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Isolation and molecular identification of *Acanthamoeba* spp. from hospital dust and soil of Khomein, Iran, as reservoir for nosocomial infection.

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ABSTRACT

Background: *Acanthamoeba* spp. are commonest opportunistic amoebae, which ubiquitous in various environmental resources. *Acanthamoeba* species are the causative agents of amoebic keratitis, granulomatous amoebic encephalitis and i.e. in immunocompromised and immunocompetent patients. Moreover *Acanthamoeba* spp. can act as reservoir and transmission agent of bacterial pathogens. Due to this issue the aim of this study was to characterized *Acanthamoeba* spp. genotypes in dust and soil of hospital samples from Khomein of Iran.
Methods: In a cross sectional study, a total of 100 soil and dust samples were collected from hospital environment of Khomein Iran, and analyzed for the presence of *Acanthamoeba* spp. based on phenotypic and molecular methods including PCR amplification and sequence analysis of 18SrRNA. A total of 5 *Acanthamoeba* isolates were sequenced, and different genotypes of isolates were detected via direct sequence analysis.
Results: The results showed that 20% of samples (20/100) were positive for *Acanthamoeba,* while only 5 cases were successfully cultured in NNM medium and were subjected to molecular assay. *A. lenticulata, A. castellanii and A. quina* were the prevalent identified species that were belonged to T4 and T5 genotypes.
Conclusions: *Acanthamoeba* spp. are the most prevalent free living amoeba in the dust and soil of hospital environment. Moreover, due to the presence of potentially pathogenic T4 genotypes in our hospital, it is recommended that in health and hygienic programs elimination of FLA should be considered.

1. Introduction

Free-living amoebae (FLA) are protozoan parasites that extensively colonized a wide variety of natural habitats including water, soil, dust, biofilms and water-air interface (Kialashaki et al., 2018; Bullé et al., 2020). In this regard some FLA such as *Acanthamoeba* spp., *N. fowleri* and *Balamuthia mandrillaris*, are opportunistic parasites which do affect the immunocompromised and immunocompetent (Król-Turmińska and Olender, 2017). Some genera especially *Acanthamoeba* spp. due to their resistant cyst stage could survive

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in harsh environmental sources such as hospital soil, dust and water. Therefore, they are the agents of granulomatous amebic encephalitis (GAE), *Acanthamoeba* keratitis (AK), pneumonia (*Acanthamoeba* pneumoniae) and cutaneous acanthamoebiasis (CA) (Król-Turmińska and Olender, 2017; La Hoz et al., 2019).

Acanthamoeba spp. in addition to directly infect human and environmental resources such as hospital environment could act as a reservoir for the storage and transmission of a variety of opportunistic pathogen bacteria that cause nosocomial infections, including *Legionella* spp., *Pseudomonas auruginusa, H. pylori, Vibrio cholera,* non-tuberculosis mycobacteria, *and L. monocytogenes* (Guimaraes et al., 2016).

Due to the living of *Acanthamoeba* spp. in hospital environmental resources including air conditioning, water distribution systems and subsequently the coexistence of pathogenic bacteria within *Acanthamoeba* spp. as well as, the present of immunocompromised patients in the hospital, in this study we aimed to assess the frequency and diversity of *Acanthamoeba* spp. species capable of survival in the hospital environment, by applying molecular and conventional methods in order to provide a better insight into their role as a reservoir for the transmission and development of nosocomial infections.

2. Materials and methods

2.1. Ethics statement

The Ethics Committee of khomein University of Medical Sciences approved this study (IR.KHOMEIN.REC.1398.006), and the study was performed in accordance with the approved guidelines.

2.2. Sampling and conventional identification

In the cross sectional study between August 2019 and July 2020, a total of 100 swab samples were collected from dust in different wards of Imam Khomeini hospital in Khomein, including internal medicine (no = 20), ophthalmology (no = 12), surgery (no = 16), emergency (no = 14), pediatrics (no = 16), ICU(no = 12), and maternal and midwifery (no = 10) wards by standard methods. The samples were transferred to the department of laboratory sciences of Khomein University of Medical Sciences and processed based on standard method (Chan et al., 2011). In brief: The swabs were vortexed in 50 ML sterile distilled water and allowed to stand at room temperature for 10 min. The suspensions were then filtered through a cellulose nitrate membrane filter (0.45 μM). Non-nutrient agar (NNA) was prepared with amoeba normal saline, which consist of 0.5 mM Na_2HPO_4, 1 mM KH_2PO_4, 2.5 mM NaCl, 40 μm $CaCl_2$-$6H_2O$ and 20 μm $MgSO_2$. $7H_2O$. The final pH of solution was adjusted to 6.9 with KOH. The filters were placed on 1% NNA medium and were incubated at room temperature for 2 weeks for detection of trophozoites or cysts of amoeba.

FLA of the genus *Acanthamoeba* were recovered by the morphological features of the trophozoites and cysts, and was sub-cultured in fresh NNA medium. Isolates for further molecular identification were kept in 2% NNA plates.

2.3. Molecular identification

Chromosomal DNA of *Acanthamoeba* spp. isolates were extracted by the method used by Golestani et al. (Golestani et al., 2018). in brief: the isolates were suspended into sterile PBS pH 7.2 and centrifugated at 1000 rpm for 15 min, the pellet was resuspended in lysis buffer (10 mmol/l EDTA, 50 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 8.0 and SDS 1%) and incubation overnight with 0.25 mg/ml proteinase K at 60 °C. The suspension was centrifuged at 20000 ×*g* for 10 min, then supernatant was transferred to another sterile microtube and precipitated DNA was resuspended in 50 μl Milli-Q water and stored at 20 °C. PCR was performed using JDPs (JDP1—JDP2)3 primer pair.

The phenotypically identified *Acanthamoeba* isolates, were analyzed for the genus and species levels using a PCR based amplification and direct analysis of sequencing of 450-bp fragment (JDP1—JDP2 primer) in 18S ribosomal RNA specific to *Acanthamoeba* species recommended by fuerst et al. PCR was performed in a volume of 50 μL containing 1.25 U Taq polymerase, 30 ng template DNA, 200 mmol/l dNTP, 1.5 mmol/l $MgCl_2$ and 0.2 mol/l of each primer. The cycling conditions were: an initial denaturing phase at 95 °C for 2 min and 30 repetitions at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s with an elongation step of 5 min at 72 °C in the last cycle (Fuerst et al., 2015). Sequencing was performed by the Pishgam Biotech (IRI), and the sequence data received were aligned manually with existing sequences of *Acanthamoeba* spp. retrieved from the GenBank database and analyzed using the Blast program in GenBank and the jPhydit program (Jeon et al., 2005). Finally *Acanthamoeba* isolates were identified at the genotype level based on the sequence analysis of the Diagnostic Fragment with other genotype sequences (Niyyati et al., 2009a).

2.4. Statistical analysis

The collected data were recorded in SPSS version 20 (SPSS Inc. Chicago, IL, USA) and analyzed by Chi square test. *P* value less than 0.05 was considered to be significant.

2.5. Accession numbers

The GenBank accessions numbers for the 18S rRNA sequencing of *Acanthamoeba* spp. isolates in this study are listed below. *A. quina* (A3, A4, A7): MT539092, *A. castellanii* (A6): MT539095 and *A. lenticulata* (A5): MT539094.

2.6. Thermo and osmotolerance assays of the positive strains for pathogenicity

The thermo and osmotolerance assay recommended by Todd et al. (Todd et al., 2015) was used to evaluate the pathogenicity of the isolates. For thermo tolerance assay, approximately 10^3 trophozoites were inoculated onto NNA medium and incubated at 30, 37 and 40 °C. For osmotolerance assay 10^3 trophozoites were inoculated in NNA medium containing mannitol 0.5 and 1 M. Positive and negative control was also applied. All plates were tested for the outgrowth of *Acanthamoeba* spp. after 24, 48 and 72 h.

3. Results

In this study, according to the morphological criteria a total of 20 FLA (20%) were isolated from 100 hospital dust and soil samples. From the 8 hospital department analyzed, *Acanthamoeba* spp. was isolated only in 4 indoor areas as shown in Table 1. Trophozoites were characterized by their irregularly renal-shape or pear- shape structures and some of them had fine pseudopodia-like extensions. Ectocysts of *Acanthamoeba* were characterized, by double-walled cysts with round external wall and wrinkled, triangular, angular and asteroid inner wall.

From 20 primary isolates, only 5 isolates were subcultured successfully after 4 weeks, subsequently molecular assay was performed only on these 5 isolates. The 18S rRNA gene sequencing of the isolates showed that all isolates had nucleotide signatures of *Acanthamoeba* spp. and belonged to T4 and T5 genotype as the most isolated type with 100% similarity with nearest validated species. Based on phenotypic and molecular data, the isolates A3, A4, A7 were identified as *A. quina*, the isolate A5 was identified as *A. lenticulata* and the isolates A6 was identified as *A. castellanii* (Table 1). The relationship between our *Acanthamoeba* spp. isolates and the validated species *Acanthamoeba* was supported by a high bootstrapping value in the phylogenetic tree constructed based on the 18S rRNA gene (Fig. 1).

The thermo and osmotolerance pathogenic assay showed that the isolates A5 and A6 (40%) which were identified as *A. lenticulata* and *A. castellanii*, belonging to T4 genotype and they showed pathogenic capability. Nevertheless, the isolates A3, A4, A7 (60%) were identified as *A. quina* belonged to T5 genotype and are not pathogenic.

4. Discussion

Acanthamoeba spp. has been isolated from numerous environmental resources including water, dust, soil, and hospital equipment's and environment (Tawfeek et al., 2016). The high frequency of *Acanthamoeba* spp. in various environment especially in hospital environmental resources, represents a high risk of infection such as skin lesions, keratitis, encephalitis, and pneumonia in immunocompromised and immunocompetent patients, which were exposed to this sources (Wopereis et al., 2020; Carlesso et al., 2010; Martinez and Janitschke, 1985). Furthermore, *Acanthamoeba* spp. could act as a reservoir and carrier for transmission of different bacterial pathogens such as *Helicobacter* spp.., *Legionella* spp. *and Pseudomonas* spp.. to the human (Borecka et al., 2020).

Microbial surveillance of the hospital environmental resources, especially in developing countries, does not include analyzing for the presence of FLA. On the other hand, in developing countries conventional tests used in medical laboratories for isolation and characterization of FLA agents are not able to identify the *Acanthamoeba* spp., hence control of these organisms in the hospital environment and setting are difficult (Trabelsi et al., 2016). However, many studies have showed that contamination of hospital equipment and supplies, traced to the persistence of FLA in dust and soil, and their high resistance to disinfectants, could be responsible for pseudo-outbreaks of infections associated with *Acanthamoeba* spp. (Carlesso et al., 2010; Marciano-Cabral and Cabral, 2003). With this regards, this study assessed isolation and molecular identification of *Acanthamoeba* spp. in hospital environment in our region. This knowledge would contribute to understanding the species diversity and circulating genotypes of *Acanthamoeba* spp. in health care setting.

Our study showed that the prevalence rate of *Acanthamoeba* spp. was 20% (20 isolate from 100 samples). In a previous study conducted in Iran and other region of the world, the isolation rate of *Acanthamoeba* spp. from hospital environment was between 17% - 51.8% (Golestani et al., 2018; Carlesso et al., 2010; Kilic et al., 2004), which is in correlation with the results of current study. In the present study, the most *Acanthamoeba* polluted ward, are the internal medicine (10%), emergency (5%), and ocular wards (5%), respectively which lacked adequate health facilities. This results showed that hospital setting could act as main reservoir of different potentially pathogenic FLA, which can be transmitted to the patient.

Isolation of *Acanthamoeba* spp. in 20% of soil and dust samples collected from hospitals environment of Khomein city of Iran, reflect

Table 1
Sampling site and molecular features of *Acanthamoeba* isolates.

Sample	Hospital units	NNA Culture /PCR	Temperature tolerance/ osmotolerance	Species	Genotype	Sequences similarity%	GenBank accession number
A3	ophthalmology	+	−/−	*A. quina*	T5	100	MT539092
A4	surgery	+	−/−	*A. quina*	T5	100	MT539092
A5	Internal medicine	+	+/+	*A. lenticulata*	T4	100	MT539094
A6	ophthalmology	+	+/+	*A. castellanii*	T4	100	MT539095
A7	Internal medicine	+	−/−	*A. quina*	T5	100	MT539092

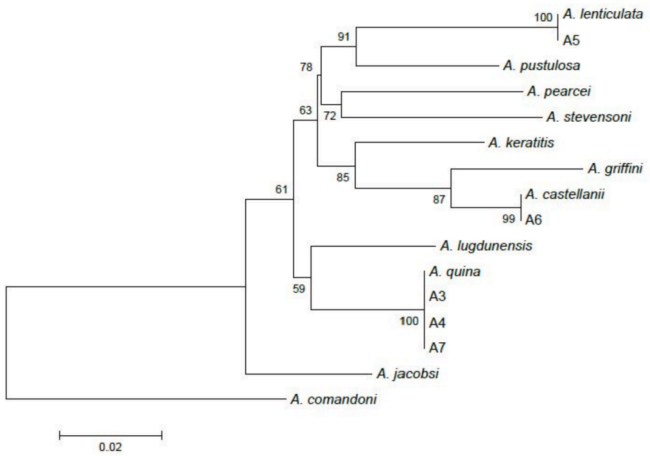


Fig. 1. 18SrRNA based phylogenetic tree of isolated *Acanthamoeba* spp. with nearest validated species with high bootstrapping value.

that soil and dust resources of the studied site are appropriate niches for outgrowth of *Acanthamoeba* spp. Overall, with regard to the high prevalence of *Acanthamoeba* spp. in hospital dust and soil, immunocompromised patients in hospital are at risk for infections with *Acanthamoeba* spp. and the pathogens within them.

In this study, the isolates A3, A4, A7 were identified as *A. quina* belonged to T5 genotype. T5 genotype explains the low infection rate, and in previous study T5 genotype has been detected in human mucosa without amoebic infection (Mirahmadi et al., 2019). The isolate A5 was identified as *A. lenticulata* and the isolates A6 was identified as *A. castellanii* belonged to T4 genotype. T4 genotypes has been predominant *Acanthamoeba* genotype. The main reason of frequency of cases due to T4 genotype is unclear and some researchers have noted that one of the reasons of its global distribution and high transmission is its dominant genotype (Fuerst et al., 2015; Mirahmadi et al., 2019).

There are only a few reports about genotypes of *Acanthamoeba* spp. in hospital environmental sources in Iran and worldwide (Niyyati et al., 2009b). In studies in Iran, most encounter *Acanthamoeba* genotype isolated from environment and patients are T3, T4 and T5, that is similar to genotypes of our isolates.

5. Conclusion

In conclusion, our result showed that soil and dust, especially in hospital environment, could be a potential transmission vehicle for *Acanthamoeba* spp. in Iran. Because of high percentage of *Acanthamoeba* spp. in dust and soil of hospital environment is a hygienic risk for public health mainly for immunocompromised patients. Therefore health agency must be conscious of *Acanthamoeba* spp. presence in hospital environments.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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