natural (lakes, rivers, springs, groundwater) and artificial (swimming pools, hydrotherapy tubs, cooling water of power plants, tap water, bottled mineral water, wastewater) water bodies (Thammaratana et al., 2016; Visvesvara, 2014; Tsvetova et al., 2004). Some amoebae are thermotolerant and thrive in naturally and artificially heated

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water used for recreation (Sifuentes et al., 2014; Moussa et al., 2013; Tung et al., 2013; Kao et al., 2012; Nazar et al., 2012; Solgi et al., 2012; Badirzadeh et al., 2011;Gianinazzi et al., 2010; Huang and Hsu, 2010; Guzman-Fierro et al., 2008; Lekkla et al., 2005; Sheehan et al., 2003; Bonilla et al., 2000; Penas-Ares et al., 1994; Rivera, 1989,1993). Thus research on this water, where people swim and bathe should include the detection of free-living amoebae capable of being pathogenic to humans (Sukthana et al., 2005).

Furthermore, it is important to learn more about the distribution of FLA in the environment, since they are organisms that can accomplish their life cycle as free-living organisms. In the environment they live as phagotrophs, feeding on bacteria and playing an integral part in the cycling of nutrients (Bonilla et al., 2004).

Since FLA are free-living organisms is necessary to know their occurrence and distribution in nature. There are several areas in Mexico with water bodies suitable for the proliferation of free-living amoebae and which are used for leisure purposes, but few studies have been carried out formally in search of FLA (Guzman-Fierro et al., 2008; Bonilla et al., 2000; Rivera, 1989, 1993). For these reasons, the aim of this study was to determine the presence and distribution of free-living amoebae in recreational and drinking water springs in the state of Morelos, Mexico. The state of Morelos has a large number of recreation centers with springs and swimming pools that are popular with visitors because of their closeness to the country’s capital.

METHODS

Sampling geographical area

The study was conducted in Morelos. The state is located in South-Central Mexico only 90 km south of Mexico City, has an area of 4,893 km² accounting for 0.25% of Mexico's total territory (Figure 1). Roughly 70% of the state has a humid and relatively warm climate. Average temperature is approximately 25 °C year round, with a rainy season from May until September. Morelos state attracts many visitors annually due to its climate, its water parks and spas and its location near Mexico City (SEP, 1982). Many people from Mexico City spend weekends in the state or own second homes there, especially in the Cuernavaca area; it is referred to as "The City of the Eternal Spring” due to its gentle climate (Romo, 2006).

The eight studied springs are located in different regions of the state of Morelos: springs A y B in the municipality of Cuernavaca, spring C in the municipality of Tlaquiltenango, spring D in the municipality of Amacuzac, spring E in the municipality of Tlaltizapan, springs F y G in the municipality of Cuautla, and spring H in the municipality Tepalcingo (Figure 1). Almost all the springs are used to recreational purpose, only spring B is used as drinking water source.

Sample collection

The samples were taken on a bimonthly basis for one year. Samples were collected where the spring gushed or emerged in sterile bottles at the surface of the water; one 1000 ml sample for free-living amoebae and other 500 ml sample for coliforms. Temperature, pH and Dissolved Oxygen were measured *in situ* as in Standard Methods (APHA-AWWA-WEF, 2012). The water samples were transported to the laboratory at ambient temperature and were processed within 4 h after sampling.

Culture and identification

The water samples were thoroughly mixed and filtered through a nitrocellulose filter (1.2 µm pore size and 47 mm diameter). Filters were placed face down on 1.5% non-nutrient agar (NNA) plates seeded with a layer of heat-inactivated *Enterobacter aerogenes* as food source of amoebae. Plates were incubated at 37 °C and monitoring daily with a Zeiss inverted microscopy to detect amoeba growth for up 7 days. Amoeba plaques emerging along the filters were picked to isolation and sub-cultured on fresh NNA-*Enterobacter aerogenes* plates.

The amoeba isolates were identified taking into account the morphological characteristics of the trophozoite, cyst, and flagellate by microscopy technique of phase contrast, using the taxonomy keys of Page (1988).

The Total Coliforms and Fecal Coliforms were analyzed according to the standardized techniques of the Standard Methods (APHA-AWWA-WEF, 2012).

Statistical Analysis

Data were analyzed using ANOVA test to determine the differences between sites and among months. Pearson correlation coefficient was used to find relationships between FLA and physicochemical and bacterial parameters.

RESULTS

Occurrence of free-living amoebae

Of the 48 samples collected from the springs, 41 (85.4%) tested positive for the presence of free-living amoebae; in 5 of the springs (A, C, D, F and H) FLA were isolated in all the months sampled, while in spring B, FLA were isolated only in 2 months (Table 1).

Figure 1. Geographical location of Morelos State and the sampling sites.

Table 1. Occurrence of free-living amoebae in the spring of Morelos, Mexico.

Spring	June	August	October	December	February	April
A	+	+	+	+	+	+
B	-	-	-	+	-	+
C	+	+	+	+	+	+
D	+	+	+	+	+	+
E	+	+	-	+	+	+
F	+	+	+	+	+	+
G	-	+	-	+	+	+
H	+	+	+	+	+	+

A total of 90 isolates were obtained belonging to 15 morphotypes, identified at the genus level according to Page (1988). Of them *Vermamoeba* with 25.5% and *Naegleria* with 18.8% were the most frequent. In contrast, 5 genera (*Willaertia*, *Echinamoeba*, *Guttulinopsis*, *Stachyamoeba*, and *Filamoeba*) were the less frequent with only 1.1% each (Figure 2).

Distribution of free-living amoebae

A significant difference ($p<0.05$) was observed in the total number of FLA isolates between springs. Springs A, E and H had the highest number of isolates and spring B the lowest (Figure 3).

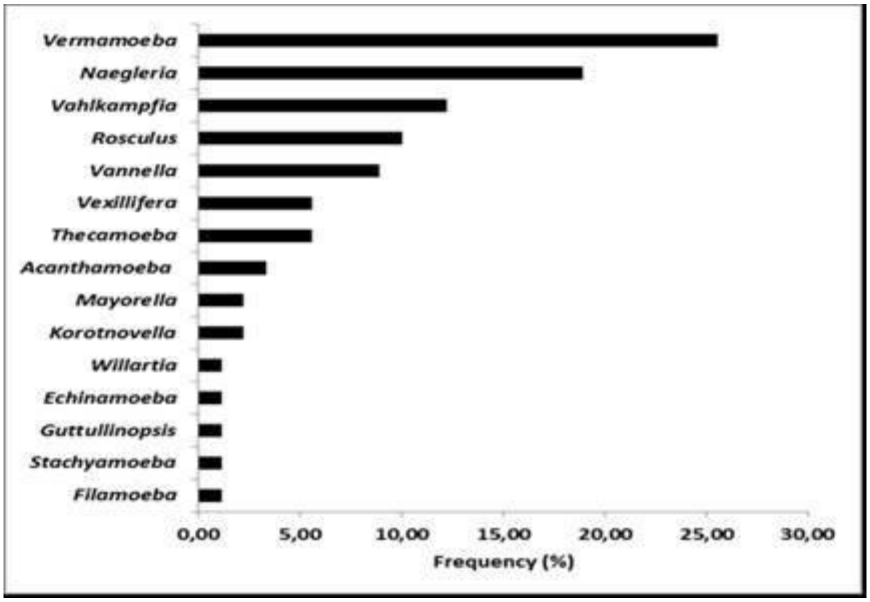

Figure 2. Frequency of FLA genera isolated from the spring.

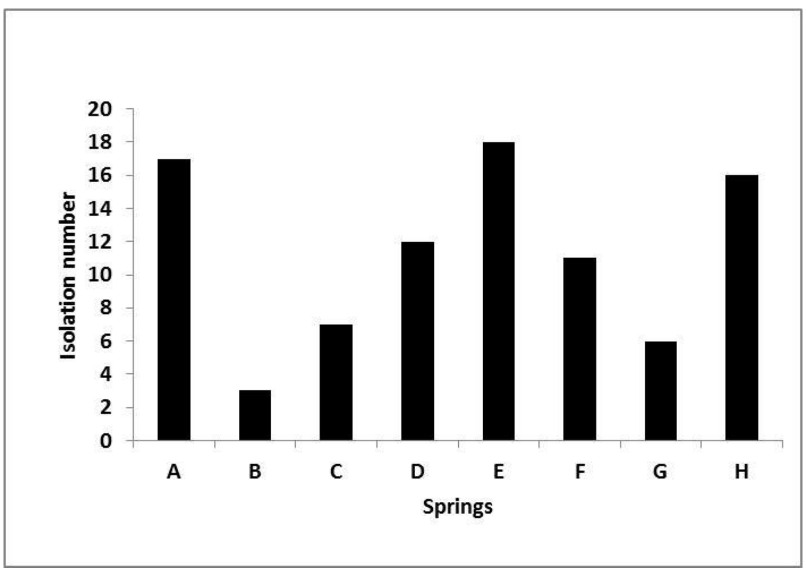

Figure 3. Spatial distribution of FLA in the springs.

Table 2 shows the genera that were isolated in each site (spatial distribution by genus). A scatter plot was made to group the amoebic genera based on spatial distribution and relative frequency. The Pearson correlation analysis showed a moderate positive degree of association between these two variables, which in general indicates that the genera with a broad spatial distribution had a high frequency value, grouping into 3 groups (Figure 4).

No significant difference (p>0.05) was observed in the number of isolates of FLA between months. February, April and June had the highest number of isolates and October the lowest (Figure 5).

Table 3 shows the genera that were isolate during each sampling month (seasonal distribution by genus). A scatter plot was made to group the amoebic genera based on seasonal distribution and relative frequency. The Pearson

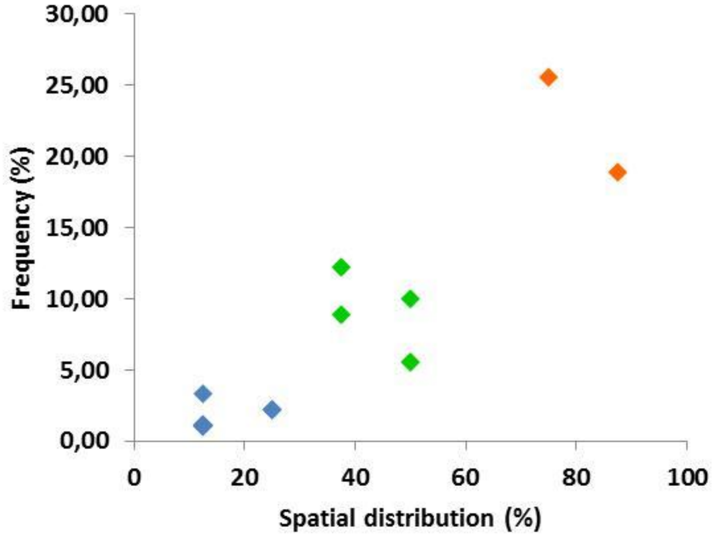

Figure 4. Grouping of FLA genera in according to their frequency and spatial distribution. Group 1 (blue) low spatial distribution (≤25%) and low frequency: *Acanthamoeba*, *Korotnovella*, *Mayorella*, *Filamoeba*, *Stachyamoeba*, *Guttullinopsis*, *Echinamoeba* and *Willaertia,* Group 2 (green) moderate spatial distribution (≤50%) and moderate frequency: *Vannella*, *Vahlkampfia*, *Rosculus*, *Thecamoeba* and *Vexillifera*. Group 3 (orange) high spatial distribution (≥75%) and high frequency: *Naegleria* and *Vermamoeba*.

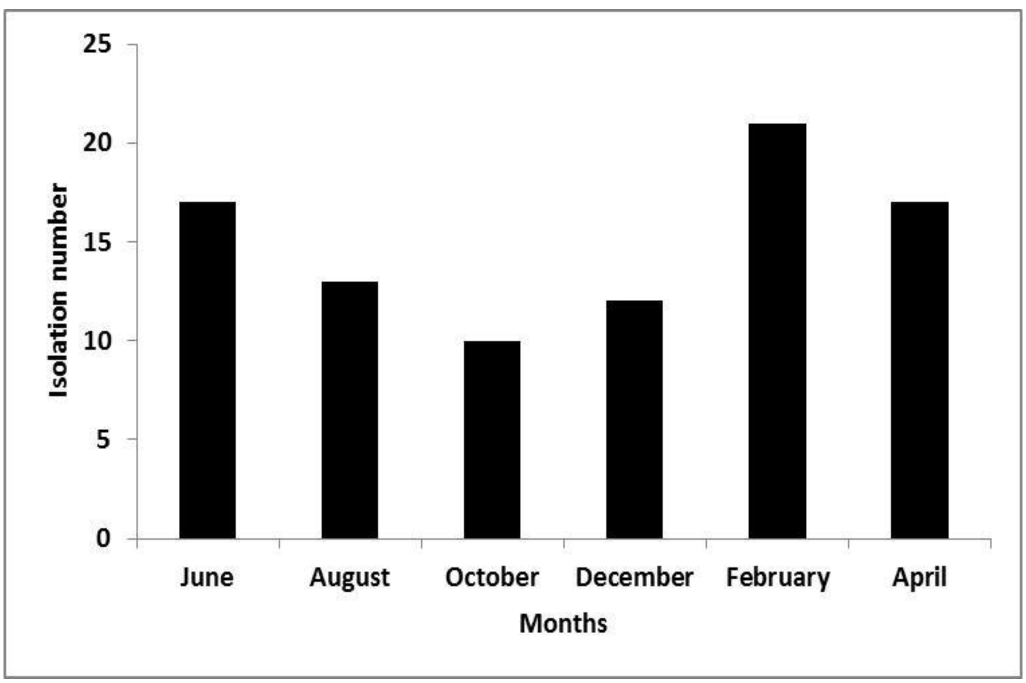

Figure 5. Seasonal distribution of FLA.

Table 2. Spatial distribution of FLA genera.

Genus	A	B	C	D	E	F	G	H	Register number	%
Naegleria	1	0	1	1	1	1	1	1	7	87.5
Vermamoeba	1	0	0	1	1	1	1	1	6	75.0
Rosculus	1	0	1	1	1	0	0	0	4	50.0
Thecamoeba	0	0	0	1	1	1	0	1	4	50.0
Vexillifera	0	0	1	1	0	0	1	1	4	50.0
Vahlkampfia	1	1	0	0	1	1	0	0	4	50.0
Vannella	1	0	0	1	1	0	0	0	3	37.5
Korotnovella	1	0	0	0	0	0	0	1	2	25.0
Mayorella	1	0	0	0	1	0	0	0	2	25.0
Acanthamoeba	0	0	0	0	1	0	0	0	1	12.5
Filamoeba	1	0	0	0	0	0	0	0	1	12.5
Stachyamoeba	0	0	0	0	1	0	0	0	1	12.5
Guttullinopsis	0	0	0	0	1	0	0	0	1	12.5
Echinamoeba	0	0	0	0	0	1	0	0	1	12.5
Willaertia	0	0	1	0	0	0	0	0	1	12.5
Total Number of Genera	8	1	4	6	10	5	3	5		

1=presence; 0=absence; Register number =number of sites where the genus was present; %= number of sites in percentage where the genus was present

Table 3. Seasonal distribution of FLA genera.

Genus	June	August	October	December	February	April	Register number	%
Naegleria	1	1	1	1	1	1	6	100.00
Vahlkampfia	1	1	1	1	1	1	6	100.00
Vermamoeba	1	1	1	1	1	1	6	100.00
Vannella	1	1	0	1	1	1	5	83.33
Rosculus	1	1	1	0	1	1	5	83.33
Acanthamoeba	0	1	0	1	0	1	3	50.00
Thecamoeba	0	0	1	1	1	0	3	50.00
Korotnovella	0	0	1	0	1	0	2	33.33
Mayorella	0	0	0	0	1	1	2	33.33
Vexillifera	0	1	0	1	0	0	2	33.33
Filamoeba	0	0	0	0	0	1	1	16.67
Stachyamoeba	1	0	0	0	0	0	1	16.67
Guttullinopsis	0	0	0	0	1	0	1	16.67
Echinamoeba	0	0	0	0	1	0	1	16.67
Willaertia	0	0	1	0	0	0	1	16.67
Total Number of Genera	6	7	7	7	10	8		

1=presence; 0=absence; Register number =number of sites where the genus was present; %= number of sites where the genus was present in percentage

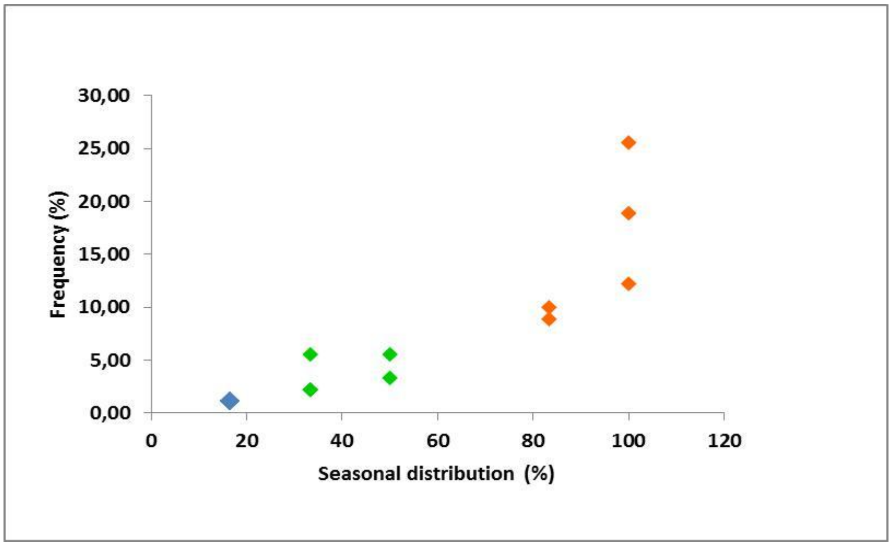

Figure 6. Grouping of FLA genera in according to their frequency and seasonal distribution. Group 1 (blue) low seasonal distribution (≤25%) and low frequency: *Filamoeba*, *Stachyamoeba*, *Guttullinopsis*, *Echinamoeba*, and *Willaertia*. Group 2 (green) moderate seasonal distribution (≤50%) and low to moderate frequency: *Acanthamoeba*, *Korotnovella*, *Mayorella*, *Thecamoeba* and *Vexillifera*. Group 3 (orange): high seasonal distribution (≥75%) and moderate to high frequency: *Vannella*, *Rosculus*, *Vahlkampfia*, *Naegleria* and *Vermamoeba.*

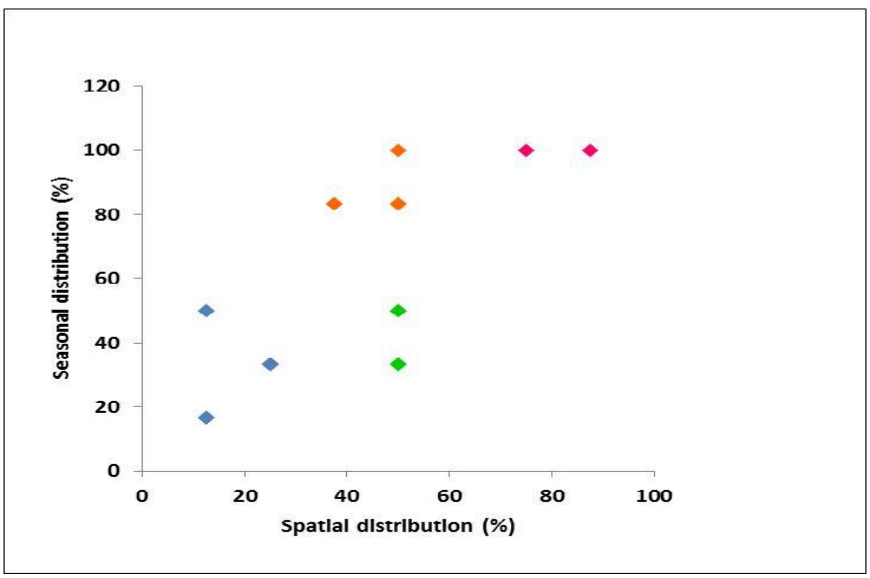

Figure 7. Grouping of FLA genera in according to their spatial and seasonal distribution. Group 1 (blue) low spatial distribution (≤25%) and low (≤25%) to moderate (≤50%) seasonal distribution: *Acanthamoeba*, *Korotnovella*, *Mayorella*, *Filamoeba*, *Stachyamoeba*, *Guttullinopsis*, *Echinamoeba* and *Willaertia*. Group 2 (green) moderate spatial distribution (≤50%) and moderate seasonal distribution (≤50%): *Thecamoeba* and *Vexillifera*. Group 3 (orange) moderate spatial distribution (≤50%) and high seasonal distribution (≥75%): *Vannella*, *Vahlkampfia* and *Rosculus*. Group 4 (pink) high spatial distribution and high seasonal distribution (≥75%): *Naegleria* and *Vermamoeba*.

correlation analysis showed a moderate positive degree of association between these two variables, which in general indicates that the genera with a broad seasonal distribution had a high frequency value, grouping into 3 groups (Figure 6).

The spatial-seasonal behavior of the amoeba genera was observed by plotting spatial distribution and seasonal distribution onto a scatter plot. According to the Pearson analysis, there was a moderate positive correlation between spatial distribution and seasonal distribution, grouping into 4 groups (Figure 7).

Table 4. Physicochemical and bacteriological parameters.

Spring	Temperature (°C)	pH	Dissolved oxygen (mg/l)	Total Coliforms (cfu/100 ml)	Fecal Coliforms (cfu/100 ml)
A	17.3	6.5	4.7	1656	486
B	17.0	6.7	4.9	290	55
C	30.3	6.8	0.7	9	4
D	29.7	7.1	2.5	66	25
E	20.8	6.6	4.5	34	12
F	18.6	5.8	5.8	0	0
G	26.0	6.2	1.3	13	0
H	32.4	6.8	1.5	2370	1315

Temperature, pH and dissolved oxygen: arithmetic mean
Total and fecal coliforms: geometric mean

Relationship of free-living amoebae with physico-chemical and bacteriological parameters.

Temperature, pH and dissolved oxygen were in ranges of 17 to 32.4 °C, 5.8 to 7.1 and 0.7 to 5.8 mg/l, respectively (Table 4). In according with the temperatures, springs were grouped into those higher than 25 °C and those lower than 25 °C.

The Pearson analysis showed a relationship between FLA and the physico-chemical parameters in some springs: a moderate negative relationship with temperature in springs A and G (-0.84 and -0.80); a moderate positive relationship with dissolved oxygen in spring G (0.82) and a strong positive in spring C (0.93); and a moderate positive relationship with pH in spring C.

The geometric means of total coliforms ranged from 0 to 2370 cfu/100ml and fecal coliforms from 0 to 1315 cfu/100ml (Table 4). No significant statistical relationship was found between FLA and coliform bacteria, but higher concentrations of total and fecal coliforms were found in springs A and H, where the highest numbers of amoeba isolations were obtained.

DISCUSSION

The number of positive samples for the presence of FLA obtained in this study was high compared to other studies conducted in springs, but it must be taken into account that in those studies the average water temperature was higher (43.6-49 °C) or the incubation temperature was higher (42-45 °C), which may have resulted in a selection of amoebae that can grow at those temperatures (Badirzadeh et al., 2011; Gianinazzi et al., 2010; Lekkla et al., 2005; Penas-Ares et al., 1994; Rivera et al., 1989).

This study considered both pathogenic and non-pathogenic amoebae, so greater diversity was found in amoeba genera than in the majority of the studies mentioned above, obtaining more non-pathogenic genera than pathogenic genera. This coincides with the findings reported by Penas-Ares et al. (1994), whose research suggests that the presence of a large number of non-pathogenic amoebae may in some way inhibit the growth of pathogenic amoebae.

Vermamoeba (*Hartmannella vermiformis*) was the genus that presented with highest frequency (25.5%). This amoeba is of direct and indirect medical importance, having been isolated from the cerebrospinal fluid of a patient with meningoencephalitis and bronchopneumonia (Centeno et al., 1996). It has also been suggested that *Vermamoeba* may cause amoebic keratitis (Lorenzo et al., 2007; Inoue et al., 1998; Aitken et al., 1996), and it has shown its ability to produce a cytopathic effect in “in vitro” keratocytes similar in magnitude and mechanism to *Acanthamoeba* (Kinnear, 2003). The indirect medical importance of *Vermamoeba* is related to its role as host of pathogenic bacteria (Santic et al., 2011).

Naegleria was the second most frequent genus (18.8%), to this genus belongs *N*. *fowleri* that is a human pathogen (Visvesvara, 2014). Sifuentes et al. (2014) mentions that the presence of *N. fowleri* in a volume of 1 L of water can be considered enough to represent a health risk, while the Mexican Standard (2010) indicates that the presence of thermophilic *Naegleria* in swimming pool water should be considered a warning sign.

It is striking that the *Acanthamoeba* genus was found with very low frequency (3.3%). This result differs from the knowledge that *Acanthamoeba* is the most widely distributed protozoa in nature (Bonilla et al., 2004; Page, 1988) and the most resistant to diverse environmental conditions such a high concentrations of organic matter (Ramirez et al., 2005, 2006). Some species of this genus can cause infections of the central nervous system and the eye, and may serve as a reservoir of pathogenic microorganisms (Scheid et al., 2014; Visvesvara, 2014; Douesnard-Malo and Daigle, 2011).

Vannella (7.7%) and *Vahlkampfia* (3.3%) have been found in waters for recreational use (Nazar et al., 2012), and have been reported in eye infections mixed with *Acanthamoeba* (Niyyati et al., 2010; Aitken, 1996). *Vannella* also has also been reported as harboring pathogenic intracellular organisms (Scheid, 2007; Michel et al., 2000).

The differences found between springs could due to that the diversity and number of organisms in a given habitat is a consequence of the relationship between the organisms and the environmental conditions. Two of the springs (A and E) with the highest number of amoeba isolates and greatest diversity (10 genera) were springs with temperatures lower than 25 °C; this temperature may favor the presence of pathogen a non-pathogen amoebae, and therefore a greater diversity; while in spring H (with a temperature >30 °C), diversity was less (6 genera), perhaps due to the selection that the temperature causes in the growth of amoebae. But, temperature was not the only factor driving amoeba diversity, in spring G with sulfurous water were found only 3 amoeba genera (*Naegleria*, *Vermamoeba* and *Vexillifera*); sulfurous water could be inhospitable for the presence of several amoeba genera. The presence of FLA in this spring is important, because it is used for curative purposes and is visited by people with ailments: arthritis, sciatica and a variety of lesions caused by practicing sports. Spring B had the lowest diversity and isolation number; even its temperature, pH and dissolved oxygen were very similar to spring A, but the concentration of bacterial coliforms was lower.

There was no difference in the number of FLA isolates between the months; which coincides with results reported by Tsvetkova et al, (2004), who did not find differences of the isolate number in the different season of the year.

The grouping of genera by spatial distribution varied slightly of grouping by seasonal distribution. In some cases the amoeba genera presented only in one spring presented in more than one month and vice versa. Taking in account both distributions, genera were grouped into 4 groups.

Acanthamoeba, *Filamoeba*, *Stachyamoeba*, *Guttullinopsis*, *Echinamoeba* and *Willaertia* had a low spatial distribution and low to moderate seasonal distribution; they presented in only one spring and in one month, with the exception of *Acanthamoeba* which presented in 3 months. It is interesting that the genera in this group were isolated only in springs with temperatures lower than 25 °C. *Vahlkampfia*, *Korotnovella*, *Mayorella*, *Thecamoeba* and *Vexillifera* had moderate spatial-seasonal distribution, occurring in 2-4 springs and 2-3 months. *Vannella* and *Rosculus* had moderate spatial and high seasonal distribution, occurring in 3-4 springs and 5-6 months. *Naegleria* and *Vermamoeba* had high spatial-seasonal distribution; they presented in almost all the springs and all sampled months.

The high distribution and frequency of *Naegleria* confirms its prevalence in this kind of water (Moussa et al., 2013; Tung et al., 2013; Solgi et al., 2012; Badirzadeh et al., 2011; Lekkla et al., 2005; Sheehan et al., 2003; Rivera et al., 1989). *Vermamoeba* proliferates better in water with low concentrations of organic matter, as the springs, and it has been reported to grow in temperatures higher than 40°C (Solgi et al., 2012).

In accordance with the water temperature most of the genera isolated (*Vahlkampfia*, *Mayorella*, *Filamoeba*, *Stachyamoeba*, *Guttullinopsis*, *Echinamoeba* and *Acanthamoeba*) were found only in springs with temperatures lower than 25 °C, two genera (*Willaertia* and *Vexillifera*) only in springs higher than 25 °C and six genera (*Naegleria*, *Vermamoeba*, *Rosculus*, *Thecamoeba*, *Vannella* and *Korotnovella*) were found in both types of spring.

The physico-chemical parameters of the water were within the reported ranges for the presence of FLA, but no general relationships were found between the amoeba isolation number and the physico-chemical parameters; which coincide with the findings of Penas- Ares et al. (1994), Moussa, et al. (2013) and Tung et al. (2013). However, some particular relationships were found. A moderate negative relationship between FLA and temperature was found in springs A and G, it means that FLA growth decreased as temperature rises; this may be explained because the majority of isolated genera are reported as non-pathogenic and therefore not thermophilic. A moderate (spring G) and strong (spring C) positive relationship between FLA and oxygen was found; it is explained because the amoebae are aerobic. A moderate positive relationship between FLA and pH was found, the pH ranged from 5.8 to 7.1, so it means that FLA preferred water with pH close to neutrality. No statistically significant relationship was found between the FLA and coliform bacteria, matching with the reported by Penas-Ares et al. (1994).

CONCLUSIONS

The study considered both pathogenic and non-pathogenic amoebae, so greater diversity was found than in the majority of studies carried out in springs, obtaining more amoeba genera reported as non-pathogen.

No general relationships were found between isolation number of FLA and the physico-chemical parameters. However, some particular relationships were observed; the diversity and isolation number of FLA in each one of monitored springs were determined by different environmental conditions; temperature, pH, oxygen dissolved, or a specific characteristic of the water as sulfurous contain.

Spatial distribution of free-living amoebae was observed, but no seasonal distribution; probably because springs are water bodies underground and are not directly affected by environmental temperature.

Grouping of the amoebae genera by their spatial distribution varied slightly of their seasonal distribution. Spatial-seasonal distribution of the amoebae allowed grouping them in 4 groups: group 1 amoebae with a low spatial distribution and low to moderate seasonal distribution, group 2 amoebae with moderate spatial-seasonal distribution, group 3 amoebae with moderate spatial distribution and high seasonal distribution, and amoebae with high spatial-seasonal distribution.

From an ecological point of view, this study contributed to knowing of the diversity of free-living amoebae in springs, and provided information about the spatial and seasonal distribution of the amoebae. Finally, considering that the springs are a popular tourist attraction and thousands of people swim in them, the wide distribution of FLA in these water bodies represent a potential risk for humans.

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