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ORIGINAL ARTICLE

OCCURRENCE OF POTENTIALLY HIGHLY PATHOGENIC FREE-LIVING AMOEBAE IN READY MADE SALADS FROM RESTAURANTS IN JATAI, GOIAS, BRAZIL

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ABSTRACT

Free-living amoebae (FLA) are part of a group of protozoa found worldwide and in the most diverse environments. They resist various temperatures and disinfection methods, and are a risk to human health. Pathogenic strains grow at high temperatures and under hyperosmolarity conditions. Some FLA genera are mainly related to primary amoebic meningoencephalitis (PAM), skin ulcerations, corneal lesions, kidney and lung infections and keratitis. Therefore, studies that assess the pathogenic potential of FLA are public health issues of great concern. We aimed to evaluate the pathogenic potential of FLA isolated in salads from restaurants *in vitro,* using osmotolerance and thermotolerance tests. Forty-five isolates were used from ready-made salads purchased in restaurants in Jatai, Goias. Twelve isolates subjected to the osmotolerance test (26.6%) showed growth in 0.5 M mannitol, 18 (40.0%) in 1.0 M mannitol and 16 (35.5%) in 1.5 M mannitol, 13 (28.8%) isolates did not show growth. Four isolates that underwent the thermotolerance test (8.9%) showed growth at 25°C, 8 (17.8%) showed growth at 30°C, 3 (6.7%) showed growth at 37°C and 30 (66.7%) did not show growth. With the indices obtained in the present study, we concluded that 15.6% of the isolates were osmotolerant and thermotolerant. Our findings highlight a public health problem once these FLA are associated with harboring or being harbored by microorganisms responsible for diseases such as diarrhea and meningitis. Measures are required to improve food hygiene and so avoid FLA-related health problems.

KEY WORDS: Pathogenicity; osmotolerance test; thermotolerance test; food contamination; free-living amoebae.

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INTRODUCTION

Free-living amoebae (FLA) are amphizoic protozoa present worldwide and in the most diverse environments, such as fresh and salt water, soil and air. The genera *Acanthamoeba, Naegleria, Balamuthia* and *Sappina* are pathogenic to humans and animals, and therefore of medical interest (Silva & Rosa, 2003). These protozoa can cause primary amoebic meningoencephalitis (PAM), granulomatous amoebic encephalitis (GAE), skin, kidney, lung and nasopharyngeal injuries, and keratitis in immunosuppressed and immunocompetent individuals (Calixto et al., 2014)

FLA are found in the trophozoite form (active, reproductive, and infective phase) and the cyst form (resistant form, observed in unfavorable conditions for the amoebae, where they become encysted). In the genera *Naegleria*, specifically, the flagellated trophozoite form is found as well, in addition to both forms mentioned above (Alves et al., 2018; Calixto et al., 2014).

Infections can be considered opportunistic once they are related to host immune status as well as to pre-existing lesions. They occur through ulcerations on the skin, lesions on the cornea and respiratory tract, subsequently flowing through the bloodstream to the central nervous system (CNS) and other organs. In addition to the associated diseases, another important point is the ability of amoebae to be hosts and carriers of other pathogenic microorganisms (Carlesso et al., 2007).

Most FLA genera feed by phagocytosis, in this mechanism some phagocyted microorganisms are able to evade the digestion process and are called Amoeba-Resistant Bacteria (ARB). These microorganisms multiply inside cysts and trophozoites, can lyse the amoebae and remain free in the organism, potentially contaminating the host (Siddiqui & Khan, 2012; Visvesvara et al., 2007).

Studies regarding disease epidemiology caused by FLA are few, due to the difficulty in obtaining a diagnosis, as they can be confused with viral, bacterial and/or fungal infections, and are often performed post-mortem, neglecting infection (Balczun & Scheid, 2017; Filho et al., 2019).

Consumption of contaminated water or food (such as salads consumed *in natura*) or even contact with these are the main sources of contamination by parasites, due to the lack of proper hygiene when handling food (Rizzi, 2018). Inappropriate cultivation factors such as irrigation with contaminated water, organic fertilization with animal manure can also lead to vegetable contamination (Silva et al., 2018).

According to a study performed by Rizzi (2018) on ready-made salads in restaurants in Jatai, Goias, a high prevalence of FLA was noted, indicating lack of hygiene when handling food in these establishments (Rizzi, 2018). Dutra (2019) observed that 76% of the samples in her study

were contaminated by FLA cysts and/or trophozoites. This also emphasizes that poor food hygiene increases the risk of infection as well as the development of opportunistic diseases (Dutra, 2019).

Pathogenic FLA strains can grow at high temperatures and under hyperosmolarity conditions. Therefore, studies that assess pathogenic potential are of great importance to public health, in order to establish therapeutic interventions as well as preventive measures (Khan et al., 2001).

This study aimed to evaluate the pathogenic potential of *in vitro* FLA isolates from ready-made salads served in restaurants in Jatai, Goias, utilizing pathogenicity tests by employing the osmotolerance and thermotolerance in the strains collected.

MATERIAL AND METHODS

The isolates were identified so that each restaurant was labeled with a number from 1 to 17 and with the letters A, B and C representing the first, second and third collection respectively. Fifty-one (51) isolates were collected and stored at the Laboratory of Clinical Biochemistry and Body Fluids at UFJ.

Primary Isolation of Free-Living Amoebae

Fifty-one isolates were utilized from samples of ready-made salads purchased in 17 restaurants in Jatai, Goias. Ten mL of sediment from the spontaneous sedimentation from the cleaning of the salad samples were transferred to Falcon tubes and centrifuged at 2500 rpm, for 15 minutes. The supernatant was discarded after centrifugation. Sowing was performed in Petri dishes containing 1.5% non-nutrient agar, using a cross with *Escherichia coli* (XL10-Gold) sown in BHI broth (Brain Heart Infusion Broth) inactivated by heat (in a water bath of 60.5ºC for 4 hours), when 500µL of the sediment were then inoculated in the center, as shown in Figure 1 (Visvesvara, 2007).

The plates were sealed with Parafilm® plastic and incubated at 36°C for 14 days. After the incubation period, identification was performed by morphological criteria in an inverted optical microscope, when 45 isolates positive for FLA were obtained. The entire seeding process was carried out in a laminar flow cabin at room temperature (Alves, 2012).

Samples that did not allow FLA identification by morphological criteria in the primary isolation were considered negative and discarded, therefore not undergoing the pathogenicity tests (Page, 1976).

The plates where FLA was identified were washed with 2 mL of saline solution (NaCl 0.9%) in order to remove the specimens present on agar surface. The material obtained was transferred to Falcon tubes, centrifuged at 2500 rpm for 5 minutes, the supernatant discarded (Figure 2A) and then 3 mL of PYG (peptone, yeast extract, glucose) liquid medium was added to provide a favorable environment to induce the transformation of the cysts into trophozoites for later use in pathogenicity tests (Figure 2B) (Khan, 2001; Alves, 2012).

Figure 1. Preparation of the plate for primary isolation of FLA. Seeding of *E. coli* (cross "+") and inoculation of the sample containing FLA in the center.

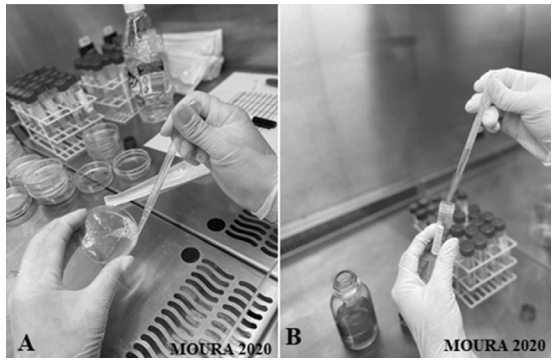

Figure 2. A – Washed samples from positive plates on microscopy for FLA. B – Addition of PYG to the washed sediment.

In vitro *pathogenicity tests.*

Osmotolerance test

According to Khan et al. (2001), the osmotolerance test shows the hyperosmolarity condition *in vitro* with different concentrations of Mannitol PA® (Dinamica Quimica Contemporanea Ltda, Indaiatuba SP, Brazil).

To evaluate the effect of osmolarity on the FLA growth, Petri dishes prepared with 1.5% non-nutrient agar, containing three different concentrations of Mannitol P.A. -Dynamic-salt (0.5 M, 1.0 M, and 1.5 M) were used.

The previously prepared plates were covered with a suspension containing *E. coli* (XL10-Gold), sealed with Parafilm® and incubated at 37°C for 24h to provide growth and colony formation.

Once the colonies had grown, the isolates were inoculated and kept for 5 days (120h) at 30°C (Figure 3).

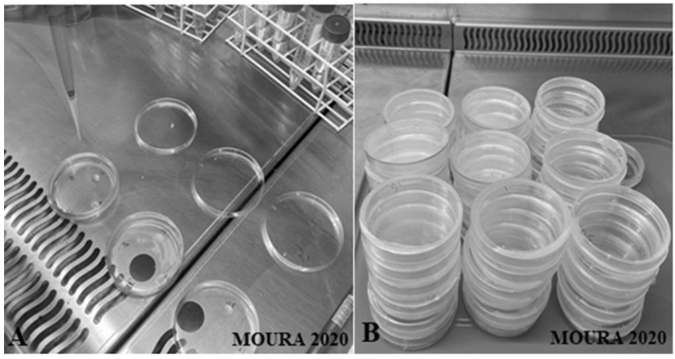

Figure 3. Osmotolerance test. A- Sample inoculation; B – Sealed plates ready for incubation.

The inoculum diameter was measured immediately after inoculation, and after the incubation period, the presence of clear zones was evaluated. To verify the pathogenic degree of the isolated FLA, the formation of clear areas (Figure 4) on the surface of the agar surrounding the inoculum was evaluated, which indicates bacterial consumption by FLA which, in turn, indicates development of the parasite, characterized as positive, and classified as osmotolerant (Winck et al., 2011; Alves, 2012). The test was performed in triplicate for each sample and concentration.

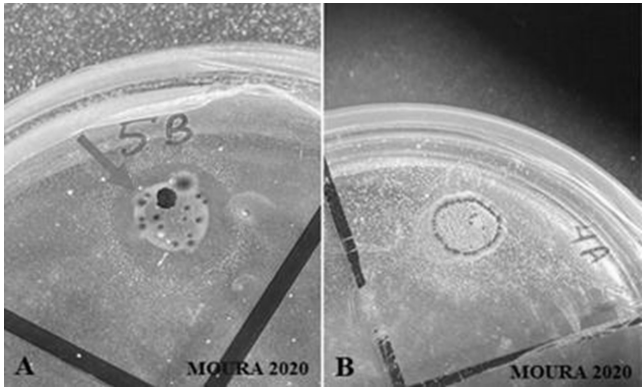

Figure 4. Bacterial consumption. A – Positive result (red arrow); B – Negative result.

Thermotolerance test

The pathogenicity of FLA strains was also evaluated regarding development at different temperatures.

The test was performed using Petri dishes prepared with 1.5% non-nutrient agar and covered with an *E. coli* (XL10-Gold) suspension. Once sowed, the plates were sealed with Parafilm® and incubated for 24 hours at 37°C for bacterial growth to occur. Next, each of the isolates cultivated in PYG liquid medium was incubated at 25°C, 30°C and 37°C for 120h.

The test was performed in triplicate, the inoculum was measured immediately after incubation and the egg white zones after the incubation period were evaluated. Bacterial consumption is measured by the presence of clear zones after 120h. The presence of clear zones on each plate was classified as positive (+) and the absence as negative (-). Those which showed growth were classified as thermotolerant (Winck et al., 2011; Alves, 2012).

RESULTS

Negative samples were identified by an asterisk (*), 45 isolates underwent osmotolerance and thermotolerance tests, as shown in Tables 1 and 2. Out of the 45 isolates submitted to the osmotolerance test, 12 (26.6%) showed growth in 0.5 M mannitol, 18 (40.0%) in 1.0 M mannitol and 16 (35.5%) in 1.5 M mannitol. Isolate 5B grew in all osmolarities, 13 (28.8%) were classified as negative and one (2.2%) did not show growth in any of the concentrations.

From the isolates submitted to the thermotolerance test, 4 (8.9%) presented growth at 25°C, 8 (17.8%) presented growth at 30°C, 3 (6.7%) presented growth at 37°C and 30 (66.7%) did not show growth, being negative at all temperatures.

Table 1. Results of the osmotolerance test in plates with FLA, in isolates from ready-made salads from restaurants in Jatai, Goias, Brazil.

Restaurants analyzed	Concentration (M)	Isolates A	B	C	Restaurants analyzed	Concentration (M)	Isolates A	B	C
1	0.5	*	-	+	10	0.5	+	-	-
	1.0	*	-	+		1.0	-	-	-
	1.5	*	-	-		1.5	-	-	-
2	0.5	-	+	-	11	0.5	-	-	-
	1.0	-	-	+		1.0	+	-	-
	1.5	-	-	-		1.5	+	+	+
3	0.5	+	+	+	12	0.5	-	-	+
	1.0	-	-	+		1.0	+	+	-
	1.5	+	+	-		1.5	-	+	+
4	0.5	+	*	-	13	0.5	-	-	-
	1.0	-	*	-		1.0	-	-	-
	1.5	+	*	-		1.5	-	-	-
5	0.5	*	+	-	14	0.5	-	*	+
	1.0	*	+	-		1.0	+	*	-
	1.5	*	+	+		1.5	-	*	+
6	0.5	-	-	-	15	0.5	-	-	-
	1.0	+	+	+		1.0	+	-	-
	1.5	+	-	-		1.5	+	+	+
7	0.5	-	-	*	16	0.5	*	-	-
	1.0	-	+	*		1.0	*	-	+
	1.5	-	-	*		1.5	*	-	-
8	0.5	-	-	-	17	0.5	+	+	-
	1.0	-	-	+		1.0	+	-	-
	1.5	-	-	-		1.5	-	-	-
9	0.5	-	-	-					
	1.0	+	-	+					
	1.5	-	+	-					

Table 2. Results of the thermotolerance test in plates with FLA, in isolates from ready-made salads from restaurants in Jatai, Goias, Brazil.

Restaurants analyzed	Temperature (ºC)	Isolates A	B	C	Restaurants Analyzed	Temperature (ºC)	Isolates A	B	C
1	25	*	-	-	10	25	-	-	-
	30	*	-	-		30	-	-	-
	37	*	-	-		37	-	-	-
2	25	-	-	-	11	25	-	-	-
	30	-	-	-		30	-	-	-
	37	-	-	-		37	-	-	-
3	25	-	-	-	12	25	-	-	-
	30	+	-	-		30	+	-	-
	37	-	-	-		37	-	-	-
4	25	-	*	-	13	25	-	+	-
	30	-	*	-		30	-	-	-
	37	-	*	-		37	-	-	+
5	25	*	-	-	14	25	-	*	+
	30	*	+	-		30	+	*	+
	37	*	+	+		37	-	*	-
6	25	-	-	-	15	25	-	-	-
	30	-	-	-		30	+	+	-
	37	-	-	-		37	-	-	-
7	25	-	-	*	16	25	*	+	-
	30	-	-	*		30	*	-	-
	37	-	-	*		37	*	-	-
8	25	-	-	-	17	25	-	-	+
	30	-	-	-		30	-	-	-
	37	-	-	-		37	-	-	-
9	25	+	-	-					
	30	-	-	-					
	37	-	-	-					

DISCUSSION

The search for a balanced, healthy diet with high nutritional value and the consumption of raw vegetables produced without the use of pesticides, the so-called organic foods, has increased. In the organic production system, the use of chemical fertilizers is replaced by the use of cattle and organic manure. Irrigation water (from rivers, wells, cisterns or reused) as well as the producer's handling might cause food contamination by parasites. Thus, the end user of this type of food is more susceptible to parasitic infections resulting from contamination in the production process and even in the final preparation in restaurants, for instance (Filho et al., 2019; Silva et al., 2018).

A study carried out in self-service restaurants in Porto Alegre RS, detected the lack of hygienic-sanitary practices in food handling in restaurants, requiring the adoption of hygiene protocols and guidance for handlers, in order to mitigate parasitic infections resulting from the consumption of contaminated food (Gonçalves et al., 2013).

The ability of FLA to grow in environments with high osmolarity and/or high temperatures is directly linked to their pathogenic potential, since such conditions are inhibitory to non-pathogenic species. Osmotolerance and thermotolerance methods have been widely used in order to observe the development and resistance of the parasite, to assess pathogenicity through its growth under these conditions (Khan et al., 2001).

The 1.0 M concentration is considered high osmolarity and studies indicate that strains that grew at this concentration were able to develop alterations in *in vivo* tests (Alves, 2012). The virulence of FLA strains has also been seen to correlate with the ability to develop at 37°C (Khan et al., 2001). The 37°C temperature resembles the human body temperature, so these isolates might be able to survive and cause infection (Duarte, 2010).

This assay inferred that 18 isolates (40.0%) showed growth at high osmolarity and three isolates (6.7%) at a high temperature (37ºC). In addition, 15.6% (7/51) of the samples from ready-made salads served in restaurants in Jatai, Goias showed growth in both analysis and can be considered potentially highly pathogenic and, therefore, a risk to human health.

The presence of FLA in food may also pose risks linked to other microbes (Van der Henst et al., 2018). Besides having pathogenic potential assessed by their ability to tolerate many conditions, FLA can be harbored by or be vectors of a variety of microorganisms (virus, bacteria, protozoa, and fungi), which suggests that finding FLA in food might indicate the presence of other microorganisms responsible for diarrhea and meningitis also transmitted by water and food (Balczum, 2017).

Food contaminated with FLA causes infections, especially in children, in the elderly and in immunocompromised individuals (Silva & Rosa, 2003). In order to prevent this, it is necessary to adopt prophylactic measures as well as making food handlers and the general population aware of the need to improve food hygiene and quality.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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