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Research Article
Molecular Identification, Isolation, Morphologic and Serologic of *Acanthamoeba* sp. Throw the Ovary of Sprague-Dawley Rats

Alaa Talal Qumsani

Department of Biology, Al-Jamoum University College, Umm Al-Qura University, 24382, Makkah, Saudia Arabia

Abstract

Background and Objective: Typically, free-living amoebae, members of the genus *Acanthamoeba* can exist in a range of biological niches, such as fresh and brackish water, filters for heating, soil, airborne dust, ventilation, in addition to air conditioning, as well as pools and hot tubs. On rare occasions, these can be linked to infections of the central nervous system in both people in addition to animals. This study aimed to identify the cause of rats' death by isolating and identifying *Acanthamoeba* sp., from the ovary of Sprague-Dawley rats. **Materials and Methods:** An amoeba was identified and cultured from the ovary of Sprague-Dawley rats that succumbed to an amoebic illness. Considering culture attributes, growth shape and immunofluorescence measures, *Acanthamoeba* sp., was determined as the infecting amoeba. **Results:** *Acanthamoeba* sp., the contaminating single adaptable cell, was discovered. Furthermore, by sequencing a symptomatic part of the atomic little subunit ribosomal RNA gene to recognize the one-celled critter as *Acanthamoeba* sp., **Conclusion:** An *Acanthamoeba* strain was demonstrated that has a wide range of genotypes and is capable of infecting people and other species can also infect rats fatally.

Key words: *Acanthamoeba*, ovary, Sprague-Dawley rats, molecular identification, isolation, morphology

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Corresponding Author: Alaa Talal Qumsani, Department of Biology, Al-Jamoum University College, Umm Al-Qura University, 24382, Makkah, Saudia Arabia

Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Protozoans called free-living amoebae are common in both manufactured samples and the natural world. The most prevalent genus of free-living amoebae[1]. *Acanthamoeba*, is known to produce *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE)[2]. There are 204 cases of GAE, which involves an infection of the central nervous system, according to estimates[3]. Immunocompromised persons are primarily affected by GAE, which is typically fatal[2]. On the other hand, AK is a corneal infection that affects people with a healthy immune system[4].

Based on morphology, over 24 different species of *Acanthamoeba* have been identified. They have primarily been divided into three groups I, II and III based on the characteristics of the cysts. Group I is nonpathogenic, but *Acanthamoeba polyphaga*, *A. castellanii*, *A. hatchetti* and *A. rhysodes* are only a few of the pathogenic species found in group II. The *A. culbertsoni*, however, is the sole pathogenic species in group III[5]. The *A. castellanii* and *A. culbertsoni* infections account for the majority of GAE cases, whilst *A. castellanii* and *A. polyphaga* infections account for AK cases[5]. Although morphology is still employed to identify isolates, the predominant technique for taxonomic characterization of *Acanthamoeba* at this time is the molecular identification technique of sequencing genes that code for the small subunit nuclear 18S rRNA[6]. To demonstrate an isolate's pathogenicity, physiological features like thermos tolerance and infectivity in mice are also examined because genotyping alone is insufficient. *Acanthamoeba* species are well established as opportunistic carriers of human and animal diseases[2,6,7]. *Acanthamoeba* can taint people's lungs, skin, nasal sinuses, eyes and produce severe sores in the focal sensory system (CNS). Furthermore, they have infected lower primates, canines, ponies, bovines, birds, reptiles, fish and invertebrates, resulting in serious illness or death[2,8-10].

In this report, the discovery of an amoeba that killed a 5 months old male Sprague-Dawley rat was done. According to cyst morphology, immunofluorescence, also molecular tests it was an *Acanthamoeba*. This study aimed to identify the cause of rats' death by isolating and identifying *Acanthamoeba* sp. from the ovary of Sprague-Dawley rats.

MATERIALS AND METHODS

Case study area: The experiment was conducted in the Department of Biology, Jamoum University College, Umm Al-Qura University, Saudi Arabia for 180 days (June to December, 2022).

Experimental animals: Five months old female Sprague-Dawley rats from the Medical Research Center, Faculty of Medicine, Umm Al-Qura University, which was then handled in accordance with the moral standards specified by the Ethics Council Committee was used. Twenty five female rats were quarantined for the customary 30 days.

Full blood count, physical examination in addition to the profile of biochemical had been all within normal ranges prior to the study with the exception of an increased creatine phosphokinase. No parasites or ova were found when the faeces were directly and flotation examined. Up until 8 days after their arrival, when 50% of the rats were discovered dead without showing any preceding clinical indications, the rats seemed to be doing well.

Two hours after death, a post-mortem examination was performed. Then ovary was obtained and cultures in both aerobic and anaerobic conditions. Samples of ovarian tissue were collected, fixed in formalin (10%, pH 7.4) with phosphate buffer, dried in ethanol of increasing strength, cleaned in toluene and mounted in molten pararplast (58-62°C)[11]. Hematoxylin and eosin were used to stain each of the following histological sections (H&E). The alterations in the ovary were examined using Leica DM750 HD digital light microscope. Intestines, lungs, kidneys and liver, in addition to spleen representative samples, had been frozen.

***In vitro* culture:** The frozen ovary was separated into little pieces, minced and isolated into three portions prior to being defrosted in a water shower at 37°C. The two parts were incubated at 37°C, one into a non-supplement agar plate with a layer of *Escherichia coli* and the other into a human lung fibroblast (HLF) cell line containing 100 g mL^{-1} gentamicin[11]. As 5% fetal cow-like serum, gentamicin and amoebae that developed on agar plates were additionally added to the fluid culture medium (PYG), yet no microbes were available[2].

Indirect immuno fluorescence: Deparaffinizing and covering each segment with a 1:200 weakening of one of three different bunny antisera made against *Acanthamoeba castellanii*, *Balamuthia mandrillaris* and *Naegleria fowleri* were utilized to perform backhanded immunofluorescence (IIF) on formalin-fixed, paraffin-inserted segments of the ovary[2,7].

DNA extraction, PCR and sequencing: An *Acanthamoeba* sp., culture (CDC: V601) produced from the ovary was utilized to collect DNA. The OSU: 08-016 was appointed to this example. The way of life test OSU: 08-016's all-out DNA was separated utilizing the DNeasy pack from Qiagen, Inc., Valencia, California. Following DNA extraction, PCR (Biometra,

Göttingen, Germany) was done to intensify the *Acanthamoeba* atomic SSU rDNA arrangements utilizing a variety of explicit preliminaries for the *Acanthamoeba* 16S rRNA (F 5-TTATATTGACTTGTACAGGTGCT-3) and *Acanthamoeba* 16S rRNA 2 (R 5-CATAATGATTTGACTTCTTCT CCT-3). This amplifies a section of the SSU r. This *Acanthamoeba* genotype identification technique compares the novel sequence to a vast library of sequences collected from many sources, including our lab. The initial method for distinguishing between various rDNA genotypes was created by Wrobel *et al.*[12]. If an isolate's complete rDNA sequences varied by 5 percent or more in comparisons of sequence alignment, this approach classified the isolate into a distinct genotype. At the time, this led to the development of two distinct *Acanthamoeba* genotypes[13]. Since then, other genotypes have been discovered using this methodology[14,15]. *Acanthamoeba* specialists can quickly learn genotype by contrasting new sequences got from this genotypically educational rDNA locale to these genotype reference successions. The following PCR conditions were used for this reaction: A 10 min initial denaturing phase at 94°C and continued via 35 cycles of denaturation at 94°C for 60 sec, annealing at 57°C for 45 sec and extension at 72°C for 60 sec; and a final extension step at 72°C for 10 min.[13,16-18]. Adhering to the maker's directions, the PCR item was sanitized utilizing the QIA quick PCR decontamination pack utilizing a microcentrifuge (QIAGEN Ltd., Crawley, UK) to group and characterize the strains. Basically, 25 mL of buffer PB and 5 mL of the PCR result were joined. At the point when the blend became yellow, it was added to a QIA quick section and centrifuged at 17900×g for the 60 sec. The combination was then additionally blended in with 10 L of 3 M sodium acetic acid derivation (pH 5.0). The item was washed with 750 L of support PE and centrifuged at 17900×g for the 60 sec after the course through was disposed of. Yet again the course through was eliminated and the QIA quick section was centrifuged for an extra 60 sec at 17900×g. From that point forward, DNA was eluted by adding 30 L of elution buffer to the focal point of the QIA quick film and setting the QIA quick column in a clean 1.5 mL microcentrifuge tube. The section was centrifuged at 17900×g for the 60 sec in the wake of being left at room temperature for 1 moment[13,16-18]. Sanger sequencing services were used to define the strains utilizing the commercially sequenced PCR results (Eurofins Scientific, Wolverhampton, UK). The sequences were obtained using the forward *Acanthamoeba* 16S rRNA (R 5-CATAATGATTTGACTTCTTCT CCT-3) primer. The *Acanthamoeba* sp., sequencing from this examination was submitted to Gen-Save money with the increasing number of GQ889265 from the *Acanthamoeba* sp., culture, OSU: 08-016. (CDC: V601).

Statistical analysis: With the use of SPSS 28-2021, the acquired data were examined using One-way Analysis of Variance (ANOVA), then Tukey's Multiple Comparison Test (V20, USA). Standard Error of the Mean (SEM) was used to show the data as means. Statistics were deemed significant at $p = 0.05$.

RESULTS

The rats were underweight in addition to losing about 20% of their body weight upon post-mortem inspection. Splenomegaly and widespread 1-2 mm white nodules were found during a gross examination of the hepatic parenchyma. While the anaerobic culture of the ovary was ineffective, the aerobic culture produced a heavy growth of tough, non-hemolytic *E. coli*.

Histopathology: In general, there was considerable necrosis and inflammation brought on by the amoebae in ovarian tissues. Nuclear chromatin clumping was present in many ovarian cells (pyknosis). Some parts of bleeding in addition to necrosis coupled via a few amoebae were detected and there were noticeably more foci that were necrotic, edematous and spongiform degraded. A few free and phagocytized amoebae were found together with severe ovarian tissue degradation (Fig. 1a-d).

Immuno fluorescence analysis: Just the amoebic creatures in tissue tests that had been treated with hostile to *A. castellanii* serum responded and emitted extreme apple green fluorescence. The amoebae had a place with the sort *Acanthamoeba* in light of the fact that they didn't respond with any different sera (hostile to *B. mandrillaris* and hostile to *N. fowleri*).

***In vitro* culture:** On agar plates hatched at both 37 and 42°C, amoebae prospered and by 72 hrs had completely covered the plate and begun to encyst after the greater part of the microscopic organisms had been eaten. The trophozoites had a major core with an immense, round nucleolus situated in the middle and were around 8-14 m in size (Fig. 2). Acanthopodia, which look like thistle-like filamentous projections that jut from the outer layer of the trophozoites, was one more trait of these organisms. The cysts were polygonal, ranging in size from 7-12 m and had two walls, the inner of which was either round or oval and the outside of which was wrinkled. *Acanthamoeba* was used to identify the amoebae and CDC: V600 was used to identify the isolate. In the cell culture flasks, amoebae also proliferated, albeit it took a few days before they were visible.

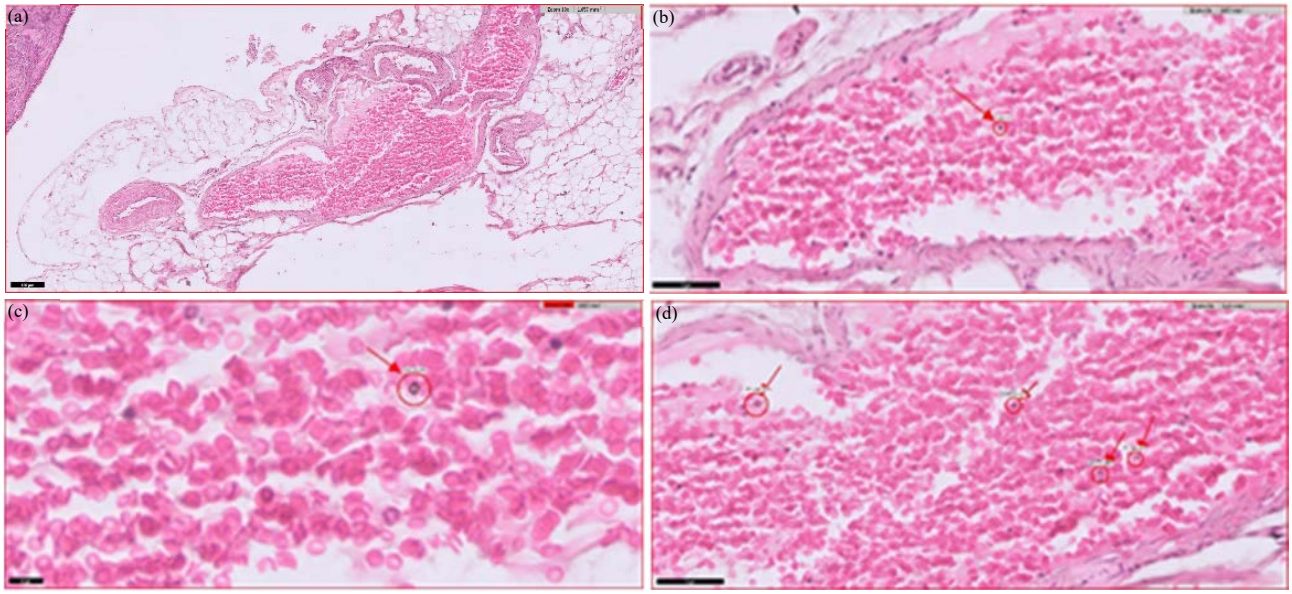

Fig. 1(a-d): Photomicrograph of the rat ovary's histological section stained with H&E. Trophozoite is shown by the (a) Number of pyknotic cells and the arrowhead, (b) A magnified version and (c-d) Macrophages engulfing a foreign body

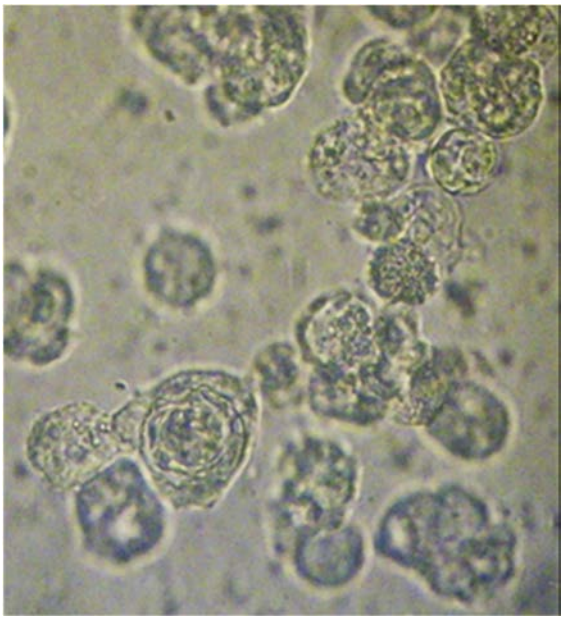

Fig. 2: Presence of *Acanthamoeba* spp., in the ovary tissue samples, shows *Acanthamoeba* trophozoites and cysts on PYG. X40 objective
Bars: 10 µm

PCR and sequencing: The partial sequencing of the 16S rRNA gene (mitochondrial subunit), DNA was successfully amplified (Fig. 3). Sequences were purchased from Eurofins Scientific, Wolverhampton, UK. With MUSCLE (v3.8.31), the sequence alignment was carried out using the default configuration and set to the greatest precision. The MEGA and the Phylogeny.fr platform were used to reconstruct the phylogenetic tree (Fig. 4). Through the use of TreeDyn, the tree was graphically depicted and altered (v198.3).

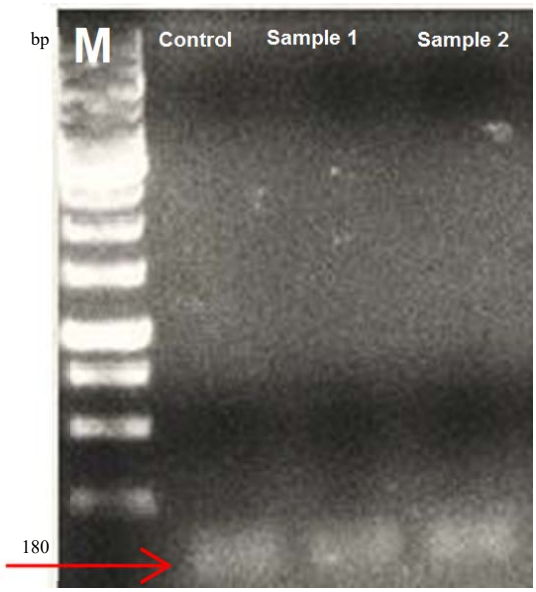

Fig. 3: Products of PCR amplified utilizing 16S rRNA primer. Bands were observed in the lane corresponding near to the expected approximately 180 bp
M: kbp DNA ladder, Control: *Acanthamoeba* (T7-control), Sample 1 and 2: Positive ovary tissue

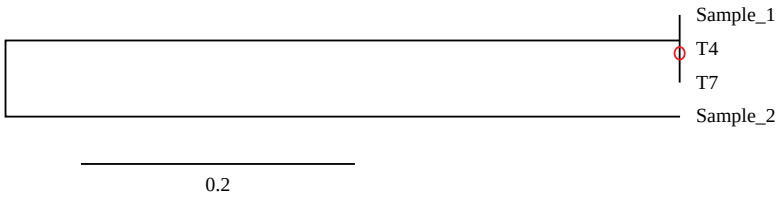

Fig. 4: Phylogenetic trees for the 16S rRNA quality utilizing information from two examples of ovarian tissue and two reference successions

DISCUSSION

In this investigation, some isolates could withstand temperatures as high as 50°C and when administered to rats, infected all of their organs and rendered them prematurely lethal. *Acanthamoeba* isolates with a high level of virulence were found in current investigation and they could actually pose a hazard to humans, were reasoned.

The *Acanthamoeba* spp., infected animal showed early infection spread in rats with high mortality. All of the examined organs in the sick animals were invaded by the most virulent isolate. The brain was the most typical location of isolation, followed by the lungs and all of the harmful amoeba isolates under study could be re-isolated from there. These findings were consistent with those of Vijayakumar[19], who discovered that the brain was the most frequently infected area after intranasal inoculation[20]. On the other hand, Kot *et al.*[21] claimed that the lung tissue was the area most severely impacted. The nose is the primary entry point for *Acanthamoeba*, where the amoebae pass through the cribriform plate and travel through the olfactory nerves to the brain[22]. The variation in virulence between isolates or even within the same isolate and the production of various virulence factors after the first infection may be the causes of the differences in *Acanthamoeba* tissue penetration.

The distribution of the lesions points to an infection that started in the cecum before spreading hematogenously to adjacent tissues. Death was attributed to the severe necrosis and inflammation that was typically present when amoebae were present in these tissues.

It has been seen that small, free-living amoebae from the genera *Acanthamoeba* and *Naegleria* live in soil and new water and eat bacteria and debris[2,7]. *Balamuthia mandrillaris* characterized by sometimes being isolated from soil[2] *Balamuthia* amoebae are thought to eat other microscopic amoebae that are prevalent in the environment rather than bacteria. There is some debate on this amoeba's dietary needs.

The CNS contaminations in people, primates like gorillas, mandrills and gibbons, as well as different vertebrates like canines, cows, bison, ponies, and kangaroos, have been connected to a few types of *Acanthamoeba* (*A. castellanii*, *A. culbertsoni*, *A. healyi*, *A. polyphaga* and *A. rhysodes*), the sole known species of *B. mandrillaris*[7]. Moreover, *Acanthamoeba* is known to initiate *Acanthamoeba* keratitis, an eye-compromising disease of the human cornea[23].

As per a new report utilizing SSU rRNA quality sequencing, a few *Acanthamoeba* secludes from fish and those connected to *Acanthamoeba* keratitis are individuals from a similar T4 phylogenetic gathering, recommending that the very attributes that permit these amoebae to contaminate animals may likewise help these amoebae taint humans[8]. Due to their capacity to coexist with free-living species in the natural world and dwell inside mammal tissue, these amoebas have been referred to as amphizoic amoebae[23].

Individuals can contract life-threatening infections from *Acanthamoeba* sp. The mortality rate for patients with *Acanthamoeba* sp., infections is quite high, ranging from 95-98%. These infections include *Acanthamoeba* sp., keratitis (AK) and granulomatous amoebic encephalitis (GAE) infection of the (CNS). Numerous factors contribute to this high mortality rate, including inadequate or delayed treatment for resistant cyst forms of *Acanthamoeba* sp. and incorrect or delayed diagnosis of the infection.

CONCLUSION

This work has demonstrated that an *Acanthamoeba* strain that has a wide range of genotypes and is capable of infecting people and other species can also infect rats fatally. The Saudi isolates of *Acanthamoeba* sp., could be determined as virulent strains. The virulence of *Acanthamoeba* sp., including Saudi isolates was enhanced by the mouse ovarian passage.

SIGNIFICANCE STATEMENT

Granulomatous amoebic encephalitis and keratitis are diseases brought on by the free-living amoebae known as *Acanthamoeba*. This study aimed to identify the cause of rats' death by isolating and identifying *Acanthamoeba* sp., from the ovary of Sprague-Dawley rats. Findings add to the body of evidence supporting the pathogenic *Acanthamoeba* sp., ability to cause death, which could be harmful to people's health. As a result, clinicians should be made more aware.

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