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RESEARCH ARTICLE

First report of detection of IgA anti-*Acanthamoeba* antibodies among Saudi population and amoeba isolation from their surroundings

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ABSTRACT

Acanthamoeba is an opportunistic protozoan pathogen which is found in diverse environment worldwide. Being ubiquitous nature of this amoeba we come across it in our daily life. *Acanthamoeba* species are recognized as human pathogens; that may cause blinding keratitis and rare but fatal granulomatous encephalitis involving central nervous system. To date, there is not a single report in literature demonstrating anti-*Acanthamoeba* antibodies among the Saudi population, and thus aim of the present study. Using ELISA, we identified the antibody level in the local population. Our results represent the secretory IgA anti-*Acanthamoeba* in mucosal secretions from 133 individuals aged 15–60 years. The anti-*Acanthamoeba* antibody prevalence rate was > 80%, and no considerable differences were observed between prevalence in males (80.28%) and that in females (80.64%). In addition, environmental sources (soil and water) from the environment of the participants in our study were evaluated for amoeba incidence. The amoeba was identified by morphological characteristics of cysts or trophozoites on non-nutrient agar plates grown with *E. coli*. Overall, 58.75% of samples from water and 32.85% of those from soil were culture positive for outgrowth of amoeba on non-nutrient agar plates. Furthermore, PCR was carried out with genus-specific primers to confirm the presence of *Acanthamoeba* DNA. Our results revealed that about 68% of cultures from water and 43% of those from soil were successfully amplified and proved to be amoeba DNA. Interestingly, a few samples yielded more than one product, which suggests that some other amoebic species may be present in the same sample (MAC-W1 and MAD-W1). To the best of our knowledge, we described for the first time the amoeba isolation from the participant's close environment and antibodies level among Saudi population. Our future studies will be focused on additional molecular characterization of isolated amoeba and their pathogenic potential which could be a possible threat for the community.

Keywords: *Acanthamoeba*, IgA, Saliva samples, Isolation, Epidemiology, Prevalence, Protozoa, Saudi Arabia.

INTRODUCTION

Free-living amoebae that belong to the genus *Acanthamoeba* are opportunistic protozoan organisms that are distributed worldwide. The amoeba has been discovered in various media of air, soil and water across the globe; therefore, it plays a pivotal role in the environment. Some *Acanthamoeba* species cause severe and deadly human infections, an example of which is a chronic granulomatous infection that involves the central nervous system (CNS) and occurs among immunocompromised individuals which always leads to death (Visvesvara *et al*., 2007). More than 200 granulomatous amoebic encephalitis (GAE) cases caused by *Acanthamoeba* spp. have been reported worldwide, but none from the Kingdom of Saudi Arabia to date (Schuster & Visvesvara, 2004). *Acanthamoeba* also causes disseminated infections including in the skin, sinuses, lungs, prostate, and uterus (Marciano-Cabral & Cabral, 2003). Furthermore, *Acanthamoeba* trigger keratitis (a painful infection of the eye which leads to blindness) mostly associated with contact lens wearers (Verani *et al*., 2009; Panjwani, 2010; Niederkorn, 2020). *Acanthamoeba* species have become increasingly recognized as important microbes. Infections caused by *Acanthamoeba* have gained attention over the years due to their presence in the ecological habitat (Rezaeian *et al*., 2008; Liang *et al*., 2010), their direct connection with humans on a daily basis, and their accountability for human diseases (Anisah *et al*., 2005; Ledee *et al*., 2009). The number of infections has

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increased, probably because of increasing numbers of contact-lens wearers, increasing populations of immuno-compromised patients, and global warming that has caused the amoeba to flourish. Of the hundreds of infections that have been caused by *Acanthamoeba*, only a few victims have survived because of delays in diagnosis due to lack of knowledge and poor empirical treatment due to lack of optimal therapy.

Due to the widespread nature of *Acanthamoeba*, it is speculated that most people have developed antibodies against this organism. Two studies have been demonstrated the incidence of anti-*Acanthamoeba* antibody titres from healthy populations (Cursons *et al*., 1980; Cerva, 1989). Of these studies, the Cerva research, which was conducted in New Zealand, shows that normal human sera possess antibodies in the ratios of 1:20 to 1:80 titres for *Acanthamoeba* spp from 100% of individuals tested (Cerva, 1989). On the other hand, of the participants in one investigation conducted in Bohemia, Czechoslovakia, it was discovered that only nine (3.2%) were seropositive (Cursons *et al*., 1980). Furthermore, 448 patients in a Prague hospital were investigated and 41 (9.1%) were found to be positive (Cursons *et al*., 1980). Among patients with CNS disorders at a psychiatric hospital, nine (3.3%) of 274 patients were reported to be positive and possessed the highest titres of positive sera at 1:160. Of note, 26 (52%) of 50 hepatitis A patients were observed to be positive for *Acanthamoeba*, with the highest titres of positive sera being 1: 640 (Cursons *et al*., 1980).

Overall, these findings reveal the contradictory nature of human contact with *Acanthamoeba*. Later studies tested for the presence of *Acanthamoeba* antibodies among 43 healthy individuals and 25 *Acanthamoeba* keratitis patients (Alizadeh *et al*., 2001; Walochnik *et al*., 2001). It is of note that all tested individuals were seropositive for *Acanthamoeba*; however, the keratitis patients confirmed considerably lower levels of sIgA compared with the healthy subjects. Here, we report our investigations into the level of anti-*Acanthamoeba* antibodies within the hitherto unexplored Saudi population, along with the prospects of isolation of *Acanthamoeba* from the surrounding environment of those who took part in our study.

MATERIALS AND METHODS

Acanthamoeba cultures

A clinical isolate of *Acanthamoeba castellanii* belonging to T4 genotype, isolated from a keratitis patient (American Type Culture Collection, ATCC 50492) was used in the present study. T4 genotype is considered to be the highly prevalent in the environment (Niyyati *et al*., 2009) and known to be the more frequently amplified in the laboratory conditions than other genotypes (Kawaguchi *et al*., 2009); thus chosen for the present investigation. *Acanthamoeba castellanii* trophozoites were grown without shaking in 15 ml of PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] in T-75 tissue culture flasks at 30°C and the media was refreshed 17–20 h prior to experiments as previously described (Imran *et al*., 2016; Rafique *et al*., 2020). This resulted in more than 99% amoebae in the trophozoite forms, which were subsequently used in experiments.

Collection of environmental samples from the participants' surroundings

Environmental samples were collected from the study participants' close environment (water from bathrooms, kitchens and mosques; soil from gardens and potted plants), using sterile polypropylene bottles and bags respectively, from June 2016 to July 2018. All sealed packed samples were tagged with the time, date, type and site of collection and stored at 4°C until used as described previously (Tanveer *et al*., 2015).

Acanthamoeba isolation using plating assays

For *Acanthamoeba* isolation, plating assays were performed as described previously (Lorenzo-Morales *et al*., 2006; Tanveer *et al*., 2013). Briefly, bacteria were grown in Luria-Bertini (LB) media, containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl overnight, and heat killed as described previously (Tanveer *et al*., 2013). Heat-killed bacteria were transferred on to non-nutrient agar plates and left for 2–3 minutes. The surplus cultures were removed and plates left to dry. Water samples (50 mL) were filtered through the nitrocellulose membrane (pore size: 0.2 µM) and filters were placed inverted on 1.5% non-nutrient agar (NNA) plates that housed an *E. coli* lawn. Plates were kept at 30°C. Plates were incubated while sealed in plastic bags to prevent dehydration and investigated with an inverted microscope. Subsequently amoebae were detected that were nourished by *E. coli* on NNA; segments of the agar that contained these amoebae were removed and relocated onto new bacteria-coated plates. After the amoebae of interest had been separated from fungal and other contaminants, they were relocated in agar cores to fresh, bacteria-lawn agar plates. Next, the plates were observed for out-growth of *Acanthamoeba* (either cysts or trophozoites or both) microscopically for up to two weeks. In contrast, soil samples (2g) were weighted and suspended in 20 mL of sterile distilled water and the soil particles were allowed to settle in the tube. Furthermore, water sample (150 µL) was taken from the tube and inoculated onto 1.5% NNA plates, as described before (Lorenzo-Morales *et al*., 2006; Tanveer *et al*., 2015).

DNA extraction

Amoebae were scraped from the NNA plates, and DNA extraction was carried out as described before (Matin *et al*., 2006; Tanveer *et al*., 2015). Briefly, amoebae were washed 3× in PBS (pH 7.4) and resuspended in 500 µL of cell lysis buffer (100 mM KCl, 40 mM Tris, 25 mM $MgCl_2$, 1% Tween-20, and 0.1 mg/mL proteinase K). After gentle mixing by inversion, the lysates were incubated at 56°C for 3h. These samples were chilled on ice for 5 min and were extracted with equal volumes of phenol-chloroform (1:1). After extraction, the aqueous and organic phases were separated by centrifugation at 15,000 rpm for 15min at room temperature (RT). Next, the supernatant was extracted with an equal volume of chloroform-isoamyl alcohol (24:1). After centrifugation at 15,000 rpm, the upper aqueous phase was collected and DNA was precipitated by adding 1/10 volume of 10 M ammonium acetate plus two volumes of cold absolute ethanol and kept at -20°C overnight. The DNA was precipitated by centrifugation at 15,000 rpm, after washing with 100 µL of 70% ethanol, then dried at RT, dissolved in 300 µL of double-distilled water, and stored at -20°C until used.

Amplification by polymerase chain reaction

The DNA amplification was carried out using genus-specific primers as described previously (Booton *et al*., 2002; Matin *et al*., 2006; Tanveer *et al*., 2013). Primer pairs include the forward primer JDP1 (5-GGCCCAGATCGTTTACCGTGAA) and the reverse primer JDP2 (5-TCTCACAAGCTGCTAGGGAGTCA). Polymerase chain reactions (PCRs) were executed in a 20 µL volume that contained 1.25 U Taq polymerase, 0.2–0.4 µg DNA, 200 µM

dNTPs, 2mM $MgCl_2$, and 2 µM primer, at 95°C for 5 min for one cycle, 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 35 cycles. There was a final elongation step of 10 min at 72°C. Amplified DNA was electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualised under ultraviolet light.

Detection of antibodies using enzyme-linked immunosorbent assays

The presence of anti-*Acanthamoeba* sIgA in mucosal secretions (saliva samples collected from healthy volunteer persons) was established using enzyme-linked immunosorbent assays (ELISA) as described previously (Brindley *et al*., 2009; Matin *et al*., 2012). Briefly, *Acanthamoeba* (50,000 amoebae per 0.2 mL/well) were inoculated in PYG growth medium in a 96-well plate at 30°C for 24 h. Consequently, the wells were air dried, and then ice cold methanol and acetone (1:1) were added for 45 min. The wells were washed twice with PBS (containing 0.05% Tween-20) to eliminate non-adherent amoebae and blocked using 3% bovine serum albumin for 1 h at 37°C. Saliva samples were obtained from 133 asymptomatic participants, and stored at 4°C until tested. The saliva samples were serially diluted from 1:1 to 1:100. The wells were rinsed and 100 µL of test samples were added to wells and incubated for 18 h at 4°C. The subsequent day, amoebae were rinsed 3× with PBS plus Tween-20 and wells were incubated with mouse anti-human IgA antibody (Abcam, Cambridge, UK). The plates were incubated for 60 min, after which the wells were rinsed again 3× as described above. Next, anti-mouse IgG antibody conjugated to horseradish peroxidase was placed in all wells. The plates were incubated for 1 h at 37°C. Finally, the wells were rinsed 3× as described above and 100 µL of substrate solution (0.1% H_2O_2 and 0.1% orthophenylenediamine in citrate buffer) was added. The reactions were permitted to develop for 15 min and finally 100 µL of 3% sulphuric acid was added to stop the reaction. The optical density of each well was established on a microplate reader at 492 nm. The optical density of *Acanthamoeba* incubated with secondary antibody in the absence of saliva samples was measured as a background. *Acanthamoeba* seroprevalence is defined here as saliva reacting with *Acanthamoeba castellanii* of the T4 genotype with an optical density above the background values.

RESULTS

ELISA detects *Acanthamoeba*-specific sIgA in mucosal secretions of Saudi population

To establish the presence of anti-*Acanthamoeba* sIgA in saliva samples, ELISA was performed as described in Materials and Methods. A total of 133 samples were collected from healthy residents of Saudi Arabia. The participants' ages varied from 16 to 65 years. The age and gender characteristics are shown in Table 1. The overall prevalence of *Acanthamoeba* was 80.64% in females and 80.28% in males. There was no significant difference in the findings between genders ($P > 0.05$, using paired T test, one tail distribution). The anti-amoeba antibodies prevalence was observed approximately 85% in the saliva samples of Saudi population among 16-29 age range which was increased to 100% among 30 years of age and onwards.

***Acanthamoeba* isolated from the nearby surroundings of all subjects tested**

To find the level of common contact of the Saudi population with *Acanthamoeba*, ecological samples taken from participants' surroundings were evaluated. These were tested to discover the incidence of *Acanthamoeba*, as described in Materials and Methods. On the basis of morphological characteristics of cysts or trophozoites, amoebae from the genus *Acanthamoeba* were recognized as positive (as shown by outgrowth of protozoa) from water samples on NNA plates after seven to 14 days of incubation when examined visually under an inverted microscope. The detached amoebae were preserved and decontaminated by sporadic cutting of a small piece of agar that contained cysts or trophozoites and transplanting it to a fresh NNA plate coated in a heat-killed bacteria lawn. Overall, 47 out of 80 (58.75%) samples from water and 23 out of 70 (32.85%) from soil were culture positive for outgrowth of amoebae on NNA plates (representative examples are displayed in Figure 1).

PCR amplification confirms existence of *Acanthamoeba* DNA in both soil and water samples

To further verify the presence of amoebae population on NNA plates, DNA was obtained from the scrapings acquired from NNA plates and exploited for PCR reactions using *Acanthamoeba* genus-specific JDP1 and JDP2 primers, as described in Materials and Methods. Most of the samples yielded a sole PCR product of fewer than 500 bp, which validated the existence of *Acanthamoeba* DNA. Interestingly, a few samples yielded more than one PCR product, which suggests that some other amoebic species may have been present in the same sample (MAC-W1 and MAD-W1). Our results revealed that 32 of 47 (68.08%) samples from water and 10 of 23 (43%) from soil were fruitfully amplified.

DISCUSSION

The genus *Acanthamoeba* belongs to a free-living amoeba family that has become progressively more distinguished as an important microscopic organism in recent years. *Acanthamoeba* are well known as human pathogens that may cause serious and fatal infections. The number of *Acanthamoeba* infections has escalated worldwide due to the existence of this amoeba in the normal ecosystem (Rezaeian *et al*., 2008; Liang *et al*., 2010; Latifi *et al.*, 2020) and its direct association with humans in everyday life and is responsible for human diseases (Anisah *et al*., 2005; Ledee *et al*., 2009). The increasing number of infections is most probably due to escalating statistics of contact-lens wearers, mounting populations of immunocompromised patients and the rise of global warming which aids the growth of the amoebae. Infections caused by *Acanthamoeba* have been

Table 1. Prevalence by age of anti-*Acanthamoeba castellanii* (T4 genotype) antibodies among males and females of the study cohort. Total number of subjects surveyed is shown in bracket

Age	Males	Females
16–19	30 (34)	24 (27)
20–24	8 (10)	7 (8)
25–29	2 (3)	2 (2)
30–34	3 (4)	1 (3)
35–39	2 (2)	2 (3)
40–44	2 (4)	2 (3)
45–49	2 (2)	1 (1)
50–54	2 (2)	1 (1)
55–59	1 (2)	2 (2)
60+	1 (1)	1 (1)
NA	4 (7)	7 (11)
Total	**57 (71)**	**50 (62)**

Table 2. *Acanthamoeba* isolation on non-nutrient agar plates from surrounding environments of the participants, amoebic DNA confirmation through PCR and antibody prevalence via ELISA of the representative areas of Saudi Arabia

S. No	Sampling from participant's close environment with sampling codes		Sampling area	Amoebic outgrowth on NNA Plates		PCR		Level of antibodies detection from participant's saliva
	Water	Soil		Water	Soil	Water	Soil	
1	ABH-W3	ABH-S4	Abha	+	+	+	+	+++
2	BIDH-W1	BIDH-S2	Buraidah	–	–	–	–	+
3	DAM-W5	DAM-S1	Dammam	+	+	+	–	++
4	KAJ-W1	KAJ-S3	Al Kharj	–	–	–	–	+
5	KHR-W4	KHR-S6	Khobar	+	+	+	–	++
6	HAI-W2	HAI-S5	Hail	+	+	+	–	++
7	HAR-BAT-W3	HAR-BAT-S1	Harar Al-Batin	+	+	–	–	++
8	HOF-W4	HOF-S2	Al Hofuf	+	–	–	–	++
9	JAF-W7	JAF-S3	Jawf	–	–	–	–	+
10	JAZN-W2	JAZN-S1	Jazzan	+	+	+	–	+++
11	JEDH-W3	JEDH-S2	Jeddah	+	–	+	+	+++
12	MAJ-W2	MAJ-S5	Majmaah	–	–	–	–	++
13	MAD-W1	MAD-S2	Madina	+	+	+	+	+++
14	MAC-W1	MAC-S1	Mecca	+	+	+	+	+++
15	NAJ-W1	NAJ-S2	Najran	+	–	–	–	++
16	QAS-W1	QAS-S1	Qaseem	+	+	+	+	+++
17	RIYH-W3	RIYH-S4	Riyadh	–	+	–	–	++
18	TABK-W4	TABK-S7	Tabuk	+	+	+	–	+++
19	TAF-W6	TAF-S3	Taif	–	+	–	–	++
20	ZUL-W3	ZUL-S2	Al Zulfi	–	–	–	–	+

Frequency of antibody level: Rare: +; Medium: ++; Significant: +++.

reported worldwide, but not previously from Saudi Arabia, perhaps due to lack of knowledge and expertise.

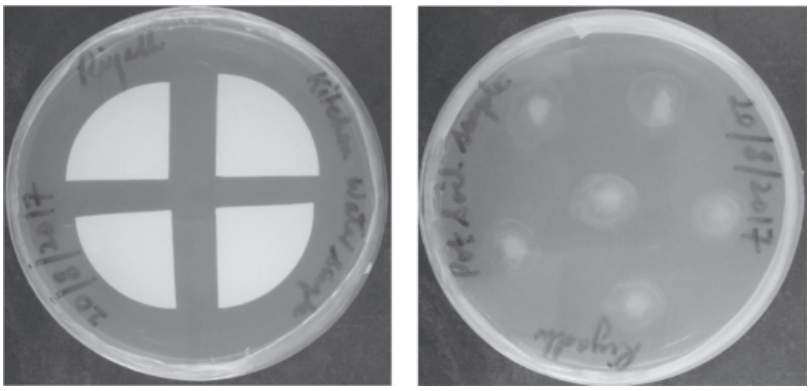
Until now, there has been no documentented proof of *Acanthamoeba* infection reported from the Gulf region (including Bahrain, Kuwait, Oman, Qatar, Saudi Arabia and the United Arab Emirates) except from Oman, where two *Acanthamoeba* keratitis cases have been reported so far (Al-Kharousi & Wali, 2009, 2012). Due to lack of expertise on this pathogen and lack of knowledge regarding this amoeba and disease, it may have gone unnoticed. Knowing the free-living characteristics of this organism, it is likely that humans interact with *Acanthamoeba* in their routine daily lives. However, it has been observed that keratitis caused by *Acanthamoeba* is frequently misdiagnosed as a herpes simplex adenovirus or other viral infection (Hammersmith, 2006).

To date there has been no documented evidence available in the literature which demonstrates the anti-*Acanthamoeba* antibody level among the Saudi population. For this purpose a noninvasive approach (sIgA in saliva) was adopted in this investigation and ELISA was carried out to establish the existence of anti-*Acanthamoeba* antibodies. It is worth mentioning that most of the participants involved in this study had undertaken at least one trip abroad (to the UK, USA or Europe) either for tourism or study and at least one trip to Makkah and Madina to perform Hajj or Ummarah. Saudi inhabitants consume filtered, bottled, mineral water which is free from pollutants (data not shown); however, the whole population uses untreated tank water to perform ablutions at least five times a day, and this could enable the interaction of this amoeba with the Saudi population either though the mouth or nose. This would explain the finding that all the participants over the age of 30 possessed antibodies against this amoeba, suggesting the local population have developed antibodies which ultimately protects from amebic infection as described previously (Leher *et al*., 1999).

Overall, the present study disclosed a high frequency (87.7%) of anti-*Acanthamoeba* antibodies, to a similar degree in males (80.28%) as females (80.64%). *Acanthamoeba* were also found in samples taken from the participants' close environment. This finding endorses the free-living nature of these pathogens to which we are exposed in our everyday lives. It has been shown that more than 80% of the healthy human population exhibits antibodies against *Acanthamoeba* (Chappell *et al*., 2001), suggesting that these organisms often come into contact with humans.

Our previous investigation to determine the presence of anti-*Acanthamoeba* antibodies among the population of Pakistan showed the highest prevalence among the tribal population of Khyber Pakhtunkhwa as compared with the rest of the country (Matin *et al*., 2012). *Acanthamoeba* has also been isolated from various environmental sources (soil and water) across Pakistan (Tanveer *et al.*, 2013, 2015). In Pakistan, the majority of the population use spring water or municipal water, as the majority cannot afford mineral/bottled water, and this could mean that the population comes across this amoeba on a daily basis. Also, Pakistan is an agricultural country; >70% of the population is engaged with agriculture, which also leads to frequent contact with this amoeba. In contrast, in another report, heterogeneous populations of 114 subjects from 37 countries and six continents were studied in the United Kingdom (Brindley *et al*., 2009). Of these 114 individuals, 100 were found to test positive for the presence of sIgA, an overall prevalence of 87.7% from all continents tested. The high prevalence of the *Acanthamoeba* antibody in ethnically and racially diverse populations suggests that (i) people come across these pathogens frequently in their normal surroundings and (ii) few diseases are caused by this organism (Brindley *et al*., 2009). Similarly, in the present investigation, of 133 participants recruited from the Kingdom of Saudi Arabia, 107 were found to be positive for the presence of sIgA, an overall prevalence of 80.45% from all regions tested. Notably, the participants from

(A) **(B)**



Labeled information on Plate A: Water sample Sampling collection site: Riyadh Water source: Kitchen Water Date of sampling: 20/08/2017	**Labeled information on Plate B: Soil sample** Sampling collection site: Riyadh Soil source: In house Pot Date of sampling: 20/08/2017

(C)

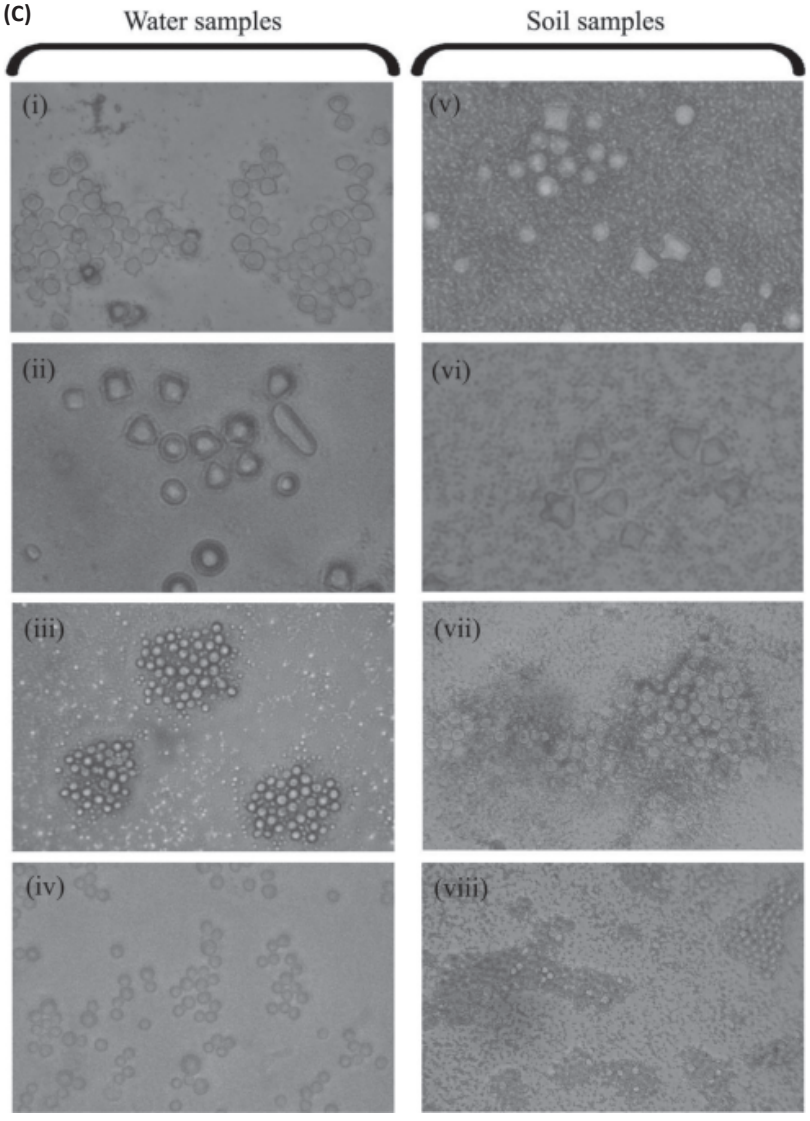


Figure 1. (a) A water sample (500 mL) was systematically stirred and filtrated via a cellulose nitrate filter and harvested on an NNA plate bedded with heat-killed *E. coli* as described in Materials and Methods. **(b)** Soil samples (2g) were suspended in 20 ml of sterile distilled water and 150 µL of each sample was harvested onto NNA plates, as described in the Materials and Methods section. **(c)** Amoebic plaque was morphologically observed on NNA plates under an inverted microscope. Plates were observed for outgrowth for up to 14 days and images (×400) taken. Images of representative water samples: (i). MAC-W1; (ii). JAZN-W2; (iii). MAD-W1; (iv). ABH-W3 and soil samples (v). QAS-S1; (vi). TABK-S7; (vii). HAR-BAT-S1; (viii). HAI-S5 are shown here.

Makkah and Madina were shown to have the maximum antibody level and amoebae were isolated from both environmental sources (soil and water) of the participants' surroundings.

The existence of free-living amoeba in environmental sources (soil and water) of Saudi Arabia is not surprising as it has been reported in the past (Al-Herrawy & Al-Rasheid, 1998; Toula *et al*., 2017). These two studies did not demonstrate much information regarding the prevalence of the amoeba in the Saudi environment. However, millions of Muslims visit Makkah and Madina for Hajj and Ummarah each year, and this could lead to the transmigration of different amoeba species from different countries to Saudi. Furthermore, thousands of animals are imported for slaughter from different countries to Makkah during the Hajj. So transportation of the amoeba through animals could also occur. Amoebic meningoencephalitis has been shown in sheep (Van der Lugt & Van der Merwe, 1990). Our research group has studied free-living amoeba since 2005; our interests are to explore the diverse environmental sources of the country to understand the epidemiology of the organism in this part of the world. Such studies have been carried out in the UK, Pakistan and Saudi Arabia. It was observed in this study that 85% of samples collected from Saudi Arabia showed growth on NNA plates that resembled morphologically the amoebic cysts, but when the same outgrowth from NNA plates was tested through amplification using *Acanthamoeba* genus-specific primers, only 42 out of 70 (60%) could be established as *Acanthamoeba*. This could be because of various factors. It is rational to expect that the non-amplified products might indicate the incidence of other protozoan cysts, since an earlier study (Arnalich-Montiel *et al*., 2013) has demonstrated the co-isolation of two different amoebae (*Vahlkampfia* and *Acanthamoeba*) from Spain. Further molecular recognition of the cysts indicated in our study could be the subject of further research.

CONCLUSION

To the best of our knowledge, this is the first comprehensive report across the Kingdom demonstrating the level of anti-*Acanthamoeba* antibodies among the Saudi population, along with isolation of amoeba from the participants' close environment (soil and water). Our current study suggests that there is an urgent need to investigate other natural/ ecological resources (air, soil and water) of the Kingdom to assess the current status of FLA (*Acanthamoeba*, *Balamuthia*, *Naegleria* and *Sappinia*), which may pose potential health threats in future. Furthermore, analysis of the isolated amoeba during this investigation is essential in order to establish their molecular identification, pathogenic potential and role in our surrounding environment. Our future studies will also be focussed on the exploration of role of environmental factors (i.e., temperature, pH, salt or mineral concentration, water & soil quality and other microbial community) which supports *Acanthamoeba* to withstand hard natural conditions and disseminate in our natural ecosystem.

List of abbreviations

bp: Base pair; CNS: Central nervous system; DNA: Deoxyribonucleic acid; dNTP: Deoxyribonucleotide triphosphate; ELISA: Enzyme-linked immunosorbent assays; *E. coli: Escherichia coli;* FLA: Free-living amoebae; GAE: Granulomatous amoebic encephalitis; IgA: Immunoglobulin A; IgG: Immunoglobulin G; ITS: Internal transcribed spacers; KCl: Potassium Chloride; LB: Luria-Bertini; *L. pneumophila: Legionella pneumophila;* MERS-CoV: Middle East Respiratory Syndrome Coronavirus; $MgCl_2$: Magnesium Chloride; mg/ml: Milligram / Millilitre; µg: Microgram; mM: Mili Molar; NNA: Non-nutrient agar; PAM: Primary amoebic meningo-encephalitis; PBS: Phasphate buffer saline; PCR: Polymerase chain reaction; PYG: Peptone yeast glucose; RT: Room temperature; sIgA: Secretory immunoglobulin A; µM: Micro molar.

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The authors are thankful to the students of Majmaah University for their tremendous support in communication with the local population. We also extend our thanks to the locals who kindly provided to us the required human body secretions and nearby environmental water and soil samples for this study. Initially, there were many issues during sample and information collection due to local cultural values and the inability of the residents to communicate in English. The students helped with communication, convinced the reluctant residents to offer samples and transported the samples in a timely and well-managed manner to the laboratory. There were fewer difficulties in the big cities such as Riyadh and Jeddah, where the people were very supportive and accommodating of our wishes. There were many discussions with the Saudi people regarding the benefits of such studies; since such studies have not been conducted in the kingdom in the past, it was difficult to communicate and convince the community of the worth of such research. In this regard 30 male students from Majmaah, Hautat Sudair and Zulfi campuses and seven female students were involved in this study. The details of the students are not disclosed at their request. These students are still involved in some of the other projects that are underway.

Competing Interests:

The authors declare that they have no competing interests.

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