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Original Article

Isolation and Molecular Identification of Vahlkampfiidae and *Vermamoeba Vermiformis* from Fresh Vegetables: A Neglected Source of Infections

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Abstract

Background: *Naegleria* spp., *Tetramitus* spp., and *Vermamoeba vermiformis* are potential pathogenic free-living amoebae (FLA) causing diseases such as keratitis, meningoencephalitis, and lung infections. We aimed to investigate the presence of Vahlkampfiidae and *V. vermiformis* in raw vegetables commonly consumed in Iran.

Methods: Totally, 70 samples of vegetables samples including watercress (22), leeks (12), parsley (10), basil (13) and mint (13) were collected from municipal markets of Tehran, the capital of Iran during June to October 2021. After washing vegetables, samples were cultivated onto 2% non-nutrient agar (NNA) medium. After morphological confirmations, DNA was extracted and identical fragments of the FLA were amplified and sequenced.

Results: Out of 70 cultured samples, 11 samples (15.71 %) were morphologically positive, of which four and seven were *V. vermiformis* and Vahlkampfiidae isolates, respectively. According to the PCR/sequencing results two, one, one, and one strains belonged to *N. australiensis*, *N. americana*, *Vahlkampfia* sp., *V. inornata,* and *T. aberdonicus,* respectively. All *Vermamoeba* genus were characterized as *V. vermiformis*.

Conclusion: The results of current study revealed the contamination of fresh raw vegetables with Vahlkampfiidae and *V. vermiformis*. In addition, to our knowledge this is the first report of *T. aberdonicus* in raw vegetables. Our findings highlight the public health importance of vegetables in transmission of FLA, as well as the potential role of FLA in transmission of potential pathogenic microorganisms via consuming of fresh raw vegetables.

Introduction

Free-living amoebae (FLA) are potentially pathogenic amoeba reported from humans and animals, including genera *Acanthamoeba*, *Naegleria fowleri*, *Balamuthia mandrillaris*, *Sappinia*, and *Vermamoeba*. These amoebae can cause amoebic encephalitis, ocular keratitis, and skin diseases in humans and animals (1). FLA are frequently found in environmental sources such as water, soil, air, and vegetables, dust, sewage, swimming pools and the sea, and hot springs (2). These protozoa have a dual life that can become parasitic if the conditions are appropriate. The majority of these amoebas in their parasitic life have two forms of trophozoites and cysts of which cysts are able to withstand adverse environmental conditions such as high/low temperature, environmental drought, and chemical agents (1, 3).

The Vahlkampfiidae family includes the genera *Naegleria*, *Willaertia*, *Tetramitus*, *Vahlkampfia* and *Paravahlkampfia*, of which *Naegleria* and *Vahlkampfia* are the most common in this family (1). So far, more than 47 species of *Naegleria* have been identified in the world (4). Various species of *Naegleria* including *N. australiensis; N. americana, N. dobsoni, N. pagei, N. polaris, N. fultoni,* and *N. gruberi* are among the most frequently reported species (5). In Iran, *Naegleria* spp., have been isolated in various water sources such as urban and drinking waters, and farm soils (5, 6) . However, there is no reports of *N. fowleri* in the environment, although a case of primary amoebic meningoencephalitis (PAM) in a 6-month-old child was reported in Iran (7). *Vahlkampfia* spp. have been isolated from environmental resources such as soil and urban water samples (8). *V. vermiformis* is a common FLA in environmental sources around the world, including soil, drinking water, and hot springs samples (5, 9, 10).

The presence of FLA in feces (11) suggests the ability of these protists to resist digestion by stomach acid and transmission by ingestion of contaminated sources such as vegetables. In addition, FLA can harbor pathogenic bacteria and carry them to the gastrointestinal tract (GT) (11). For example, *V. vermiformis* was diagnosed in a nasal swab of an HIV patient harboring *Mycobacterium chelonae* as endosymbiont (12).

It is worthy to mention that Vahlkampfiids including *Vahlkampfia* and *Paravahlkampfia* and *Vermamoeba vermiformis* can lead to severe disease's such as keratitis and encephalitis with poor prognosis (4,7, 12).

There are few studies focusing the contamination of fresh vegetables with free live amoebae using morphological criteria. Our previous study showed that vegetables could be contaminated by pathogenic *Acanthamoeba* genotypes (13). However, regarding the reports of clinical outcomes due to *Vermamoeba* and *Vahlkampfia* in the literature (14), and the importance of FLA for carrying pathogenic microorganisms as Trojan horse, we aimed to investigate the presence of Vahlkampfiidae and *Vermamoeba* spp., in raw vegetables, commonly used in Iranian meals.

Materials and Methods

Processing of the samples and cloning

Seventy fresh vegetable samples including: garden cress (22), chives (12), mint (13), parsley (10), and basil (13) were collected from municipal markets of Tehran city, Iran during June to October 2021 (13). These samples were collected from five different district (north, south, west, east and middle) in Tehran, Iran. Samples were processed as previously described (13). Briefly, 250 g of each vegetable sample was vigorously agitated in 250 mL of sterile PBS (pH= 7-8). After three-times washing by sterile PBS (pH= 7-8), the materials were then transferred to a 1-L graduated cylinder and incubated for 24 h at 25° C for more precipitation. Afterwards, supernatant was filtered by a 47-mm nitrocellulose membrane filters (0.45 µm pore size; Sartorius,

Goettingen, Germany). The membranes were then cultured onto non-nutrient agar (NNA) plates, covered with inactivated *Escherichia coli* bacteria. The plates were incubated at room temperature, and were followed by daily examination of the cultures under light microscope to investigate the presence of trophozoites and cysts of amoeba. The positive plates for Vahlkampfiidae and *V. vermiformis* were purified using continues cultivation.

DNA extraction, PCR, sequencing and alignments

To extract genetic materials, trophozoites and cysts of *V. vermiformis* and Vahlkampfidae were washed and collected from the plates surface. DNA was extracted using a commercial total DNA extraction kit (Yekta Tajhiz Azma, Iran). To amplify the characteristic fragments, primer pairs (ITS1,2 primers: F5'-GAACCTGCGTAGGGATCATTT-3' and R 5'TTTCTTTTCCTCCCCTTATTA-3) for Vahlkampfiidae and (NA1, 2 primers: Hv1227F 5'-TTACGAGGTCAGGACACTGT-3' and NA2, Hv1728R 5'-GACCATCCGGAGTTCTCG-3' for *V. vermiformis* were employed (15). The PCR reaction was performed using 2X red PCR master mix (Ampliqon, Denmark), 10 ϱM of each primers, DNA, and distilled water. The cycling condition was set as pre-denaturation step for 5 min at 94°C, followed by 35 repetitions at 94°C for 45 s, annealing steps were at 56°C for 45 s, and 72°C for 45 s. The extension time was prolonged for 10 min at 72°C. The PCR products were separated by 1.5% agarose gel, stained with a solution of ethidium bromide, and detected by UV transilluminator. PCR products were sequenced in one direction and by forward primers.

Molecular and phylogeneic analyses

To characterize genus/species, all sequences were mannually edited and subjected to the BLAST online software. Sequences were submitted to the GenBank database with accession numbers: MZ708947-MZ708957. To explore the genetic correlation of isolates, obtained sequences were aligned and mannually trimmed by BioEdit (v. 7.2.6), and a phylogenetic tree was constructed for using MEGAX software based on Maximum Likelihood (ML) and Tamura 3-parameter with bootstrap 1000 replicants (16). Bootstrap higher than 75 % were considered as significant.

All procedures performed in this study were in accordance with the ethical standards released by Ethical Review Committee of the Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1401.243).

Results

Vahlkampfiidae and Vermamoeba purification

11/70 (15.71 %) of commonly used vegetable samples were positive for the FLA based on the morphological page keys (Fig. 1). Accordingly, the positive vegetable samples were garden cress (4/11; 36.3%), chive (3/11; 27.2%), parsley (2/11; 18.1%), mint (1/11; 9%), and basil (1/11; 9%) (Table 1).

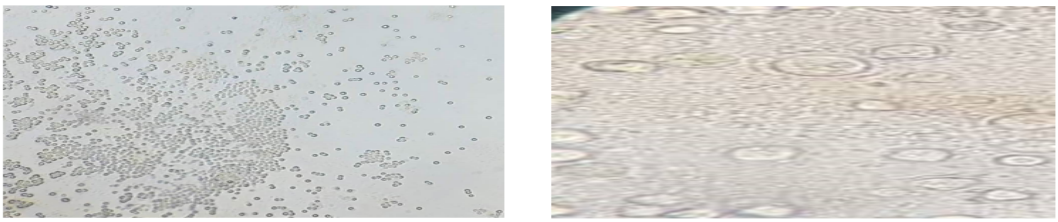

Fig. 1: Photograph of Vermamoeba vermiformis (Left) and Vahlkampfiids (right) cysts using plate culture (x10 and x40). (Original)

Table 1: Contamination rate of vegetable samples to *Vermamoeba* and Vahlkampfiidae using morphological method

Vegetables	*Total No.*	*No (%) of positive samples*
Garden cress	22	4 (36.3)
Chives	12	3 (27.2)
Parsley	10	2(18.1)
Mint	13	1 (9)
Basil	13	1 (9)
Total	70	11

Molecular identification and genotyping

PCR test showed that four (5.71%) and seven (10%) of samples were positive for *Vermamoeba* and Vahlkampfiidae, respectively. The results of gene sequencing showed that Vahlkampfiidae belonged to *N. australiensis* (isolates val.v6 and val.v7), *N. americana* (val.v3), *Vahlkampfia* sp. (val.v1 and val.v2), *V. inornata* (val.v11), and *T. aberdonicus* (val.v4). In addition, all Vermamoeba were identified as *V. vermiformis* (isolates H.v1, H.v6, H.V5, H.V9) (Table 2). Val.v7 was isolated from Garden cress and showed high homology and query coverage (100%) with a strain isolated from Pool water in Iran (Acc. No: MT292609.1). Furthermore, Val.v1 was isolated from garden cress and showed high homology and query coverage (100%) with the Acc. No: MW031120.1. An isolate designated as H6 was isolated from chive and showed high homology and query coverage (100%) with Acc. No: JQ271687.

Table 2: Data regarding the sources and the isolated species/genotype of *Vermamoeba* and Vahlkampfiidae in vegetable samples.
NA: Not assigned.

Isolate codes	*Vegetables*	*Genus / Species*	*Detection method*	*Identity/Query coverage*	*Similar Acc. No.*	*Acc. No.*	*Sources of ref*
Val.V1	Garden cress	*Vahlkampfia* sp.	PCR	100/100	MW031120.1	MZ708947	NA
Val.v2	Mint	*Vahlkampfia* sp.	PCR	99.24/96	MW031120.1	MZ708948	NA
Val.v3	Basil	*N. americana*	PCR	98.84/92	LC128777.1	MZ708949	Fish gills
Val.v4	Parsley	*T. aberdonicus*	PCR	100/90	AJ698842.1	MZ708950	Water
H.v5	Chive	*V. vermiformis*	PCR	97.22 /92	HE617185.1	MZ708951	Biofilm
Val.v6	Parsley	*N. australiensis*	PCR	100/98	GU597033.1	MZ708952	Biofilm in hot spring water
Val.v7	Garden cress	*N. australiensis*	PCR	100/100	MT292609.1	MZ708953	Pool water(iran)
H.v9	Chive	*V. vermiformis*	PCR	98.10/92	KT185625.1	MZ708955	Culture of A. castellani
Val.v 11	Garden cress	*V. inornata*	PCR	96.95/82	AJ698838.1	MZ708954	Water
H.v1	Garden cress	*V. vermiformis*	PCR	99.78/99	JQ271687.1	MZ708956	River
H.v6	Chive	*V. vermiformis*	PCR	100/100	JQ271687.1	MZ708957	River

Phylogenetic analysis

Phylogenetic tree was employed to clear the relationship between our isolates and reference isolates. Accordingly, our isolates were clearly clustered with reference genes (Fig. 2).

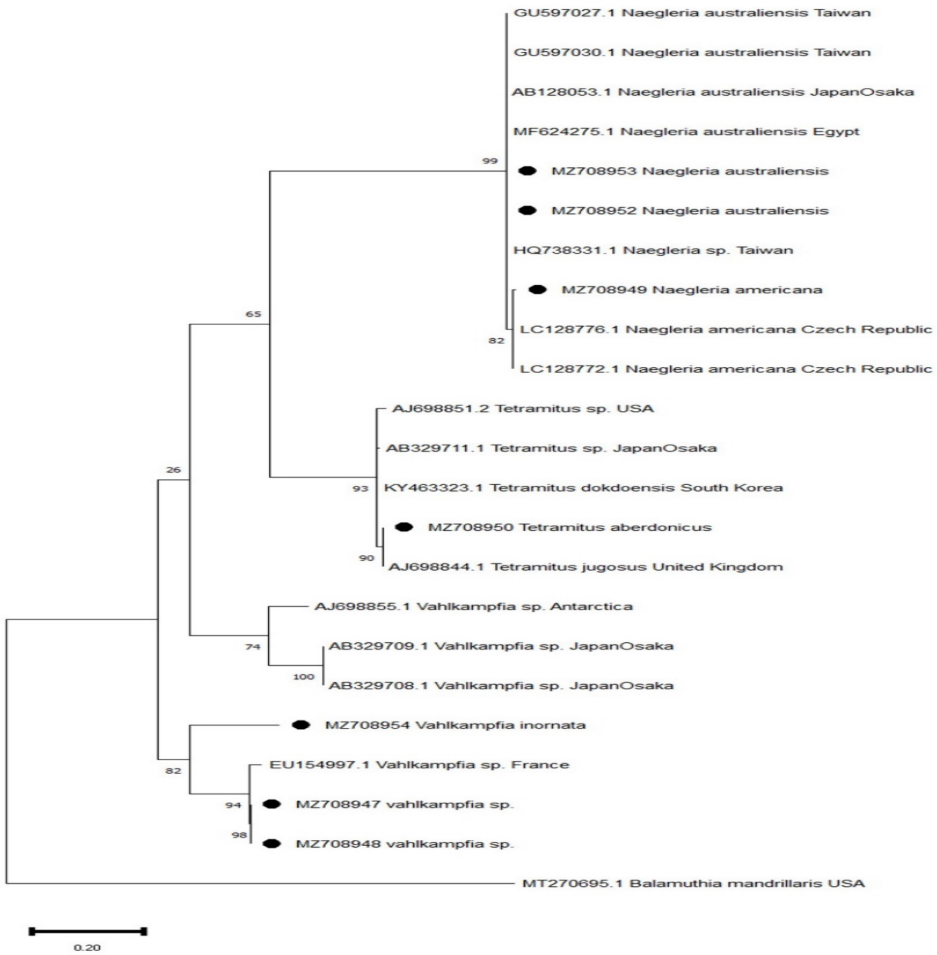

Fig. 2: Phylogenetic tree of the 18S rRNA gene of Vahlkampfiidae isolated from vegetable samples together with reference sequences. The phylogenetic tree represents that all identified strains were cluster with the reference genotypes. The phylogenetic tree was drawn using the maximum-likelihood method and the Tamura 3 parameter model. Bootstrap support (%) values of >75% are indicated above the branches. Asterisks indicate reference genotypes

Discussion

The present study is the first study isolating and characterizing Vahlkampfiidae and *V. vermiformis* from commonly raw used vegetables including leeks, parsley, watercress, basil, and mint. In addition, to the best of our knowledge, this is the first study describing *T. aberdonicus* and *V. inornata* from vegetables.

Vegetables can carry a couple of pathogenic parasites including FLA (13, 17, 18). One of the pioneer studies was conducted by Vaerewijk et al (19), who recovered *A. polyphaga* in

vegetable boxes and on the inside surface of domestic refrigerator samples. More recently, Soler et al., (17) analyzed 40 organic cabbage, lettuce, spinach, and strawberry samples, which all were positive for *Acanthamoeba* sp., and *V. vermiformis*. In this regard, our team investigated vegetable samples collected from municipal markets and isolated potentially pathogenic *Acanthamoeba* sp. from samples (13). However, concerning the role of FLA in carrying a couple of potentially pathogenic microorganisms, the presence of them in fresh products increases the public health concerns. For example, Moreno-Mesoero evaluated lettuce samples for the presence of *Helicobacter pylori*. Interestingly, although *H. pylori* was not detected neither by cultivation nor molecular tests from lettuce samples, all isolated FLA were positive for internalized *H. pylori*, and 5/25 (25%) were viable (18). Soler et al., (17) investigated the microbiome pattern of FLA isolated from fresh products and reported *Pseudomonas* and *Flavobacterium*, as dominant bacterial genus. The presence of human pathogenic bacterial taxa such as *Arcobacter*, *Klebsiella*, *Mycobacterium*, *Salmonella*, and *Legionella,* beside *Pseudomonas* and *Flavobacterium* highlighted the role of FLA in transmission of pathogenic microorganisms (17). It was suggested that fresh vegetables could be infected by FLA via irrigation with contaminated water resources and/or during harvest and packaging processes (17, 20, 21). The reports of FLA from effluent wastewater discharged by refinery wastewater treatment facility, which is employed for irrigation of vegetable farms, not only supports the fact that FLA are resistant to harsh conditions, but also signify the role of irrigation by contaminated sources in transmission of FLA to vegetables (3).

Naegleria spp., are commonly isolated from hot springs (4), however, this genus has been reported from farmlands (5, 22). Contamination of farmlands to FLA seems to happen via irrigation of fresh products with FLA-contaminated water sources. In this regard, Pazoki et al., (22) investigated the presence of FLA from water canals used for irrigation of farmlands, and isolated *N. philippinensis* and *N. americana* from samples. In addition, *Naegleria* spp., may carry a couple of human-infecting microorganisms. Huang et al., isolated *N. lovaniensis*, *N. australiensis*, *N. clarki*, *N. americana*, and *N. pagei* from hot springs, while 5.7% of the water samples were positive for both *Naegleria* and *Legionella* species (*L. pneumophila* and *L. erythra*) (23). Denet et al., showed coexistence of *Micrococcus luteus*, *Kocuria rhizophila*, or *Brevibacterium iodinum* with FLA genera such as Naegleria (24). Therefore, the presence of *Naegleria* spp. in fresh vegetables not only signifies the chance of transmission of potentially pathogenic species to humans, but also increases the risk of secondary infection due to the pathogenic endosymbionts.

As a finding, *T. aberdonicus* was isolated for the first time in vegetable samples. *T. aberdonicus* was firstly isolated from water samples in the United Kingdom (25). This genus has been isolated from cold water, salt lakes, hot springs, soils, and more recently refinery wastewater (3, 26-28). Except for *T. vestfoldii*, which is isolated from Antarctic salt lake and grows at 5 °C (28), this amoeba usually grows at room temperature (27, 29). *T. lobospinosus*, *T. entericus*, and *T. wacamawensi* are the species, have been isolated from feces or contaminated soil and can grow at 37 °C (29). In the current study, *T. aberdonicus* was isolated from NNA at room temperature (25 ° C), which is similar to the conditions, reported from our previous study (3). Notably, *Tetramitus* was isolated as the predominant FLA from agriculture and garden soil, while bacteria such as *Micrococcus luteus* and *Bacillus* sp., *Enterococcus faecium*, *Paenibacillus* sp., *P. aeruginosa*, and *Stenotrophomonas maltophilia* were characterized as its endosymbionts (24).

V. inornata is a less known FLA, which has been isolated from water resources (30, 31). This FLA is known as a non-pathogenic species; however, the presence of *V. inornata* in fresh vegetables highlights the role of water

resources in contamination of downstream farmlands.

V. vermiformis is frequently reported from water resources, wastewaters, and soil samples, which its association with pathogenic bacteria has been increasingly considered (32-35). Pagnier et al., (34) described *V. vermiformis* as the predominant FLA in hospital water samples and suggested association between this protozoan with *L. taurinensis*. Recently, Nisar et al., (36) showed that *V. vermiformis* was the major FLA isolated from domestic and hospital water system. They suggested the presence of *L. pneumophila* inside the isolated *V. vermiformis* using qPCR and fluorescence in situ hybridization (36). *V. vermiformis* was isolated from nasal swap of a HIV/AIDS patient in Peru carrying pathogenic bacteria, *M. chelonae* (12). The presence of *V. vermiformis* in organic fresh vegetables (37) and its association with potentially pathogenic bacteria (17) increase the public health concern due to this protozoan.

The limitations of this study was that during the Corona era, we sometimes faced the closure of educational centers.

Conclusion

Our results suggested the presence of Vahlkampfiidae and *V. vermiformis* in fresh raw vegetables. In addition, to our knowledge this is the first report of *T. aberdonicus* in raw vegetables. Regarding the presence of potentially pathogenic FLA, our findings highlight not only the chance of transmission of pathogenic FLA to humans via fresh products, but also the public health importance of vegetables due to transmission of potential pathogenic microorganisms to humans via consuming of fresh raw vegetables.

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Conflict of interest

The authors declare that they have no conflict of interest

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