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PROTOZOOLOGY - SHORT COMMUNICATION

# Molecular identification and genotyping of *Acanthamoeba* spp., in bronchoalveolar lavage fluid from immunocompetent patients with chronic respiratory disorders (CRD)

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## Abstract

This study aimed to investigate the presence and genotyping of *Acanthamoeba* spp., in the bronchoalveolar lavage fluid (BALF) of immunocompetent patients with chronic respiratory disorders (CRD). In this study, 211 BALF samples were collected from patients with CRD during the COVID-19 pandemic who were candidates for fiberoptic bronchoscopy (FOB) at Imam Khomeini Hospital, Sari, Mazandaran Province, northern Iran and investigated for *Acanthamoeba* spp., by PCR. A total of 211 FBAL samples were examined; 5 (5/211; 2.36%) were positive by using the PCR test for Acanthamoeba spp. According to sequence analysis, three strains belonged to the T4 genotype and one strain to the T2 genotype. Our data demonstrate that the presence of *Acanthamoeba* (T4 and T2) in BALF specimens of patients with respiratory infections. However, it is important to note that these findings may be merely accidental. Our findings suggest further investigation to fully understand the role of *Acanthamoeba* spp. in the pathogenesis of lung infections.

**Keywords** Bronchoalveolar lavage · *Acanthamoeba* · PCR · T4 genotype · T2 genotype

## Introduction

*Acanthamoeba* spp., are opportunistic parasite, and among the most abundant and widespread free-living amoebae, present in diverse ecological environments, including fresh and brackish water, mineral water, soil, air, dust, sewage samples, and plant surfaces (Landell et al. 2013; Niyyati et al. 2016; Saberi et al. 2019). There are two stages to the *Acanthamoeba* life cycle: replicative trophozoites and resistant cyst forms (Marciano-Cabral and Cabral 2003). Based on the morphological characteristics, *Acanthamoeba* species have been classified into three distinct morphological groups: group I (stellate endocyst and well-separated ectocyst), group II (polymorphic endocyst with arms and usually wrinkled ectocyst), and group III (round endocyst without arms and usually smooth ectocyst) (Page 1988). According to sequence variations in the nuclear small subunit 18S ribosomal RNA gene, *Acanthamoeba* spp., are divided into 23 different genotypes (T1–T23), in which the most predominant genotype is T4 (Corsaro 2020; Putaporntip et al. 2021). *Acanthamoeba* spp., are capable of causing rare but devastating diseases (Król-Turmińska and Olender 2017; Trabelsi et al. 2012). *Acanthamoeba* spp. are the causative agents of granulomatous amoebic encephalitis (GAE), a serious infection of the brain and spinal cord, amoebic keratitis (AK), an infection of the eye that typically occurs in healthy individuals, and amoebic pneumonitis (AP). They also cause chronic rhinitis, sinusitis, lymphadenitis, and rheumatoid arthritis (Lau et al. 2021; Lorenzo-Morales et al. 2015; Visvesvara 2013). *Acanthamoeba* spp., infection of the lungs occurs mostly in immunosuppressed patients (Kaul et al. 2008). According to previous studies, 19 cases of AP or disseminated acanthamoebiasis with lung infection

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were reported (Kot et al. 2021). These patients had a history of acute and chronic marrow leukemia, acute myeloblastic leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia, atypical pneumonia, and organ transplant recipients (Kot et al. 2021). However, according to the literature review, Vernon et al. reported a case of *Acanthamoeba* infection in a lung transplant patient, who presented with rhinosinusitis (Vernon et al. 2005). Moreover, Oliva et al. reported a case of disseminated acanthamoebiasis in a single-lung transplant patient, and Van Hamme et al. also reported a fatal case of acanthamoebiasis in a lung transplant patient (Oliva et al. 1999; Van Hamme et al. 2001).

Fiberoptic bronchoscopy (FOB) with direct examination of the visible airways is a frequently used invasive method for the diagnosis of pulmonary pathologies (Haas et al. 2010). FOB candidates have a persistent cough, inflammation, and infections such as tuberculosis (TB), pneumonia, fungal or parasitic lung infections, lung tissue mass, and other abnormalities visible on a chest X-ray or other tests (Minassian et al. 2018).

Little information is available regarding the isolation and characterization of *Acanthamoeba* spp., in patients suffering from respiratory infections/diseases. There have been some reports of *Acanthamoeba* in bronchoalveolar lavage fluid (BALF) taken from immunocompromised patients (Lanocha et al. 2009; Newsome et al. 1992). Nevertheless, there is no evidence of the presence of *Acanthamoeba* in BALF specimens of immunocompetent subjects. Thus, the present study aimed to detect *Acanthamoeba* spp., in BALF specimens of immunocompetent patients with respiratory disorders (CRD) using polymerase chain reaction (PCR) and determine their genotypes.

## Patients and methods

### Ethical statement

This study was approved by the ethical principles and the national norms and standards for conducting medical research in Iran (IR.MAZMS.REC.1400.9241). Written informed consent was obtained, and their clinical and demographic data were recorded using a structured questionnaire.

### Sample collection

Two hundred and eleven frozen BALF specimens (about 2–3 mL) were taken from patients with CRD who were candidates for FOB at Imam Khomeini Hospital, Sari, Mazandaran Province, northern Iran, throughout 2020–2021. The patient's age ranged from 1 to 87 years. The mean age of the patients was 53.7 years. The majority of the patients were males (131; 56.7%). All BALF specimens (stored in the BAL Bio bank) were collected from each candidate in sterilized containers, and they were kept in the Iranian National Registry Center for Lophomoniasis (INRCL) at Imam Khomeini Hospital during the COVID-19 pandemic at − 20 °C until used.

### DNA extraction and PCR amplification

DNA was extracted using phenol–chloroform–isoamyl alcohol (25:24:1), and the PCR was set up in a total volume of 25 µL, which included 12.5 µL of the Taq DNA Polymerase 2 × Master Mix RED (Ampliqon), 1 µL of each forward (JDP1) (5′ GGCCCAGATCGTTTACCGTGAA 3′) and reverse (JDP2)) 5′ TCTCACAAGCTGCTAGGG AGTCA 3′) specific primers that amplified partial sequences of the 18S rRNA (rDNA) gene, 3 µL of the extracted DNA and 7.5 µL of distilled water. The PCR cycle profile consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 1 min, and then a final extension at 72 °C for 5 min. Positive (T4 genotype, Acc. No: MN339660.1) and negative (distilled water) controls were always used in the reactions. The amplified PCR products were observed using agarose gel electrophoresis on a 1.5% agarose gel (Invitrogen, Life Technologies GmbH, Germany) stained with SYBR® Safe Stain (Invitrogen®).

### Sequencing and genotyping of Acanthamoeba strains

The identification of *Acanthamoeba* genotypes has been determined by molecular phylogeny based on complete sequences of the nuclear SSU rRNA gene (18S rDNA) (Corsaro 2020). Unfortunately, with full sequence limitation in the current study, PCR products were sequenced based on JDP fragments by the Sanger method in both directions, with specific primers using an ABI PrismTM 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) by the Macrogen Company (Seoul, South Korea). The *Acanthamoeba* sequence was edited with Chromas and BioEdit software. The sequences were then compared with those of other similar *Acanthamoeba* sequences which are available in GenBank using the Basic Local Alignment Search Tool (BLAST) search engine.

## Results and discussion

In this study, 2.36% (5/211) of the collected BALF samples from patients were positive for *Acanthamoeba* spp. The past medical history of all patients was CRD, such

as asthma and chronic obstructive pulmonary disease (COPD). Three out of the five positive cases were farmers, and one of them was a worker. The infant case suffered from low birth weight (LBW) at birth. A summary of demographic and risk factors for patients is shown in Table 1. The result of PCR displayed a single approximately 460 bp fragment of the 18S rRNA (rDNA) gene, which was consistent with the product size of the *Acanthamoeba* genus. Despite several trials, among five PCR products which were chosen to be sequenced, one failed repeatedly, thus, four samples were sequenced and deposited in the GenBank using BankIt under accession number: MZ542841-44. BLASTn analysis showed that *Acanthamoeba* isolates obtained from patients who were suitable candidates for FOB belonged to the T4 ($n=3$), and T2 ($n=1$) genotypes. A summary of demographic and risk factors for patients is shown in Table 1.

Our preliminary study was persuaded by limited previous evidence on AP, or disseminated acanthamoebiasis with lung infection. The concept of evidence-based research has a significant impact on the design of studies; thus, the purpose of this evidence-based study was to attempt to detect and genotype *Acanthamoeba* spp., in the BALF samples from patients with CRD undergoing FOB in the Mazandaran Province, northern Iran, during the COVID-19 pandemic.

Amoebae have repeatedly been reported from the upper respiratory tracts of individuals (Król-Turmińska and Olender 2017). In the Siripanth study, the first cases of early detection and double infections of *Naegleria* spp. and *Acanthamoeba* spp. were reported in the sinus cavity of a symptomatic patient (Siripanth et al. 2005). In our study, based on the results obtained from PCR, 5 (2.36%) of the samples were found to be positive for *Acanthamoeba* spp. One study presented morphologic features of *Acanthamoeba* species following cytocentrifugation and staining procedures, including hematoxiline and eosin, trichrome, and Papanicolaou (Newsome et al. 1992). The authors stated that this method could be used to identify *Acanthamoeba* species in BAL specimens (Newsome et al. 1992). However, the limitation of this method compared to molecular analysis is that it cannot determine the species and genotypes of *Acanthamoeba*. In the current study, the genotyping data based on partial sequences of the 18S rRNA gene showed T4 and T2 genotypes. Previous studies showed that T4 is confirmed as the predominant genotype, which is most common in human infections (Maciver et al. 2013; Mirjalali et al. 2013; Saberi et al. 2021). On the other hand, following T4, genotype T2 predominates in environmental samples in Iran (Maghsood et al. 2005; Shokri et al. 2016). Interestingly, in the Walochnik study, the *Acanthamoeba* was detectable by PCR from the BAL sample of a human immunodeficiency virus-negative patient and was identified as genotype T2, which is consistent with the results of our study (Walochnik et al. 2008). Note that this was the first case of GAE involving genotype T2 (Walochnik et al. 2008). GAE occurs mostly among immunocompromised patients, and the mortality rate of GAE is around 97–98% (Kot et al. 2018). Altogether, 75 cases of patients with GAE caused by *Acanthamoeba* spp. have been reported (Kot et al. 2021).

Another study examined the occurrence of potentially virulent strains of amoebae in 130 clinical samples from patients with symptoms of pneumonia in Poland (Lanocha et al. 2009). The presence of *Acanthamoeba* was detected in two broncho-aspirate fluid samples taken from patients after chemotherapy, and in two BAL samples taken from patients with respiratory deficiency (Lanocha et al. 2009).

It should be noted that the samples of patients who were found to be positive for *Acanthamoeba* spp., were negative for cancer, COVID-19, and bacterial and fungal infections. Because of the endosymbiont debate, we cannot claim that *Acanthamoeba* spp., caused lung infections in these subjects. However, these patients seem to have typical or atypical pneumonia, which can be caused by *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* (Jones 2010). These bacterial pathogens can take shelter inside *Acanthamoeba* spp. as endosymbionts and be safe from drugs. Consequently, further investigations are needed to better clarify

**Table 1** Demographic data regarding the positive *Acanthamoeba* spp., patients using molecular identification

| Isolate code | Isolated amoebae/genotype | Accession number | Age (year) | Gender | Fiberoptic bronchoscopy reason | Past medical history |
|---|---|---|---|---|---|---|
| S 449 | *Acanthamoeba*/T4 | MZ542841 | 43 | Male | Persistent cough with hemoptysis | CRD |
| S 380 | *Acanthamoeba*/T4 | MZ542842 | 40 | Male | Persistent cough | CRD |
| S105 | *Acanthamoeba*/T4 | MZ542843 | 1 | Female | Productive cough with bronchiectasis | CRD |
| S107 | *Acanthamoeba* T2 | MZ542844 | 31 | Male | Persistent cough and dyspnea | CRD |
| S 96 | *Acanthamoeba* spp. | NA | 43 | Female | Persistent cough and wheezing | CRD |

*NA*, not applicable.

*Acanthamoeba*–bacteria interactions, their pathogenesis and pathophysiology in lung infections potentially leading to pneumonia.

Moreover, based on some evidence, although anti-*Acanthamoeba* antibodies have been detected in a high number of healthy human individuals, they do not have any lung infection or other organ involvement (Khan 2006). The high abundance of *Acanthamoeba* spp. in diverse environmental resources makes contact with this opportunistic parasite is non-preventable. Due to the ability of *Acanthamoeba* to act as a reservoir host (allowing bacteria to survive and multiply) or Trojan horse (allowing bacteria to survive without multiplying) and or carrier (allowing attachment to the surface) for a variety of microbial pathogens (as endobionts) such as bacteria, yeast, and viruses (Khan 2006; Jones 2010). *Acanthamoeba* is of particular medical relevance for patients with immunosuppression and or chronic underlying diseases (Khan 2006). Possibly, in this study, the detected *Acanthamoeba* strains (T2, T4) played a role as reservoirs and or Trojan horses for pathogenic bacteria. Thus, *Acanthamoeba,* this pathogenic amoeba, may pose a serious risk to CRD patients.

In our study, out of the five positive patients, three were farmers, one was a worker, and the fifth was an infant with LBW. Farmers and workers have more exposure to the soil and environmental resources, so they may be at risk of infection due to *Acanthamoeba*. Also, it has been shown that babies with a history of LBW were at an increased risk of hospitalization for lung infections (Walter et al. 2009; Miller et al. 2012). Overall, since *Acanthamoeba* is universally present in water resources, air, and soil, susceptible hosts, particularly those with a past medical history of CRD, possibly are at risk of *Acanthamoeba* infections in the respiratory system, and *Acanthamoeba* may also act as a shelter for microbial lung pathogens.

## Conclusion

The molecular analysis allowed us to investigate the presence of *Acanthamoeba* (T4 and T2) in BALF samples of patients with acute and chronic respiratory infections in particular. However, we declare that these findings may be purely accidental. Our findings warrant further investigation among patients who had chronic respiratory infection to fully appraise the role of *Acanthamoeba* spp., in the future. It is worth mentioning that a comprehensive survey should be conducted to determine the distribution of *Acanthamoeba* pathogenic strains and identify their endosymbiont microbial pathogens among patients having chronic respiratory infections. Thus, preventative and therapeutic strategies to reduce biofilm formation certainly help to avoid serious complications of microbial infections.

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## Declarations

**Conflict of interest** The authors declare no competing interests.

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