The *Acanthamoeba* and *Sappinia* inflammation Induction in Cancer Patients' Oral Cavity

Shaymm Abd al Hussein Khalaf [1*], Bassad A. AL-Aboody [1]

Abstract

Background: Free-living amoebae (FLA), including Acanthamoeba and Sappinia, pose threats to immunocompromised individuals, causing lung infections and granulomatous encephalopathy. Cancer, marked by uncontrolled cell proliferation, shares similarities with parasitic infections, both challenging the host's immune response. Method: This study aimed to determine the prevalence of FLA among cancer patients in Thi-Qar, Iraq. Oral cavity samples were collected from 40 cancer patients and 20 healthy individuals in December 2023. Isolated strains were cultured on non-nutrient agar with Escherichia coli. Molecular and morphological analyses were conducted to identify Acanthamoeba and Sappinia spp. Result: Acanthamoeba and Sappinia were detected in 7.5% and 5% of immunocompromised patients, respectively. Acanthamoeba exhibited higher pathogenicity, especially the T4 and T32 genotypes, compared to Sappinia. Conclusion: The study highlights the prevalence of FLA among cancer patients, with Acanthamoeba showing greater pathogenic potential. These findings underscore the importance of understanding FLA infections in immunocompromised populations for effective management strategies.

Significance | This study showed the presence and prevalence of Free-living Amoebae (FLA), particularly Acanthamoeba and Sappinia, in immunocompromised cancer patients is crucial for disease management and prevention.

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Editor Md Shamsuddin Sultan Khan, And accepted by the Editorial Board Mar 31, 2024 (received for review Feb 6, 2024)

Keywords: Acanthamoeba, Sappinia, Cancer patients, Oral cavity, Protozoan infections

Introduction

A free-living protozoan, Acanthamoeba spp., is commonly found in various environments, including air, soil, dentistry offices, hospitals, and home water supplies (AL-Abody, 2021). Another free-living, single-celled amoeba present in the environment is Sappinia, which comprises two species: S. diploidea and S. pedata (AL-Abody, 2021). Microbial life often coexists closely with other species, forming advantageous relationships (Anwar-Sabah, 2023). Free-living amoebae like Acanthamoeba and Sappinia spp. play a significant role in environmental ecosystems by acting as carriers or reservoirs for numerous prokaryotic species, including bacteria, fungi, and viruses, through a process known as Trojan horse formation.

Human infections with Acanthamoeba and Sappinia, particularly in immunocompromised patients, pose challenges due to their resistance to macrophage function, serving as a "training ground" for pathogens (Çelik and Şimşek, 2022). Parasites and cancer cells share characteristics such as resistance to apoptosis, ability to multiply independently of external stimuli, and evasion of host immune responses. Consequently, complete elimination of these organisms from the body can be challenging (Çelik and Şimşek, 2022). Parasitic agents can have both tumoral and antitumoral effects through immune response regulation, angiogenesis and metastasis prevention, inhibition of proliferative signals, and management of inflammatory responses leading to cancer development (Çelik and Şimşek, 2022).

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Please cite this article.
Shaymm Abd al Hussein Khalaf, Bassad A. AL-Aboody (2024). The Acanthamoeba and Sappinia inflammation Induction in Cancer Patients' Oral Cavity, Journal of Angiotherapy, 8(3), 1-7, 9647

Free-living amoebae (FLA) such as Acanthamoeba spp. and Sappinia spp. are recognized as opportunistic and harmful protozoans (Visvesvara et al., 2007). Acanthamoeba spp. and Sappinia spp. are considered the two most significant agents of FLA in medicine, veterinary medicine, and natural ecosystems (Visvesvara et al., 2007). Acanthamoeba spp. undergoes two stages in its life cycle: cyst and trophozoite, with the trophozoite being the active form and the cyst forming under adverse environmental conditions (Hady, 2022). The cyst, due to its extreme resistance to UV radiation, detergents, and dehydration, is the most common mode of infection transmission (Dudley et al., 2005).

Various genotypes of Acanthamoeba spp. exhibit a worldwide distribution trend and are isolated from diverse clinical and environmental sources (Dudley et al., 2005). The T4 genotype is considered the most prevalent and harmful species in many settings (Putaporntip et al., 2021). FLA, particularly Acanthamoeba, have been isolated from soil and water sources in regions like Thi-Qar and Al-Qadisiyah (Zahraa, 2023). Human infections with Acanthamoeba spp. can lead to conditions such as amoebic keratitis, granulomatose encephalitis, and cutaneous acanthamoebiasis, with initial manifestations through the mouth, respiratory system, or wounded skin (Khan et al., 2006).

Sappinia spp., consisting of S. pedata and S. diploidea, are found extensively in environments like decomposing forest litter and soil when isolated from fresh water sources. Although rare, Sappinia spp. can cause amoebic encephalitis, as seen in an immunocompetent adult male, with uncertain modes of infection believed to occur through hematogenous dissemination to the brain or inhalation through the nasopharynx (Gelman et al., 2001).

Materials and Methods

Sample Collection

Samples were obtained from 40 cancer patients' oral cavities, representing various cancer types (breast, liver, brain, and lung), with ages ranging from 13 to 60 years. The samples were collected from Al-Haboobi Teaching Hospital for cancer patients.

Bacterial Isolation

An E. coli isolate was obtained from the bacteriology laboratory of the College of Science, University of Thi-Qar. It was cultured overnight at 37°C in 100 milliliters of nutrient broth. Following autoclaving of the culture for 15 minutes at 121°C, the broth containing the dead bacteria was centrifuged at 10,000 rpm/min for 10 minutes. After discarding the supernatant, the bacterial pellet was washed twice with sterile distilled water and suspended in 10 milliliters of the same water to serve as a nutrient source.

Sample Processing

Sterile cotton swabs were used to collect samples from cancer patients' oral cavities, including the roof of the mouth, inner sides of the mouth, and tongue. The swabs were cultured on non-nutritional agar medium (NN-agar medium), prepared according to Al-Maliky's (2014) instructions.

Incubation and Monitoring

The medium with E. coli surface was incubated at 26°C and 37°C and monitored weekly for two weeks using wet mount slides (Moker, 2017).

Amoebae Subculturing and Identification

Subculturing of Sappinia and Acanthamoeba spp. was conducted for two weeks using an activation solution such as Page Amoebic Saline (PAS). Morphological features, including the size and shape of cysts and trophozoites, were examined using the Pussard and Pons detection classification key (Page, 1988), aiding in accurate identification of observed amoebae structures.

Results

The current study identified FLA (Free-Living Amoebae), including Acantamoeba spp. and Sappinia spp., in 6 out of the total 40 samples tested. Both cysts and trophozoites were observed using the culture method, and these findings were confirmed through microscopic and molecular examination. Interestingly, this led to the diagnosis of a specific type of cancer affecting humans.

Comparing the oral cavity samples of cancer patients with those of healthy individuals, the study found a positivity rate of 10% (6 out of 60 samples) for FLA, with 8.33% (5 out of 60 samples) positive for Acanthamoeba spp. under microscopic examination and 5% (3 out of 60 samples) positive via PCR. In the case of Sappinia spp., the total positivity rate for cultivation and PCR was 3.3% (2 out of 60 samples).

Morphological diagnosis of amoebae isolates

After detecting amoebic growth on culture media, samples were carefully mounted on microscope slides using sterile cotton swabs. Subsequently, the samples were examined under a microscope at both 10x and 40x magnifications to identify trophozoites and cysts, distinct stages of amoebae, following the diagnostic criteria outlined by Page (1988).

Several distinguishing characteristics were observed upon direct examination on agar using a light microscope at 10x and 40x magnifications:

Acanthamoeba spp. Trophozoite Stage: Trophozoites of Acanthamoeba spp., with diameters ranging from 25 to 40 µm, were analyzed in this study. After seven days of cultivation, these trophozoites displayed an indistinct cytoplasmic boundary, a single nucleus, and delicate acanthopodia protruding from the body surface. The trophozoites exhibited an irregular or elongated oval shape (see Fig 1).

Identification of Acanthamoeba spp. Cysts: Various characteristics, including the number, size, shape, and arrangement of cyst pores, as well as the overall morphology and size of the cysts, were utilized

Table 1. Occurrence of Acanthamoeba spp. and *Sappinia spp.* from cancer patients' oral cavities, as determined by PCR molecular analysis and microscopy.

Groups under study	NO. samples	Positive cultivation of FLA		Positive cultivation *Acanthamoeba* spp.		Positive PCR of *Acanthamoeba spp.*		Positive cultivation of *Sappinia spp.*		Positive PCR *of Sappinia spp.*	
		NO.	%	NO.	%	NO.	%	NO.	%	No.	%
Oral cavity of cancer patients	40	6	15%	5	12.5%	3	7.5	2	5%	2	5
Healthy person	20	0	0	0	0	0	0	0	0	0	0
TOTAL	60	6	(10%)	5	(8.33%)	3	(5%)	2	3.3	2	3.3

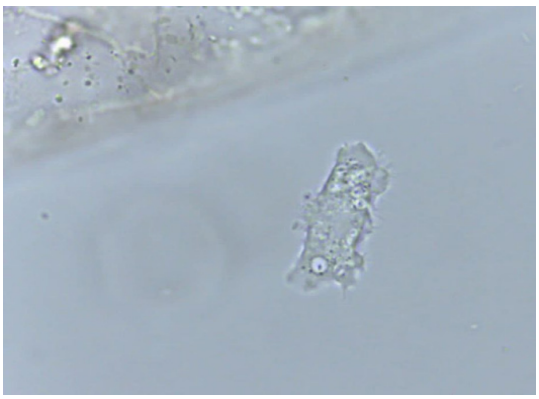


Figure 1. *Acanthamoeba spp.*trophozoites 100X.

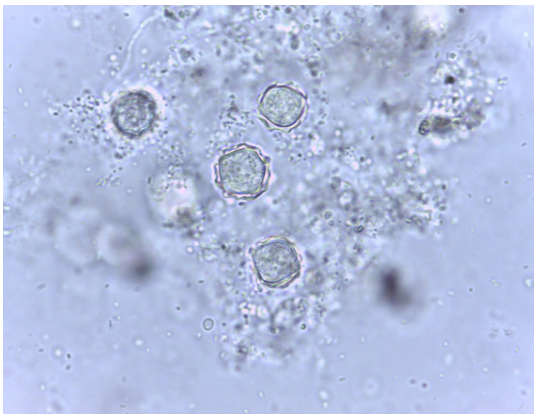


Figure 2. *Acanthamoeba spp.*cyst 100X.

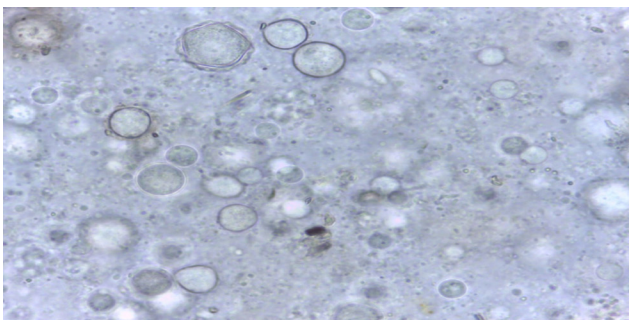


Figure 3. This figure show *Acanthamoeba spp.*with white arrow and *Sappinia spp*. with red arrow from oral cavity of cancer patients sample.

for Acanthamoeba spp. cyst identification. The cysts typically had diameters ranging from 13 to 20 µm. Their robust resistance to environmental factors was attributed to their double-layered cell wall, consisting of an endocyst and an ectocyst (see Fig 2).

Detailed characteristics of Acanthamoeba spp. trophozoite and cyst stages were as follows:

Trophozoite: Oval-shaped with dimensions of 30-45 µm and binucleate, featuring a distinct double nucleus closely positioned along a flattened surface. The trophozoite displayed a clear ectoplasm and translucent cytoplasm responsible for pseudopodium formation during movement. Additionally, granular endoplasm, contractile vacuoles, and food particles were observed.

Cyst: Rounded with a diameter of 15–30 µm, the cysts typically lacked a distinct median line. Within the same culture, single, double, triple, and tetra-nucleate cysts were observed, indicating variability. (See Fig 4).

Molecular detection

DNA extraction, polymerase chain reaction and sequencing:

Genomic DNA from cell cultures of Acanthamoeba spp. and Sappinia spp. was extracted using the gSYNC TM DNA Extraction kit from Geneaid, Korea, following the manufacturer's instructions. After morphological characterization, the identities of Acanthamoeba spp. and Sappinia spp. were genetically confirmed using conventional PCR.

For Acanthamoeba spp., a set of two primers specific to Acanthamoeba spp. were utilized, as developed by Schroeder et al. (2001). The forward primer JPD1 (5'GGCCAGATCGTTTACCGTG 3') and reverse primer JPD2 (5' TCTCACAAGCTGCTAGGGAGTCA 3') were employed. Similarly, for Sappinia spp., a set of two primers specific to Sappinia spp. were used, as designed by Qvarnstrom et al. (2009). The forward primer (3' -5' TCTGGTCGCAAGGCTGAAAC) and reverse primer (5' -3' GCACCACCACCCTTGAAATC) were employed.

PCR amplification for Acanthamoeba spp. and Sappinia spp. followed similar protocols. It involved an initial denaturation step of 10 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 35 seconds, annealing at 56°C for 35 seconds, and extension at 72°C for 40 seconds, with a final extension step at 72°C for 10 minutes.

The PCR products were then electrophoresed on a 1.5% agarose gel and exposed to UV light. The resulting bands were compared to ladder DNA, with the Acanthamoeba spp. yielding an amplification band size of 450–500 bp (see Fig 5), and the Sappinia spp. producing a 160 bp band (see Fig 5).

To confirm the genetic identity, the nucleotides from the PCR products were sequenced, and BLAST analysis was performed against global isolates in GenBank.

Acanthamoeba spp. and Sappinia spp. were detected in samples obtained from the oral cavities of cancer patients. Out of 40 samples, 3 (7.5%) tested positive for Acanthamoeba spp. cysts and trophozoites, while 2 (5%) tested positive for Sappinia spp. cysts and trophozoites.

Table 1 presents the distribution of Acanthamoeba and Sappinia spp. species identified in samples from cancer patients' oral cavities, along with the number of positive cultures and PCR results.

Sequencing analysis of the positive samples revealed that all Acanthamoeba spp. isolates from cancer patients' oral cavities exhibited a 98% homology identity with Acanthamoeba jacobsi (genotype T 32) isolate PRZTOO3's 18S ribosomal RNA gene (accession number MG945000.1). Notably, one isolate was found to be closely related to a strain isolated from hot baths in Turkey.

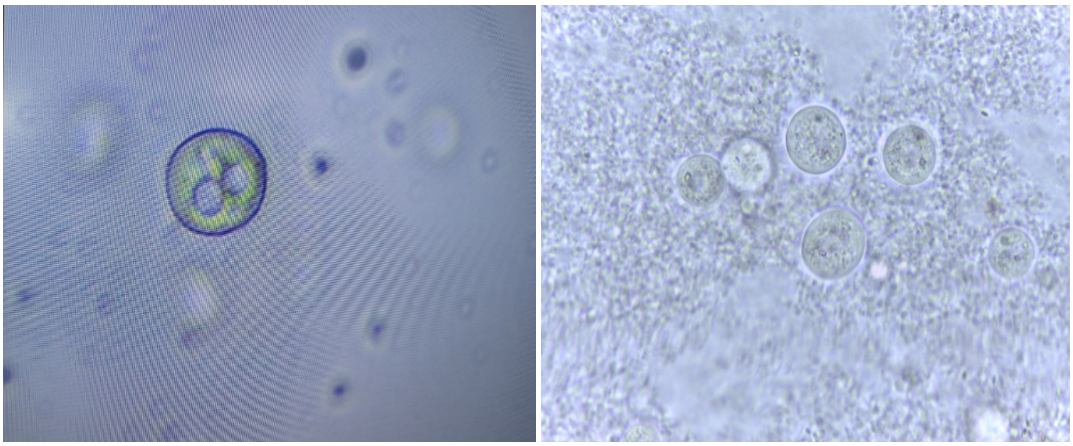
Similarly, sequencing analysis of the positive samples showed that all Sappinia spp. isolates from cancer patients' oral cavities exhibited a 95% homology identity with the 18S rRNA gene of Sappinia spp. CW-2015 (accession number kp165392.1).

Discussion

The presence of Free-living Amoebae (FLA) species, particularly Acanthamoeba spp. and Sappinia spp., raises concerns about the emergence of zoonotic diseases. FLAs can pose a severe threat when they penetrate the central nervous system (CNS), leading to potentially fatal conditions such as meningitis (Greub and Raoult, 2004). The increasing population of immunocompromised individuals, including those with conditions like AIDS, cancer, diabetes, hepatitis, or undergoing organ transplantation and steroid medication, are particularly vulnerable to opportunistic infectious agents, including FLAs (Fung et al., 2008).

Acanthamoeba strains have been implicated in up to 20% of infectious keratitis cases among contact lens wearers (McCallum et al., 2009). In this context, our investigation identified Acanthamoeba spp. and Sappinia spp. strains in clinical samples, including those from the oral cavities of cancer patients in Thi-Qar province, Iraq, employing morphological assessment, PCR analysis, and sequencing. Notably, this study marks the first documentation of Acanthamoeba spp. isolation from cancer patients' oral cavities in Iraq.

Our findings are consistent with previous studies conducted by Mohsen et al. (2023), who identified Acanthamoeba from the oral cavities of cancer patients undergoing chemotherapy and healthy individuals in Iran, as well as Tayebeh Taghipour's work from 2019 to 2020. There were no significant disparities between our findings and those of previous research. Moreover, our data indicate that 6.6% of cancer patients exhibited Acanthamoeba spp. infection,



(A) **(B)**

Figure 4. (A) and (B)*sappinia diploidae cyst* 100X unstained.

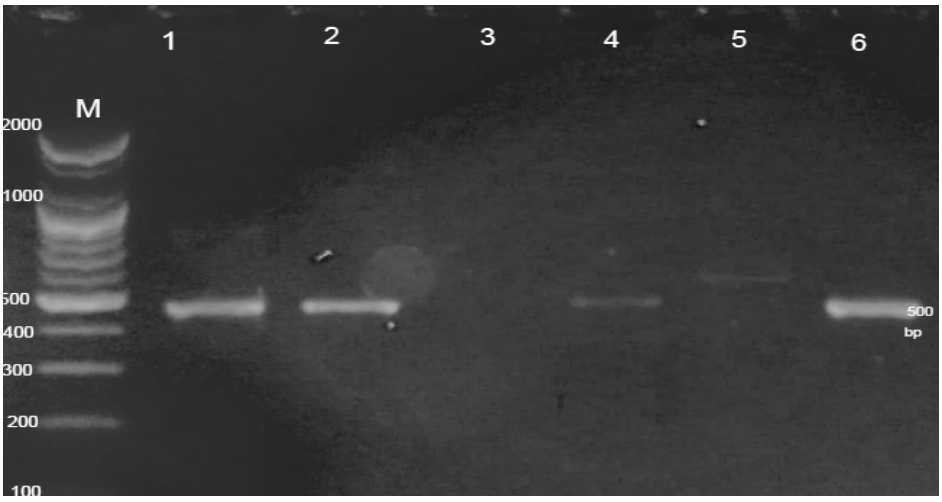


Figure 5. The Results of the Gel Electrophoresis of the Culture positive Samples of *Acanthamoeba spp.* Note. M: marker (2000 -100 bp) lance 1,2,6 positive and 3,4,5 negative samples and the PCR product analysis of 18S ribosomal RNA gene from genomic DNA of *Acanthamoeba* spp. from oral cavity of cancer patients.

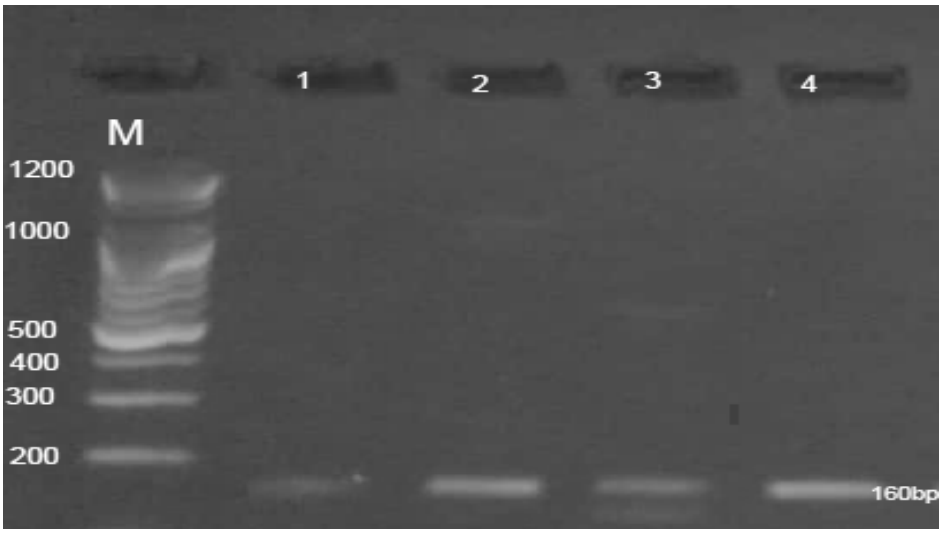


Figure 6. The Results of the Gel Electrophoresis of the Culture positive Samples of *Sappinia spp.* Note. M(100-1200)bp marker. Positive samples for *Sappinia spp* 2,4, and 1, 3 nective band.This image shows fragments with lengths 160 bp.

which deviates from the expected prevalence of such infections in this population.

This study represents the first survey to isolate and identify potentially harmful genotypes of Acanthamoeba spp. and Sappinia spp. from the oral cavities of immunodeficient cancer patients in Thi-Qar. Our findings contribute to the understanding of FLA infections in vulnerable populations and underscore the importance of further research in this area.

Conclusion.

In this study, we investigated samples from the oral cavities of immunocompromised cancer patients to assess the presence of free-living amoebae (FLA). Our findings revealed the presence of several amoeba species, including Acanthamoeba spp. and Sappinia spp., in the oral cavities of these immunocompromised cancer patients. Interestingly, these species were notably absent in healthy individuals. This highlights the opportunistic nature of these amoebae, particularly in individuals with compromised immune systems.

The diminished immunity in immunocompromised individuals significantly increases their susceptibility to infections caused by these free-living amoebae, which are particularly prevalent among this population. Therefore, it is imperative to exercise caution and implement necessary treatments and preventive measures to mitigate the risk of FLA-related diseases in immunocompromised cancer patients. Further research and vigilance in this area are crucial for better understanding and management of these infections.

Author contributions

S.A.A.H.K. conceptualized, analyzed data, B.A.A.A. wrote and drafted the manuscript.

Acknowledgment

Authors were grateful to each research participant and their guardians.

Competing financial interests

The authors have no conflict of interest.

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