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Isolation and Characterization of *Vermamoeba vermiformis* from Swimming Pools in Lahore, Pakistan

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ABSTRACT

Background. Free-living amoebae (FLA) are common in aquatic environments and their interaction with humans can lead to significant public health risks. Many of these amoebae are opportunistic pathogens, causing infrequent yet severe diseases. *Vermamoeba vermiformis*, a widely distributed FLA, has been associated with keratitis infection, often in conjunction with *Acanthamoeba*. Furthermore, *V. vermiformis* can serve as a host for pathogenic bacteria, such as *Legionella pneumophila* and *Stenotrophomonas maltophilia*, amplifying potential health risks. This study aimed to investigate the presence of FLA in three (3) swimming pools situated in Lahore, Pakistan.

Methodology. A total of eighteen (18) water samples were collected from the swimming pools and filtered using 0.45µm cellulose acetate filter papers. The filter papers were carefully placed upside down on non-nutrient agar (NNA) plates seeded with heat-attenuated *E. coli*. A pure culture of FLA was obtained through repeated subculturing on NNA plates seeded with E. coli, ensuring the results' reliability and validity.

Results. Samples from all three (3) pools exhibited the presence of FLA. The isolated FLA was identified as *V. vermiformis* based on its morphological appearance under the light microscope, and molecular characterization was performed using the SSU rRNA gene sequence. The trophozoites of *V. vermiformis* were elongated and cylindrical, with a single pseudopodium, giving them a limax shape. The cysts of *V. vermiformis* had a double-walled oval and round structure. A clear hyaloplasm was observed at the anterior end of the pseudopodia of actively moving *V. vermiformis* under a light microscope.

Conclusion. SSU rRNA, gene-based, molecular characterization confirmed isolated FLA as a local isolate of *V. vermiformis.* Phylogenetic analysis indicated its close homology with *Echinamoeba*. The presence of *V. vermiformis* in swimming pool water poses a potential threat to human health, as it is an opportunistic pathogen and a well-known host of different pathogenic bacteria.

Keywords: bacteria, cysts, free-living amoebae (FLA), molecular characterization, swimming pools, opportunistic pathogens, swimming pools, trophozoites, *vermamoeba vermiformis*, water-brone

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Highlights

1. Water samples from swimming pools were analyzed for the presence of FLA.
2. Trophozoites and cysts of FLA were observed on NNA plates.
3. Molecular characterization based on the SSU rRNA gene confirmed the species as *V. Vermiformis.*

1. INTRODUCTION

The growing demands of the rapidly growing global population have driven the exploitation of natural resources for various benefits. However, this has also made the human population more vulnerable to natural pathogens. Many disease outbreaks are believed to be a consequence of human intervention, with AIDS and COVID-19 serving as prime examples[1, 2]. This is primarily because many living organisms are natural hosts for several pathogens. Any direct contact can lead to transmitting these disease-causing agents to human beings.

Free-living amoebae (FLA) is a diverse group of protozoa primarily engaged in consuming organic matter, bacteria, algae, and fungi[3–5]. These amphizoic protozoans inhabit many environments, including freshwater, seawater, soil, and air [6, 7]. The lifecycle of amoebae comprises two distinct stages, namely trophozoite and cyst. Trophozoites represent the active, motile, phagocytic, and reproducing phases, while cysts are characterized by thick walls and dormancy, with no active division[8]. Cysts typically possess two layers, that is, an outer ectocyst and an inner endocyst [9]. These layers exhibit remarkable resistance to desiccation, changes in pH, temperature fluctuations, and disinfectants[7, 10]. Several common FLAs, including *Naegleria fowleri*, *Acanthamoeba* spp., *Hartmannella* spp., *Balamuthia mandrillaris*, and *Sappinia* spp., are known to be pathogenic to vertebrates, including human beings. They can cause severe infections affecting the nervous system, respiratory tract, and skin [11–17].

Vermamoeba vermiformis, a widely distributed FLA of class Tubulinea, is characterized by its cylindrical body and wide pseudopodia[18–20]. Initially, it was identified as *Hartmannella vermiformis* due to its worm-like shape, high length-to-width ratio, and cylindrical form [21]. However, subsequent molecular studies revealed that *H. vermiformis* and *Echinamoeba* constitute a distinct clade separate from other members of the Hartmannellidae family [22]. The differences in both morphology and genetics led to the reclassification of *H. vermiformis* into the family Vermamoebidae, and the species was officially renamed *Vermamoeba vermiformis* [18]. It has been isolated from water samples collected from hospitals[23, 24], drinking water resources[25, 26], surface water [15, 27], rainwater [28], heating and cooling units [29], and soil [30].

V. vermiformis has gained attention due to its direct or indirect association with various diseases [31]. Its trophozoites have been identified in the cerebrospinal fluid of individuals suffering from amoebic encephalitis and bronchopneumonia [32]. *V. vermiformis* infects contact lens wearers, which leads to mixed keratitis infection when coexisting with *Acanthamoeba* [33]. The cytopathic changes caused by *V.*

vermiformis, including symptoms such as redness, satellite foci, and ciliary injection, are similar to *Acanthamoeba* [33–35]. Reports also link it to painful ulcers near the eye [36] and tissue lesions in fish during infections [37]. Additionally, *V. vermiformis* has been implicated in disease outbreaks affecting the gills of rainbow trout in southwestern Germany [38]. Another critical aspect is its role as a host for opportunistic pathogens such as *Legionella pneumophila*, the causative agent of Legionnaires' disease [39].

Pakistan's central and southern regions experience warm climatic conditions from March to October, creating an ideal environment for the proliferation of FLA. The presence of *V. vermiformis* in the country's water bodies remains relatively unexplored. The current study reports its detection in three swimming pools located in Lahore. It is imperative to investigate its prevalence in local aquatic ecosystems to estimate the risk of amoebic infections and associated pathogenesis. The identification of *V. vermiformis* was achieved through both morphological and molecular methods. This study also underscores the potential health risks for individuals using swimming pools and other recreational water facilities during the hot summer months.

2. MATERIALS AND METHODS

2.1. Sampling

A total of eighteen (18) water samples were collected in sterilized bottles from three (3) swimming pools in Lahore (Figure 1) in the summer month of June, according to the methodology of Ithoi et al. [40]. The samples were promptly brought to the laboratory and processed on the same day.

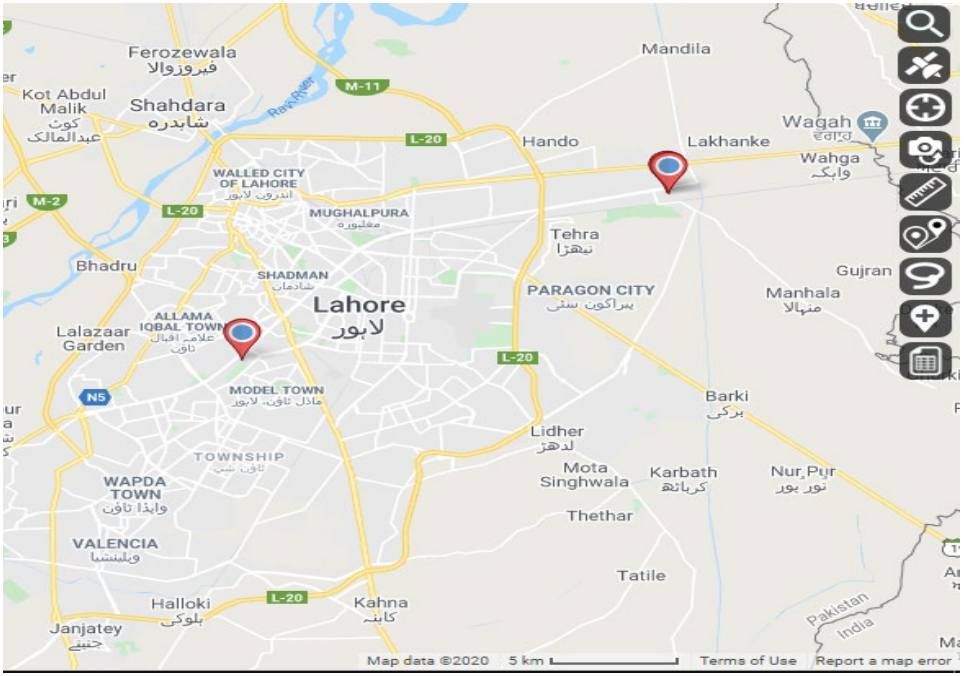

Figure 1. Location of the Selected Swimming Pools in Lahore

2.2. Isolation of FLA

All the water samples from the three (3) swimming pools were filtered under a weak vacuum using sterile cellulose acetate filter papers of 0.45µm pore size. These filter papers were put upside down on the non-nutrient agar (NNA) plates seeded with heat-inactivated *E. coli* and incubated at 27ºC for three (3) weeks [25]. Filter papers were removed after 24 hrs, and the plates were observed daily for FLA growth under an inverted microscope at 100X [41]. Plates with no amoebae were marked negative after 20 days.

2.3. Culturing and Purification

Plates positive for FLA growth were further used to obtain a pure culture. The purification step involved subculturing trophozoites and amoeba cysts with the lowest contamination onto fresh NNA plates seeded with heat-killed bacteria. This subculturing process was repeated until contamination-free pure culture was obtained. The pure culture of isolated amoeba was subsequently grown in a suspension of heat-inactivated *E. coli*. This culture was observed under the light microscope on alternate days.

2.4. Growth Characteristics

Approximately 30 cysts of isolated FLA were cultured in triplicate in 20 ml saline seeded with heat-killed *E. coli* at 27±2ºC. Growth was observed daily under the microscope. Three (3) drops, each of 5µl cultures, were placed on a clean glass slide for examination. The number of trophozoites in each drop was carefully counted, and the average count was used to draw the growth curve of FLA.

2.5. Morphological Characterization

The trophozoites and cysts of FLA on NNA plates were observed using an inverted microscope. For samples in the liquid medium, the drops of FLA culture were taken on a glass slide after scraping and vigorously shaking the flask to dislodge trophozoites from the glass walls. The slides were carefully observed under a light microscope at 100X and 400X. Flagellation test was also performed according to Lares-Villa and Hernandez-Pena [42].

2.6. Molecular Characterization

Molecular characterization of the isolated FLA was based on the SSU rRNA gene sequence. Genomic DNA was isolated following the method described by Costa et al. [43]. Briefly, the cells were scrapped off the NNA plate using a sterile cell scraper. The suspension was transferred to an autoclaved microfuge tube and centrifuged at 10,000 x g for 10 mins. The pellet was resuspended in 200µl of TEN buffer (10 mM Tris-Cl pH 8.0, 10 mM ETDA, 400 mM NaCl) with the addition of 2% [v/v] Triton X100 and 1% SDS, followed by incubation at 60ºC for 4 hours. Subsequently, DNA extraction was performed using the phenol-chloroform method. The SSU rRNA gene was amplified using universal primers for FLA [44]. The PCR reaction mixture contained 1 μg of genomic DNA, 200 μM of each dNTP, 0.4 pmol of each primer, 1.5U of Taq DNA polymerase, and 1.5mM $MgCl_2$ in the presence of 1X reaction buffer. The PCR amplification cycle consisted of an initial denaturation at 94ºC for 5 minutes, followed by 35 cycles of 94ºC (45sec), 65ºC (1 min), and 72ºC (55 sec), respectively, with a final elongation of 72ºC for 10 mins. The nucleotide sequence of the amplified SSU rRNA gene was submitted to NCBI GenBank under accession number MF112024.

2.7. Phylogenetic Analysis

The homologous sequences of the amplified DNA were retrieved from NCBI using the Nucleotide BLAST search tool. All these sequences were aligned using Clustal Omega. The phylogenetic tree was constructed based on the neighbor-joining method of base changes number per site through Mega 6.0 [45].

3. RESULTS

Microscopic examination of the culture plates revealed that seven (7) out of eighteen (18) plates from all three (3) swimming pools tested positive for Free-Living Amoebae (FLA).

3.1. Growth Characteristics

The growth pattern of the isolated FLA was studied in *E. coli* supplemented saline medium. The number of cells observed under the microscope indicated that the log phase started after three (3) days of inoculation. Whereas the growth optima (>500 cells per ml) was achieved after seven (7) days, followed by a stationary phase. A rapid decline was observed after nine (9) days (Figure 2). Maximum Growth was observed after one week of vaccination

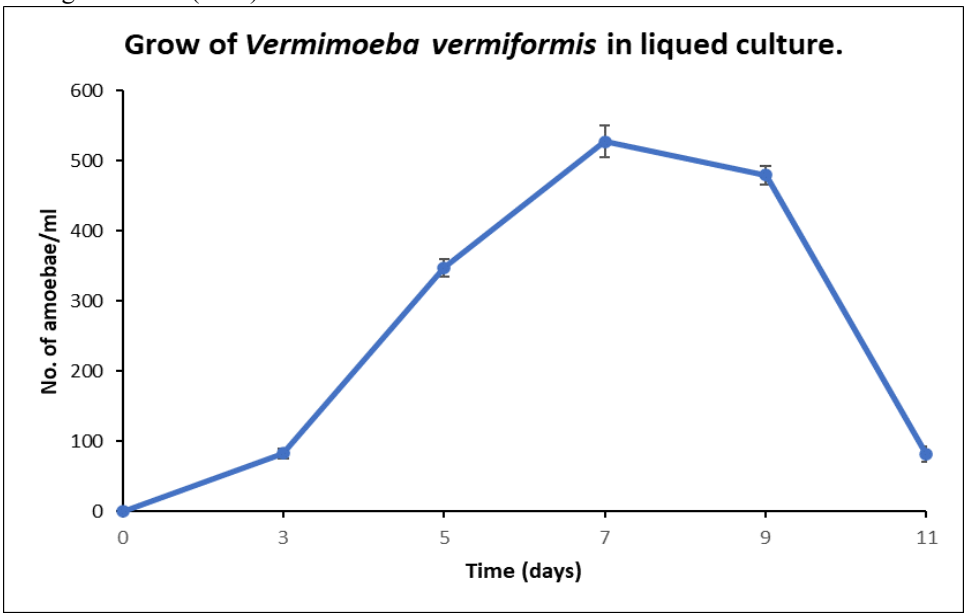

Figure 2. Growth Curve of *V. vermiformis* in Liquid Culture Supplemented with Heat-Killed *E. coli*

3.2. Morphological Characterization

The FLA trophozoites on NNA plates displayed irregular shapes (Figure 3); however, in liquid culture, they exhibited a limax shape with a single pseudopodium. The motile trophozoites showed a distinct hyaline cap at the anterior end of the newly formed pseudopodium. The nucleus and vacuolar structures were visible under a light microscope at 400X magnification. Although the trophozoites were observed after three (3) days, cysts could be seen

after nine (9) days of culturing. The cysts were typically round (Figure 4), smooth, double-walled, and without ostiole. They predominantly appeared in clusters, although isolated cysts were observed occasionally. No flagellated forms of *V. vermiformis* were detected.

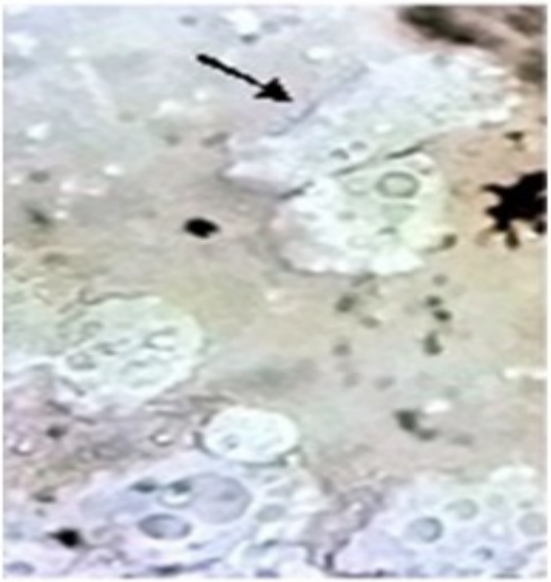

Figure 3. Irregular-Shaped Trophozoites of *V. vermiformis* on Non-Nutrient Agar

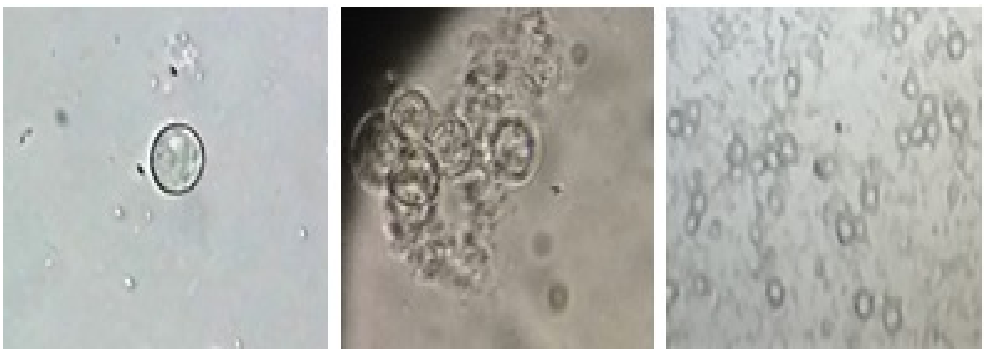

Figure 4. Cysts of *V. vermiformis* Appear Round with Smooth Double Walls

3.3. Molecular Characterization

The SSU rRNA gene fragment of approximately 800 bp was PCR amplified by universal FLA primers from the genomic DNA of the isolated FLA. DNA sequencing and BLAST analysis confirmed that the isolated amoeba is a local isolate of previously reported *V. vermiformis*, exhibiting 100% similarity. The phylogenetic relationship elucidated by constructing a phylogenetic tree utilizing the neighbor-joining method revealed that the isolated FLA and the previously reported *V. vermiformis* strains

share a close evolutionary relationship with *Echinamoeba*. Both organisms belong to the same class, Tubulinea, within the subphylum Lobosa (Figure 5). Evolutionary history inferred through the neighbor-joining method shows a close homology of *V. vermiformis* with *Echinamoeba*.

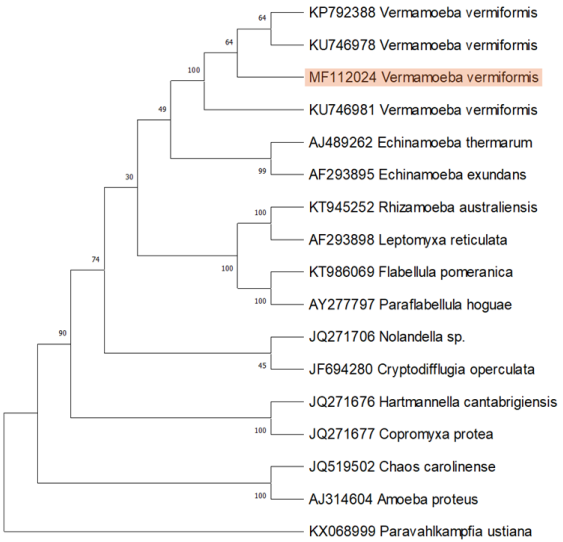

Figure 5. SSU rRNA-Based Phylogenetic Analysis of Locally Isolated *V. vermiformis* with Other Members of the Tubulinea Class.

4. DISCUSSION

Free-living amoebae (FLA) are opportunistic pathogens associated with some severe diseases, such as Primary Amebic Meningoencephalitis [46] and Amoebic Keratitis [33]. However, literature on their pathogenic role and prevalence in the ecosystem remains scarce. Hence, there is a pressing need for extensive research on all facets of FLA to understand better and control their pathogenic impact.

During the scorching summer months in Lahore, many individuals (often accompanied by their families, including children) flock to freshwater recreational areas. This increases their chances of coming into direct contact with waterborne pathogens. The presence of FLA is well-documented in aquatic environments[47–49]. Latifi et al. [50] conducted a study on their occurrence in hot water springs and along the beaches of the Caspian Sea. Out of the total sample collected, 54% were positive for FLA. Similarly, Gianinazzi et al. [48] reported the presence of 17 waterborne FLA in various aquatic sources, including rivers, lakes, and swimming pools in Switzerland. These findings highlight the importance of further exploring their role in ecosystems and their potential pathogenicity, especially their impact on animals and human beings.

The trophozoites of *V. vermiformis* exhibit a limax shape in the liquid medium. However, they can take on various forms on NNA plates, including limax, oval, or irregular shapes (Figure 4).

Many other researchers have also reported the typical limax shape appearing in liquid culture and irregular forms emerging on solid agar [21, 51, 52]. The current observations align with this pattern since it was found that irregular shapes were more prominent when *V. vermiformis* formed aggregates before encystment. This irregular appearance likely indicated the onset of the resting stage (cyst formation) as elongated trophozoites transitioned into rounded cysts.

The trophozoites of *V. vermiformis* typically have a single pseudopodium, although they occasionally produce two pseudopodia. The newly formed pseudopodium features a transparent hyaline cap at the anterior end. The hyaline cap contains clear hyaloplasm, distinguished by its lack of granular material, thus making it easily distinguishable from granuloplasm. While the hyaline cap is visible at the anterior end of pseudopodia, it is not observable in dorsal ridges when viewed from the top. Additionally, hyaloplasm and granuloplasm remain separate from ectoplasm and endoplasm, with the latter terms referring to the cytoplasm's viscosity [53].

Cysts of *V. vermiformis* appear as round, double-walled structures and possess a smooth outer surface. These cysts lack an ostiole, a feature consistent across both liquid medium and non-nutrient agar cultures. The presence of soft, double-walled cysts without an ostiole, which distinguishes them from *Acanthamoeba* cysts, has been reported in many studies [21, 52, 53]. The ostiole serves as the point of excystment during favorable conditions [54].

The widespread presence of *V. vermiformis* is reported in various man-made water bodies frequently visited by the public[6, 55]. In particular, it is more than just an opportunistic pathogen capable of causing amoebic encephalitis and keratitis [39]. Although, like most FLA, it feeds on bacteria, some pathogenic bacteria known as amoeba-resisting bacteria survive in its body. Thus, FLA functions as a Trojan horse for these pathogenic bacteria against disinfectants and increases their pathogenicity [56]. *V. vermiformis* could potentially serve as a reservoir for *L. pneumophila* within aquatic environments, posing a substantial public health concern [23]. *L. pneumophila* is a significant human pathogen transmitted through water sources [57]. This bacterium is responsible for legionellosis, commonly known as Legionnaires' disease, a severe form of pneumonia in immunocompromised persons [58]. So, the presence of *V. vermiformis* in swimming pools means more chances for an FLA attack and a higher population of pathogenic bacteria.

The presence of *V. vermiformis* in swimming pools is not uncommon, considering this amoeba genus is one of the most abundant. However, this may adversely impact human health with changing climatic conditions, particularly global warming. *V. vermiformis* enhances the resilience of amoeba-resistant bacteria to disinfectants and other antimicrobial agents, potentially leading to drug resistance and pathogenicity [59]. So, water bodies such as swimming pools must undergo proper disinfection procedures to minimize the risk of potential waterborne outbreaks. The resistance to disinfection, as observed in treatment plants [60, 61], and the inadequate cleaning of water pipelines within the pool may account for its presence [44].

4.1. Conclusion

This study marks the first report on *V. vermiformis* in aquatic water bodies in Lahore, Pakistan. *V. vermiformis* is an opportunistic pathogen and also hosts some pathogenic bacteria. Its presence in aquatic water bodies, especially swimming pools, may be alarming for immunocompromised persons. The rising summer temperatures and the increasing global warming may exacerbate this issue [62]. The detection of *V. vermiformis* in swimming pools highlights the need for proper disinfection to control the growth of opportunistic FLAs.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

FUNDING DETAILS

No funding has been received for this research.

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