Molecular detection of *Entamoeba histolytica* in fresh vegetables and irrigation water

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ABSTRACT

Entamoeba histolytica (*E. histolytica*) is the causative agent of human amebiasis. As *E. histolytica* remains an important reason of morbidity and mortality in developing countries, it can cause up to 100,000 deaths/year worldwide. In this study, a survey was carried out on contamination of some common freshly eaten vegetables and their irrigation water with *Entamoeba histolytica*. Fresh vegetable samples were collected from public markets and agriculture field in Nahia and Saft areas, Giza, Egypt. A total of 255 vegetable and irrigation water samples were separately examined by PCR technique using *Entamoeba histolytica* species specific primers. The results showed that *Entamoeba histolytica* was found in 6.4% (7/110) and 3.7% (4/109) of field and market vegetable samples, respectively. *Entamoeba histolytica* was detected in 5.6% of 36 irrigation water samples collected from the same agriculture fields. A seasonal pattern of the presence of *Entamoeba histolytica* was observed with a high prevalence during cool seasons. In conclusion, contamination of freshly eaten vegetables may represent a risk to the health of consumers. Dill is the most contaminated vegetable by *Entamoeba histolytica*.

INTRODUCTION

Entamoeba histolytica is considered a protozoan parasite of infantile public health importance (Haque *et al*., 2003). *Entamoeba histolytica* is an invasive intestinal pathogenic protozoan belonging to sarcodines (Singh *et al*., 2009). In developing countries, *Entamoeba histolytica* is an important reason of morbidity and mortality in babies (WHO 1996 and 2005). About 80-90% of entamoebic infections are asymptomatic and are likely due to the nonpathogenic species such as *E. dispar* or *E. moshkovskii*. Therefore, the worldwide incidence of *E. histolytica* was nearly estimated to be 5 million cases annually (Ben Ayed and Sabbahi, 2017).

The life cycle of *Entamoeba histolytica* is simple and consists of an infective cyst stage (10 to 15μm in diameter) and a multiplying trophozoite stage (10 to 50μm in diameter) (Lebbad, 2010).

About 6 varied species belonging to Genus *Entamoeba* (*E. moshkovskii, E. dispar, E. histolytica, E. coli, E. polecki* and *E. hartmanni*) are detected in the human intestinal lumen (Philips *et al*., 2018). These species are accepted as commensals,

except for *E. histolytica* (Leber and Novak 2011). Morphological identification cannot differentiate between non-pathogenic *Entamoeba dispar*/*Entamoeba moshkovskii* and pathogenic *Entamoeba histolytica,* but molecular techniques can do (Fotedar *et al*., 2007).

The intestinal protozoan *Entamoeba histolytica* is responsible for up to 100.000 deaths per year worldwide, with especial reference to developing countries (Wiwanitkit and Assawawitoontip, 2002). *Entamoeba histolytica* is the causative agent of amoebiasis with or without clinical manifestations (WHO, 1997). The prevalence of amoebiasis depends on socioeconomic conditions of the population; whereas up to 50% of the affected populations were in areas with poor sanitary conditions (Caballero-Salcedo *et al*., 1994). Comparatively, amoebic colitis predominated in Egypt, while amoebic liver abscess prevailed in South Africa (Stauffer *et al*., 2006). Amoebiasis, in the majority of infected persons is symptomless. In some cases and after few months, the asymptomatic persons having *E. histolytica* cysts may provoke colitis. The common symptoms caused by *E. histolytica* dysentery are diarrhea, tenderness and abdominal pain (watery, bloody, or mucous). Amoebic diarrhea can cause fever in some patients and recurrent bowel movements (about 10 or more per day). Patients are often reluctant to eat, and may lose some weight (Haque *et al*., 2003).

Fresh vegetables can be contaminated with intestinal parasites, during production, collection, transport, preparation and processing (Erdogrul and Sener, 2005) . Freshly consumed vegetables constitute a conventional portion of the eating habit of many people. When eaten in raw or without peeling, vegetables can easily transmit food-borne protozoan parasites (Hassan *et al*., 2012). Microscopic examination is a simple low cost method and still the most common and routinely used technique for identification of intestinal parasites as this technique solely depends on differences in morphologic criteria between different organisms (Kebede *et al*., 2004). However, microscopic examination has been shown to offer a low sensitive method depending, largely, upon the skills of persons carrying out the analysis (Verweij *et al*., 2004). PCR methodology became very important and vital tool for differentiation, genotyping and sub-typing of enteric protozoa. The previously developed PCR assays targeted one or more gene loci for one specified enteric protozoan. As previously proved that the sensitivity and specificity of molecular techniques are greater than traditional microscopic methods (EL-Sabbagh, 2010), so, the aim of the present work was to evaluate the prevalence of *Entamoeba histolytica* on freshly eaten vegetables and their irrigation water by using PCR techniques.

MATERIALS AND METHODS

Study area:

The present study was carried out in Nahia and Saft areas located in Giza, Egypt (Figure 1). Choosing the areas of study depended on the type of water by which the agriculture lands were irrigated. Nahia areas have agriculture fields irrigated with two types of irrigation water; surface freshwater from Maryotia canal (for Nahia1) and ground water (for Nahia2) Saft area has agriculture fields irrigated only with ground water. Fresh vegetables and irrigation water were collected from the same agriculture field in Nahia and Saft areas. Samples from cultivated freshly-eaten vegetable in these agriculture lands and from public markets in Giza, Egypt were

separately collected. In addition, irrigation water samples were also collected. All samples were collected from December 2014 to November 2016.

Fig. 1: A map showing the field study areas (Nahia and Saft) in Giza, Egypt, which are signed with yellow stars.

Sample collection:

About 110 and 109 fresh vegetable samples were collected from agriculture fields and public markets, respectively. In addition, 36 field irrigation water samples were collected from the same agriculture fields from which vegetables were collected. The main vegetables grown in these examined agriculture fields were Dill (*Anethum graveolens*), parsley (*Petroselinum crispum*), watercress (*Nasturtium officinale*), tomatoes (*Solanum lycopersicum*), lettuce (*Lactuca sativa*), carrot (*Daucuscarota*), white radish (*Raphanussativus var. Longipinnatus*), green onion (*Allium cepa*), and cucumber (*Cucumis sativus*).

All fresh vegetable samples (500g of each) were separately collected in clean transparent nylon bags. Irrigation water samples were collected in 20L sterile polypropylene containers. All collected samples were separately labeled with stickers having date, name of sample, name of collecting area and type of irrigation water. Samples were transferred to Environmental Parasitology Laboratory, National Research Centre, Dokki, Giza, Egypt at the same day of collection.

Processing of collected samples:

Each vegetable sample was washed twice, firstly with 2L distilled water and secondly with a detergent solution consisting of 10mL of Tween 80 diluted in two liters of physiological saline solution (0.85% NaCl) (Luz *et al*., 2017) with vigorous shaking for 15 min (Al-Shawa and Mwafy, 2007) and the used washing water was managed the same as irrigation water samples.

Each water sample was filtered through stainless steel pressure filter holder (Sartorius) using nitrocellulose membrane (0.45μm pore size and 142mm diameter) (Brandonisio *et al*., 2000). After filtration, the membrane filter was washed 3 times (each time with 100ml sterile physiological saline). The obtained washing solution was then centrifuged at 2000rpm for 5min for the collection of debris (Kwakye-nuako *et al*., 2007 and WHO, 2000). Supernatants were discarded and the pellets were separately collected in an eppendorf tube and kept at -20ºC for PCR techniques.

Molecular examination by PCR:

The obtained concentrate from each sample (that was kept at -20ºC) was separately subjected to DNA extraction using QIAamp DNA stool mini kit according to the manufacturer instructions. The extracted DNAs were used as templates in the

PCR reaction. The selected forward primer was: Enta (ATGCACGAGAGCG AAAGCAT) and the reverse primer was EhR (GATCTAGAAACAATGCTTCTCT) (Hamzah *et al.*, 2006). The primers used in this study were checked for their specificity by conducting BLAST searches on the GenBank DNA sequence database (http://www.ncbi.nlm.nih.gov). Primers showed 100% specificity for the target gene loci and a high number of copies for gene target (18S rDNA) within the organism were accepted. The target PCR product for *E. histolytica* was 166bp. PCR amplifications were carried out by using BIOER Little Genius thermal cycler apparatus, USA. PCR amplification was performed in a total volume 20µL containing 1µl of DNA, 1X green buffer (Promega), 1.5mM $MgCl_2$ (Promega), 0.2mM dNTPs (Promega), 1U of Go Taq Hot start polymerase (Promega) and 10 pmol of each primer. PCR cycling conditions began with initial denaturation at 95°C for 3min, followed by 35 cycles of 95°C for 40sec, 55°C for 40 sec and 72°C for 40sec. A final extension step was performed at 72°C for 10min (Hamzah et al., 2006). The PCR product was analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide and photographed under UV transillumination using the Gel Doc 1000 image analysis system (Bio-Rad, Hercules, CA, USA).

RESULTS

Entamoeba histolytica was detected by PCR in 7 out of 110 (6.4%) vegetable samples collected from agriculture fields. Also, 2 (5.6%) out of 36 field irrigation water samples collected from the same fields were also positive for *Entamoeba histolytica*. Moreover, 4 (3.7%) out of 109 vegetable samples collected from markets were positive for *Entamoeba histolytica* (Table 1).

Table 1: Molecular detection of *Entamoeba histolytica* in collected vegetables and irrigation water samples.

Sample source	Total samples	Positive samples Number (%)
Field vegetables	110	7 (6.4%)
Market vegetables	109	4 (3.7%)
Irrigation water	36	2 (5.6%)

The highest prevalence of *Entamoeba histolytica* was recorded in vegetables (12.9%) collected from Nahia1 area (irrigated by surface water), followed by 4.3% and 3.6% of vegetable samples from Saft area (irrigated by ground water) and Nahia2 area (irrigated by ground water), respectively. Concerning field irrigation water, it was found that *Entamoeba histolytica* contaminated 16.7% of surface irrigation water in Nahia1 area. On the other hand, no contamination with *Entamoeba histolytica* was detected in ground irrigation water of both Nahia2 and Saft areas (Table 2, Fig. 2).

Table 2: Prevalence of *Entamoeba histolytica* on vegetable and irrigation water samples from different field areas by PCR.

		Samples sites		
		Nahia1*	Nahia2**	Saft**
Vegetables	Total	31	56	23
	Positive	4	2	1
	%	12.9	3.6	4.3
Irrigation water	Total	12	12	12
	Positive	2	0	0
	%	16.7	0	0

*= Field irrigated with surface water   **= Field irrigated with ground water
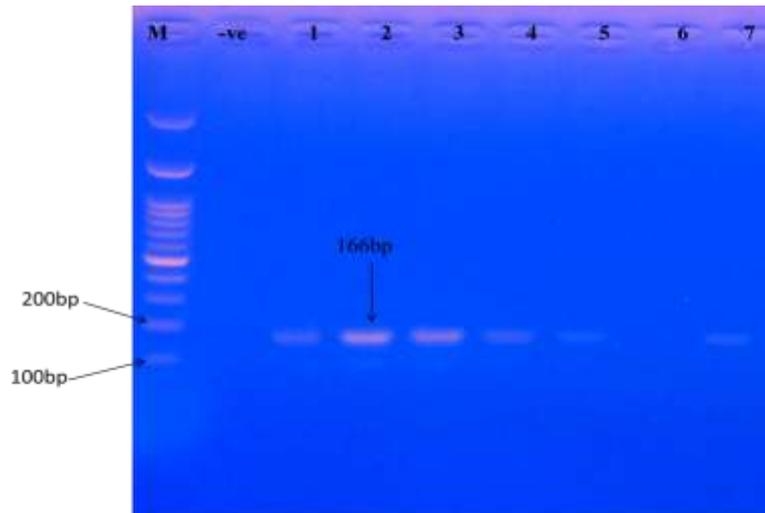
Fig. 2: Agarose gel electrophoresis for PCR products of *Entamoeba histolytica*. M: Marker; -ve: negative control; 1, 2, 3, 4, 5 and 7: positive samples; 6: negative sample.

In field vegetables, the highest prevalence rate of *Entamoeba histolytica* was found in 21.4% of dill samples, followed by 14.3%, 9.1% and 8.3% in parsley, lettuce and watercress samples, respectively. No contamination with *Entamoeba histolytica* was noticed in white radish, green onion, tomatoes, carrot and cucumber collected from field. In market vegetables, the highest prevalence rate of *Entamoeba histolytica* was detected in 16.7% of dill samples, followed by white radish (9.1%) and tomatoes (6.3%) samples. No contamination with *Entamoeba histolytica* was detected on lettuce, parsley, watercress, green onion, carrot and cucumber (Table 3).

Table 3: Prevalence of *Entamoeba histolytica* on different types of examined vegetables.

Sample type	Field vegetables		Market vegetables	
	Examined	Positive (%)	Examined	Positive (%)
Lettuce	11	1 (9.1)	12	0 (0)
Parsley	14	2 (14.3)	11	0 (0)
Watercress	12	1 (8.3)	10	0 (0)
Dill	14	3 (21.4)	12	2 (16.7)
White radish	12	0 (0)	11	1 (9.1)
Green onion	11	0 (0)	12	0 (0)
Tomatoes	12	0 (0)	16	1 (6.3)
Carrot	12	0 (0)	13	0 (0)
Cucumber	12	0 (0)	12	0 (0)

Concerning seasonal variation in field vegetables, the highest prevalence rate of *Entamoeba histolytica* reached 17.2% in winter, followed by 3.7% and 3.4% in autumn and spring, respectively, but no contamination with *Entamoeba histolytica* occurred in summer. With respect to seasonal variation in market vegetables, the highest prevalence rate of *Entamoeba histolytica* reached 7.4% in autumn, followed by 3.7% and 3.6% in summer and spring, respectively, while no contamination was detected in winter. In field irrigation water, the prevalence of *Entamoeba histolytica* reached 11.1% in each of winter and spring, but no contamination with *Entamoeba histolytica* was detected in both autumn and summer (Table 4).

Table 4: Seasonal prevalence of *Entamoeba histolytica* on examined samples by PCR.

Sample types	Season	Total examined samples	Positive samples	
			No.	%
Field vegetables	**Autumn**	27	1	3.7
	Winter	29	5	17.2
	Spring	29	1	3.4
	Summer	25	0	0
Market vegetables	**Autumn**	27	2	7.4
	Winter	27	0	0
	Spring	28	1	3.6
	Summer	27	1	3.7
Irrigation water	**Autumn**	9	0	0
	Winter	9	1	11.1
	Spring	9	1	11.1
	Summer	9	0	0

DISCUSSION

Diagnostic methods based on microscopic examination cannot differentiate between *Entamoeba histolytica* and / or *dispar*, while molecular techniques were used for species differentiation (Hemmati *et al*., 2015). Therefore, in the present study we used PCR for detection of *Entamoeba histolytica* in environmental samples to obtain an accurate consequence of contamination with that pathogen. WHO has put emphasis on the need to develop improved techniques for the species-specific diagnosis of *E. histolytica* infection as the light microscopy cannot differentiate between *Entamoeba* spp. and inaccurate outcomes were obtained (WHO, 1997). The distinction between *E. histolytica*, *E. dispar,* and *E. moshkovskii* has led to some confusion in epidemiological studies of amoebiasis (Hooshyar *et al*., 2012).

Globally, few reports concerning the prevalence of *E. histolytica* on vegetables and irrigation water samples were documented by using PCR technique. The present study indicated that the contamination with *E. histolytica* in surface irrigation water of Nahia1 area was found to be 16.7% by using PCR. Other researchers in Turkey recorded a higher percentage (32%) of *E. histolytica* in Ankara river by PCR (Bakir *et al*., 2003). In Rasht city located in Iran, *Entamoeba* was present among 4 samples out of 49 surface water samples by microscope while only one sample was confirmed for *E. histolytica* by PCR (Hemmati *et al*., 2015) .

In the current study, the prevalence of *Entamoeba histolytica* in market vegetables reached 3.7% by PCR. Many epidemiological surveys on the prevalence of intestinal amoeba based on microscopy were published worldwide; all of them showed a higher prevalence of infection than the present study such as Egypt (7.1%) (Hassan *et al.,* 2012), Sudan (42.9%) (Mohamed *et al*., 2016), Syria (8.75%) (Alhabbal, 2015), and Nigeria 20% and 5.6% (Simon-Oke *et al*., 2014 and Auta *et al*., 2017). Results from the previous studies seemed to be not accurate because the *Entamoeba histolytica* is similar with *Entamoeba dispar* in morphological characters.

The present study showed that, *Entamoeba histolytica* was detected in washing water of dill, parsley, lettuce, watercress, white radish and tomatoes collected from the market and/or the field, with the highest prevalence in dill, followed by lettuce. The highest prevalence of parasitic contamination on vegetables may be due to the rough surface and leaf folds of this vegetable (ex. dill) which may retain dirt that cannot be easily washed off (Islam *et al*., 2004). Also, the large surface area and a compact structure (ex. lettuce) that can provides better fixation and permanence of infective parasitic stages (Adamu *et al*., 2012). It is believed that the main source of

contamination of field vegetables collected from Nahia1 was the contaminated irrigation water (surface). On the other hand, the ground water sources irrigating the Nahia2 and Saft areas were free from *Entamoeba histolytica*. It is thought that ground water was not the source of contamination, so it is supposed that the animal and human manure composts used as fertilizers were the main source of contaminations with this parasite and this was supported by other workers (Islam *et al*., 2004; Budu-Amoako *et al*., 2012).

In Egypt, *Entamoeba histolytica* was morphologically detected in coriander, cucumber, pepper, and radish (Hassan *et al*., 2012). In India, *Entamoeba histolytica* was detected only in cabbage samples by PCR technique (Rai *et al*., 2008). In Syria*, Entamoeba* spp. was detected by using light microscopy in spearmint, lettuce, coriander and parsley collected from different markets of Alqalamoun region, with a high prevalence in lettuce (Alhabbal, 2015). In Iran, *Entamoeba histolytica* cysts were detected by using microscope only in tomatos collected from markets (Yagoob and Mohammad, 2015) and in radish, leek and water cress (Saki *et al*., 2013) and in sweet basil, wild leek, garden cress, scallion, coriander, parsley, and peppermint collected from markets (Ebrahimzadeh *et al*., 2013). In Saudi Arabia, *Entamoeba* spp. was detected using microscope in watercress and lettuce (Alhabbal, 2015), and garden rocket, parsley, green onion and lettuce collected from the markets (Ammar and Omar, 2013). Transport, handling and exhibition at the point of sale can also influence the parasitological contamination of vegetables (Takayanagui *et al*., 2006). In environmental study conducted in Pakistan, the contamination with *Entamoeba* spp. using light microscope was detected in lettuce, cabbage, carrot, radish, coriander, beet, cucumber, tomato and chili collected from major markets (Shafa-ul-Haq *et al*., 2014). In Iraq, *Entamoeba histolytica* was detected using light microscope in fresh vegetables including celery, rocket, leek, cress, green onion and lettuce collected from markets (Saida and Nooraldeen, 2014), (Ali and Ameen, 2013) . In Bangladesh, *Entamoeba histolytica* was detected using light microscope in carrot, tomato, okra, women finger, coriander, cucumber and betel leaf (Nadia, 2014). The presence of the *Entamoeba* spp. on vegetables samples could be due to inappropriate agricultural practices during cultivation, and direct contact with soil and water that is contaminated with human and animal feces (Silva, 2014). Environmental studies conducted in Nigeria found that the contaminations with *Entamoeba histolytica* cysts were detected in cabbage and spinach (Akyala Ishaku *et al*., 2013), and in carrot, spinach, pumpkin and waterleaf collected from markets (Idahosa, 2011) . *E. histolytica* is the only species in *Entamoeba* complex clearly related with pathogenicity and neither culture methods nor microscopy are able to discriminate between different *Entamoeba* species, but the use of molecular methods is the golden standard that can solve this problem (Fotedar *et al*., 2007).

CONCLUSION

Molecular methods are a necessity to discern the different species of the *Entamoeba* complex. There is a high risk of infection with *Entamoeba histolytica* in the freshly eaten vegetables. The vegetable contamination with pathogens including *Entamoeba histolytica* is significant hence; consumers should be informed and educated with regard to food safety, good distribution practices and improvement of sanitary conditions in vegetable markets. The present study identified parasitic contaminants on pre-harvest vegetables associated with the use of contaminated

surface water; suggesting the fact, that *E. histolytica* may pose occupational risk of infection to the farming communities. Dill was the most contaminated vegetable by *Entamoeba histolytica.*

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ARABIC SUMMARY

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الكشف الجزيئي عن طفيلي *Entamoeba histolytica* في الخضروات الطازجة ومياه الري

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يعتبر طفيلي الإنتاميبا (*E. histolytica*) هو المسبب الرئيسي لمرض الدوسنتاريا الأميبية في الإنسان والحيوان , حيث ينتشر هذا المرض عالميا وبصفة خاصة في البلدان النامية ويمكن أن يتسبب في وفاة ما يصل إلى ١٠٠,٠٠٠ شخص على مستوى العالم.

في الدراسة الحالية تم إجراء مسح على بعض الخضروات الطازجة ومياه الري الخاصة بها وذلك للكشف عن طفيلي *E. histolytica*. حيث تم جمع عينات الخضار الطازجة من الأسواق العامة والحقول الزراعية في منطقتي صفط اللبن وناهيا بمحافظة الجيزة، مصر. تم غسل عينات الخضروات كل نوع على حده ثم جمع مياه الغسيل ومياه الري بشكل منفصل وفحصها باستخدام تقنية تفاعل البلمرة المتسلسل باستخدام البريمر الخاص بطفيلي *E. histolytica*.

أظهرت النتائج وجود الإنتاميبا في ٦.٤٪ (١١٠/٧) و٣.٧٪ (١٠٩/٤) من عينات الخضروات الحقلية وعينات الخضروات من الأسواق، على التوالي. كذلك أوضحت النتائج وجود الإنتاميبا في ٥.٦٪ من ٣٦ عينة مياه ري تم جمعها من نفس الحقول الزراعية التي تم جمع الخضروات منها. ولوحظ وجود انتشار عالي للإنتاميبا خلال المواسم الباردة. مما سبق يتضح أن الخضروات الطازجة عامة والملوثة بطفيلي الإنتاميبا تمثل خطرًا على صحة المستهلكين وأن نبات الشبت من أنواع الخضراوات الأكثر تلوثا بها.

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