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Different Detection and Treatment Methods for *Entamoeba histolytica* and *Entamoeba dispar* in Water/Wastewater: A Review

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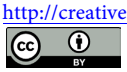


Abstract

Entamoeba histolytica is an anaerobic parasitic protozoan and well known as a human pathogen, while its close relative, *Entamoeba dispar*, also possesses similar characteristics as an infectious agent. These microorganisms are generally transmitted in fecal-contaminated water. However, *E. dispar* present in industrial wastewater is also capable of creating biofilms that can cause adverse impacts in piping networks. Therefore, it is important to detect both of these protozoan species in water and to find a cost-effective technique for inactivation or management control. This review article summarizes the available detection methods in water and wastewater matrices along with feasible disinfection techniques.

Keywords

Entamoeba histolytica, *Entamoeba dispar*, Detection Methods, Water and Wastewater Treatment

1. Introduction

There are six species of the genus *Entamoeba* that are found in the human intestinal lumen including *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba poleki*, *Entamoeba coli*, and *Entamoeba hartmanni* [1]. Among the six species, *Entamoeba histolytica* is considered to be a human pathogen [2]. Infection leads to the disease known as amebiasis, which is a common cause of death due to parasitic infestation, second only to malaria [3]. Symptoms include diarrhea with cramping, lower abdominal pain, low grade fever, releasing blood and mucus containing stools, and flask-shaped ulcers [4], as shown in **Figure 1**.

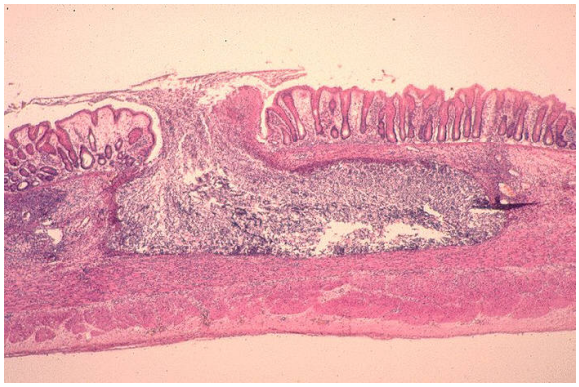


Figure 1. Flask-shaped ulcers developed due to amebiasis [5].

Amebiasis is responsible for about 100,000 deaths each year [6] [7]. The National Institute of Allergy and Infectious Diseases (NIAID) has classified *E. histolytica* as a category B priority biodefense pathogen [8]. Although humans are the only notable hosts for *E. histolytica*, it can be a zoonotic parasite. Ai *et al.* [9] investigated the feces of different animals and found *E. histolytica* in horses and other *Entamoeba* species in camels, yaks, sheep, and goats.

Although morphologically identical to *Entamoeba histolytica*, *Entamoeba dispar* was until recently considered a non-pathogenic parasite. Several studies [10] [11] [12] have reported intestinal symptoms in patients infected with *Entamoeba dispar*. In addition, Graffeo *et al.* [3] mentioned a rare case of enteritis in Italy, a non-endemic country. In addition, Oliveira *et al.* [13] stated that both *Entamoeba histolytica* and *Entamoeba dispar* infect 12% of the world's population, where the former is responsible for only 1% of the infections. Epidemiological surveys mentioned in Oliveira *et al.* [13] indicated that most of the asymptomatic infections are caused by *Entamoeba dispar*.

Moreover, a DNA extraction analysis of diarrheal stool samples in Northwest Ethiopia revealed that about 42.2% of infections were caused by *Entamoeba dispar* [14]. In the study of Calegar *et al.* [15], 57.1% of the infected samples contained *Entamoeba dispar*, 23.8% *Entamoeba histolytica*, and 14.3% possessed both the parasites. According to Sukprasert *et al.* [10], one of the major environmental transport systems of *E. dispar* is via water. Infection of the parasite into the human body is most likely to occur by drinking fecal-contaminated water or ingesting food irrigated with fecal-contaminated water [4]. Particularly in developing countries, drinking water is supplied to residents without proper treatment necessary to inactivate *Entamoeba* sp. [10].

Entamoeba dispar does not only infect human and non-human hosts, but it is also capable of being a pioneering agent to create biofilms. Meeroff *et al.* [16] investigated the microbial community structure formed along the inner walls of an industrial wastewater deep injection disposal well at the Solid Waste Authority (SWA) of Palm Beach County, Florida and identified the most abundant microorganism in the biofilm to be *Entamoeba dispar* (>30%). The formation of the biofilm reduced the injectivity rate by nearly 40% from 1.46 - 1.48 m^3/hr/kPa

to 0.86 - 1.02 m^3/hr/kPa. Currently, there are over 150,000 deep injection well facilities in the United States at an average cost of approximately $8 - 9 million per facility. Reduction of injectivity increases the cost of pumping and may eventually cause catastrophic failure resulting in replacement. Therefore, the objectives of this review article are to 1) Describe the occurrence of *Entamoeba* species in nature; 2) Review different methods of detection for *Entamoeba* species, particularly in water/wastewater samples; 3) Discuss disinfection options to control the population of *Entamoeba* species in water and wastewater matrices.

2. Occurrence in Nature

Entamoeba dispar is found to occur in nature 10 - 14 times more often than *Entamoeba histolytica* [1] [17]. Ayed *et al.* [18] indicated that the most common sources of *Entamoeba dispar* include human feces and consequently, raw sewage and septic tanks. It is also commonly found in cold regions along with tropical and sub-tropical regions containing contaminated wastewater [19]. **Table 1** summarizes the occurrence distribution of *Entamoeba* species in the natural environment.

In addition, *Entamoeba* species are also documented in Yemen, Northern South Africa, Southwestern China, Bangladesh, Vietnam as well as in Central and South America [35]-[40].

Entamoeba species can also be spread among different environmental media mainly through surface runoff, where it is transported from contaminated soil to surface water [19] [41]. In addition, leakage from underground storage and septic tanks can carry the parasite species to groundwater [42] [43]. Moreover, seepage of contaminated water through subsoil surfaces can transport the *Entamoeba* species to well water [25]. Survival of *Entamoeba* species in different environments is highly dependent on temperature. **Table 2** summarizes the effects of temperature on the survival of the parasitic cysts. However, in extreme cold or warm conditions such as temperatures below 5˚C or over 40˚C, cysts inactivate rapidly [19].

Table 1. Occurrence of Entamoeba species in natural environments.

Environments	Countries/Regions	References
Surface water	Egypt, Spain, Thailand, Iran	[4] [20] [21] [22]
Well water	Egypt, Nigeria, Taiwan	[23] [24] [25]
Refuse dumps, Soils, vegetable farms, school playgrounds	Egypt, Nigeria, Sub-Saharan Africa, South-Asia, Japan	[20] [26] [27] [28]
Sandy beaches	Brazil	[29] [30]
Drinking water	Iraq, India, Japan, Iran	[27] [31] [32] [33]
Sewage water	Tunisia, Taiwan	[25] [34]

Table 2. Effect of temperatures on survival of Entamoeba species in the environment [19].

Environments	Temperature Range (˚C)	Survival (days)
Feces and soil	28 - 34	8 - 10
Water and sewage sludge	0 - 4	60 - 365
Surface water and wastewater	20 - 30	Up to 15 days
Cultures	20 - 30	Up to 10 days

3. Morphology and Biology

The genus *Entamoeba* falls in the phylum of Sarcomastigophora and Lobosea class of the protozoan sub-kingdom where its order and family are Amoebida and Endamoebida, respectively. *Entamoeba* is closely related to other types of parasites such as Rhizopoda and Amoebozoa. Cysts of both *Entamoeba histolytica* and *Entamoeba dispar* are morphologically indistinguishable and range in size from 10 - 20 μm (typically 12 - 15 μm) in diameter, while the shape is spherical in bright-field microscopy [44]. Mature cysts are characterized by 4 nuclei, where immature cysts possess only 1 or 2 nuclei [1] [45]. Similar to the cysts, trophozoites of both the species are also identical. In general, the size of a trophozoite is around 10 - 60 μm and contains a single nucleus [44]. The life cycle of *Entamoeba* species and its relationships with the host is shown in **Figure 2**.

From the US Centers for Disease Control and Prevention [44], the life cycle of *Entamoeba* starts when cysts and trophozoites are released to the environment via human feces from infected individuals with diarrhea. However, cysts can be released even in formed stools [44]. In general, cysts are able to survive in an open environment for up to one month [19] before getting mixed with water, soil, crops, etc. from where transmission can occur. On the other hand, trophozoites get destroyed relatively quickly (on the order of days to weeks) once they are released outside of the human body [44].

Ingestion of mature cysts occurs through the drinking of contaminated water and food that travels through the stomach to reach the small intestine. If trophozoites are ingested, they will not be able to survive the gastrointestinal environment. Once in the small intestine, trophozoites are released from the cysts (excystation) and then travel to the large intestine where they multiply by binary fission and eventually produce new cysts, which are passed in feces to continue the life cycle in search of another host [44].

Inside a human host, pathogenic trophozoites inhabit in the gut lumen and once in the colon, pathogenic trophozoites degrade the mucosal layer to bind with epithelial cells [46]. This path is known as commensal colonization. Only in 10% of infections [47] [48], amoebiasis occurs. The mechanisms of the infection of hosts caused by pathogenic trophozoites are summarized in the following flowchart (**Figure 3**).

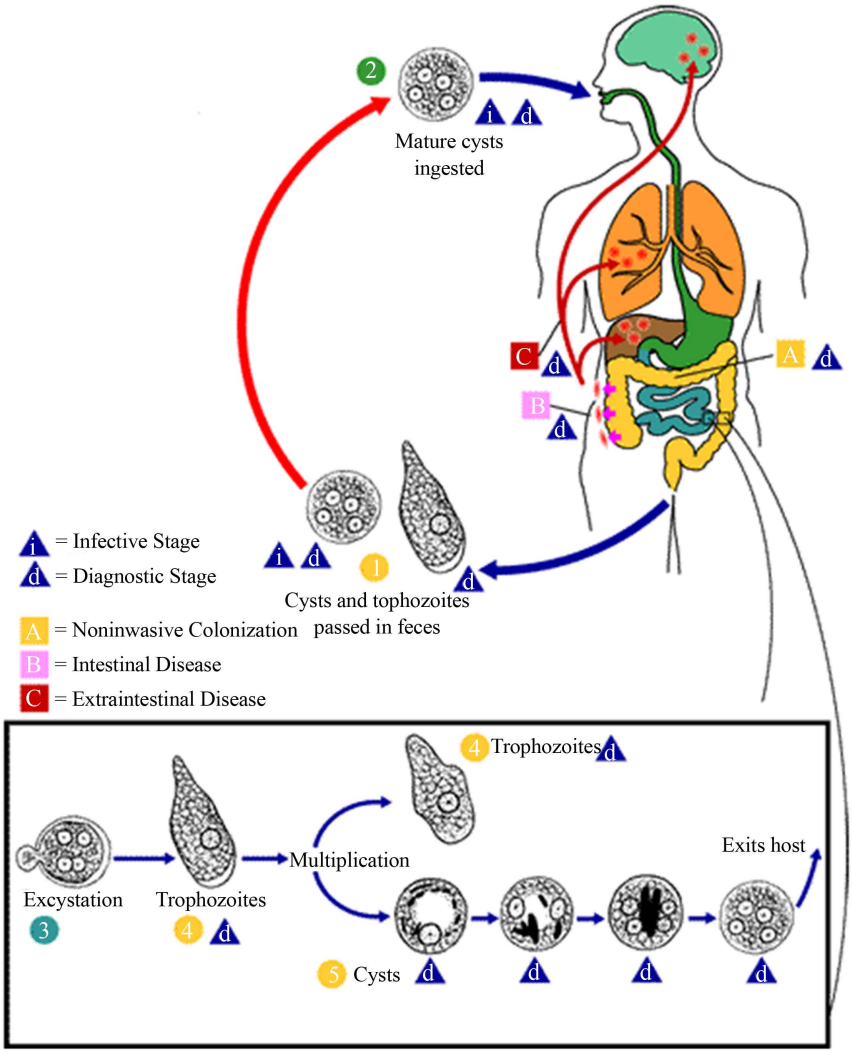


Figure 2. Human-parasite relationship for *Entamoeba* species [44].

4. Laboratory Diagnostic Techniques

Several diagnostic procedures have been reported to detect *Entamoeba* species in water samples including microscopic analysis, PCR techniques and ELISA. They are discussed briefly as follows.

4.1. Microscopy

Microscopy analysis to identify *Entamoeba* sp. in feces include wet preparation, concentration, and permanently stained smears [1]. According to Fotedar *et al.* [1] and Huston *et al.* [49], microscopic analysis of direct saline mounts is usually conducted on a fresh specimen, but it is an insensitive method (10%). It is recommended by Fotedar *et al.* [1] to analyze samples within 1 hour of collection to explore motile trophozoites that may carry red blood cells. In general, the concentration method is enough to identify cysts, but the use of permanently stained smears is an essential process to recover and detect *Entamoeba* species (Fotedar *et al.*) [1].

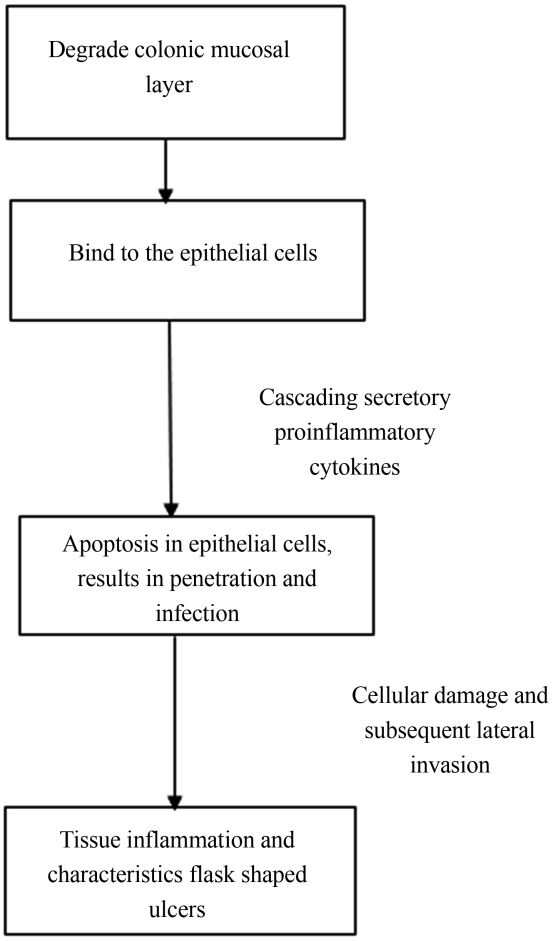


Figure 3. Flowchart of the host's infection mechanisms by pathogenic trophozoites of *E. histolytica*.

Different types of staining have been used in various studies to detect the parasite species in stool and blood samples. Wright-Giemsa stains and acid-fast stain (modified Ziehl-Neelsen stain) are two commonly used stains. Ezenwa *et al.* [50] used Giemsa stain to detect *E. histolytica* from blood samples. The study used diluted Giemsa's stain (1:10) with pH 7.2 buffer to cover the fixed blood smears for 30 minutes. Then, each slide was bottle dried and air dried by keeping at a vertical position in a dust free environment. Abdel-Hafeez *et al.* [51] used both acid-fast stain and Giemsa stain to detect *E. histolytica* from stool samples. In the case of water samples, Al-Khalidy and Jabbar [31] employed modified Ziehl-Neelsen stain for the microscopic analysis.

Fixative processes should be employed to prevent the degradation of the trophozoites of the parasites. Schaudinn's fluid, merthiolate iodine-formalin, sodium acetate-formalin (SAF) are some of the common fixatives used for the concentration procedure [1]. In general, SAF fixative consists of 5% formalin and 2% acetic acid. **Figure 4** and **Figure 5** represent microscopic images of *Entamoeba histolytica*/*dispar* trophozoites and cysts with different types of staining respectively.

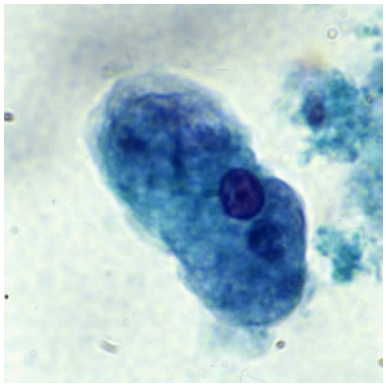
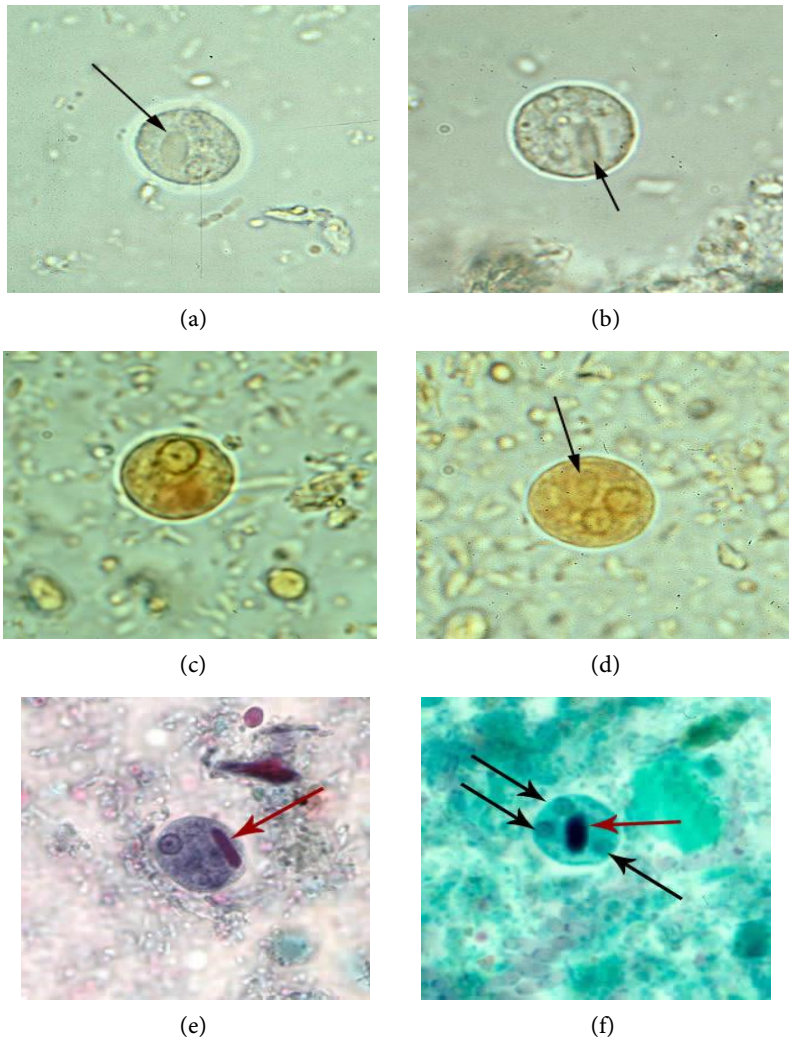


Figure 4. *Entamoeba histolytica*/*dispar* trophozoites stained with trichrome [52].



(a) (b) (c) (d) (e) (f)

Figure 5. Photographs taken of *Entamoeba histolytica/Entamoeba dispar cysts*. (a) Cyst in an unstained concentrated wet mount of stool; (b) Cyst in an unstained concentrated wet mount of stool; (c) Cyst in a concentrated wet mount stained with iodine; (d) Cyst in a concentrated wet mount stained with iodine; (e) Cyst stained with trichrome. Note the chromatoid body with blunt ends (red arrow); (f) Cyst stained with trichrome. Three nuclei are visible in the focal plane (black arrows), and the cyst contains a chromatoid body with typically blunted ends (red arrow) [52].

Al-Nihmi *et al.* [53] collected treated sewage water to detect the presence of *Entamoeba* sp. At first, the wastewater specimens were prefiltered to remove coarse particles. Next, the sample was settled for 2 hours to allow sediments and large particles to be removed by gravity. Next, 90% of the supernatant was discarded, and the remaining 10% sediments were centrifuged at 1000 G for 15 minutes before the pellets were suspended in 10 mL buffer solution (pH = 4.5). Next, the prepared solution was centrifuged at 1000 G for 1 minute. The pellets were suspended in 6 mL of 10% formol water followed by addition of 4 mL dimethyl ether and vortexing to obtain a uniform mixture. The whole mixture was centrifuged at 1000 G for 1 minute. The supernatant was discarded, and formed pellets were suspended in saline. A small portion of the pellets were transferred to a slide for the microscopic analysis. The study found an average of 22.2% of the tested samples as positive for *E. histolytica* cysts or trophozoites over a three-month surveillance period.

In Tunisia, Sabbahi *et al.* [34] analyzed 5 L of raw sewage or partially treated wastewater and 10 L of treated wastewater with secondary biological treatment for the presence of *Entamoeba* sp. Samples were settled to remove sediments at ambient temperature, and the supernatant was removed using a pump. Then, the prepared sediment was centrifuged in 15 - 50 mL centrifuge tubes with lids at 1000 G for 15 minutes. Next, the pellets were suspended in equal volumes (double the amount of the pellet) of acetoacetic acid buffer (pH = 4.5) followed by the addition of ethyl acetate. After mixing the sample for 10 minutes, it was centrifuged at 1000 G for 15 minutes, resulting in the formation of three distinct layers (e.g. a black layer on top, a turbid layer in the middle, and a sediment layer at the bottom). After that, the suspended layer was resuspended in five volumes of zinc sulfate solution with a specific gravity of 1.18 (density 33%) and mixed thoroughly. Then, 5.0 µL of resuspended material is placed on a slide for microscopic detection magnifying by ×100 and ×400. The number of protozoan cysts per liter is calculated using the following mathematical expression [34]:

$$N = AX/PV \tag{1}$$

where, N = number of cysts per liter, A = number of cysts counted in microscopy analysis, P = volume used for the microscopy examination, and V = initial sample volume.

In another study conducted in Germany [45], raw sewage and wastewater samples were passed through a 0.3 mm sieve for the removal of coarse solids. Then, the sample was centrifuged at 4500 G for 30 minutes followed by filtration using 0.22 µm nitrate cellulose membranes. After filtration, the sample was stained and examined in light microscopy for the identification of *Entamoeba* species. Microscopy techniques for identifying *Entamoeba* are not as reliable as cultures and isoenzyme analysis, as the sensitivity of the method is very poor (60%) [1]. It is very difficult to differentiate *Entamoeba histolytica* and *Entamoeba dispar* using microscopy, as these two species are morphologically iden-

tical to each other [54] [55] [56]. Pillai *et al.* [57] reported that the accuracy of *Entamoeba* identification was only 9.5% compared to that of other methods such as the PCR and ELISA. Microscopy is only used to identify *Entamoeba* species, while Polymerase Chain Reaction (PCR) is usually followed to differentiate *Entamoeba histolytica* and *Entamoeba dispar* [58].

4.2. PCR Techniques

PCR is a molecular technique to detect the presence of target microorganism by increasing the number of copies of DNA. The approach requires DNA templates, primer pairs, DNA polymerases and dNTPs to increase the copies of the nucleic acids by denaturation, annealing, and extensions. The steps are controlled by a thermocycler, which varies the temperature for specific times.

There are three types of PCR tests available: conventional PCR (C-PCR), nested PCR (N-PCR) and real time PCR assay (RT-PCR). Kim *et al.* [59] discussed the comparison of the three types of PCR assays for *Vibrio vulnificus*. In C-PCR, only a single set of primers are used. N-PCR is a modified PCR test that employs two sets of primers to increase sensitivity. For N-PCR, two successive PCR tests are conducted, where the amplified products from the first reaction are used as templates for the second reaction [60]. In the case of RT-PCR, fluorescent dyes are used for measuring amplification to evaluate the number of DNA copies and can be conducted by using 18 S rRNA as the PCR target [61]. To conduct the PCR analysis, a reference sequence is needed for each of the target microorganism. In most of the cases, the PCR target sequences are derived from the NCBI GeneBank database. For *E. histolytica* and *E. dispar*, different types of reference sequences were used in different studies. A brief summary of the reference sequences employed in the previous studies are provided in **Table 3**.

For the detection of *Entamoeba* species present in wastewater samples, it is required to purify/concentrate the specimens prior to nucleic acid extraction. **Table 4** describes several purification/concentration procedures and DNA/RNA extraction kits employed in previous studies to recover nucleic acids from *E. histolytica* and *E. dispar* in water and wastewater samples.

The next step is to design the primer sequences for the target genomes. A number of online software packages are available to design the primers including Primer Blast from NCBI, Primer 3. Primerselect, Dansis Max, NetPrimer,

Table 3. Summary of the reference sequences used in the previous studies.

Target Microorganisms	Reference sequences (NCBI GeneBank Accession Number)	References
E. histolytica	X56991	[61]
	X75434.1	[62]
E. dispar	KP722600.1	[61]
	Z49256	[62] [63]

Table 4. Purification/concentration procedures and nucleic acid extraction kits used for detection of *Entamoeba* species.

Species	Purification/Concentration	Nucleic Acid Extraction Kit	References
Entamoeba histolytica	1) Filtration using 0.3 mm sieve to remove coarse materials. 2) Filtration of the liquid through 0.22 µm nitrate cellulose membrane filters. 3) Centrifugation of the filtered specimens at 4500 G for 30 minutes.	DNA isolation kit (Macherey Nagel GmBH, Germany)	[45]
Entamoeba histolytica	1) Filtration using 800 µm filter paper to remove sediments. 2) Centrifuged at 5000 G for 10 minutes.	QIAamp DNA mini kit (QIAGEN, Hilden, Germany)	[64]
Entamoeba dispar	1) Centrifuge at 4000 G at 4˚C for 30 minutes. 2) Resuspension in phosphate buffer solution (pH 7.4) and additional centrifugation at 4300 G at 4˚C for 15 minutes. 3) Resuspension in 280 µL of Buffer AL (Qiagen, Hilden, Germany) and 20 µL proteinase-K (Qiagen) and incubate at 56˚C for 60 minutes. 4) Repeat at −80˚C for 30 minutes.	MagNa Pure LC Total Nucleic Acid Isolation Kit with the MagNa Pure LC 2.0 Instrument (Roche Diagnostics, Basel, Switzerland)	[65]
E. histolytica & *E. dispar*	1) Filtration of 5 L through 1.2 µm filters. 2) Wash concentrated samples with 50 mL phosphate buffer solution. 3) Centrifuge at 1500 G for 5 minutes. 4) Perform immunomagnetic separation and sucrose flotation on the produced supernatant.	QIAamp DNA minikit	[22]

Array designer 2, Fast PCR, Oligo 7, Prime designer 4, Gprime and others. The ideal length of a primer is between 18 - 24 bp. The primer works as a pair, and the differences between annealing temperatures of primers in a pair should be less than 3˚C. Several designed primer pairs, selected genome types, amplifying product sizes, preparation procedures for amplification and cycles used in previous studies are described in **Table 5**. Some primers were designed to be species-specific for *Entamoeba histolytica* and *Entamoeba dispar* separately; where in other cases, the primers were designed for both (genus-specific).

4.3. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is another technique to identify the presence of the *Entamoeba* species in environmental water samples or from stool or blood samples. For *Entamoeba* species, ELISA is generally conducted to detect serum IgG antibody against the microorganisms [70] as it is demonstrated to have improved sensitivity [71]. A number of commercially available test kits have been used including *Entamoeba histolytica* II test kits, RIDASCREEN *E. histolytica* IgG, *Entamoeba* Celisa Path and others [55] [70] [72] [73]. The US Environmental Protection Agency (USEPA) established standard procedures to detect *Cryptosporidium parvum* and *Giardia lamblia* in wastewater by using Immuno-Magnetic Separation (IMS) (USEPA Method 1623). Although, it is a popular detection method for *Entamoeba* species, it has lower sensitivity than that of the PCR assays. In general,

Table 5. Designed primer pairs, selected genome types, amplifying product size, amplification procedure and followed cycles from previous studies.

Protozoa species	Primers used	Preparation for amplification	Total cycles	References
E. histolytica & *E. dispar*	Forward: 5'-TAAGATGCACGAGAGCGAAA-3' Reverse: 5'-GTA CAAAGGGCAGGGACGTA-3' Target genome: 18 S rRNA Amplifying product size: 900 bp	Final volume of 25 µL, containing 12.5 µL of 2× PCR kit master mix (Ampliqon ApS, Literbuen 11, DK-2740 Skovlunde, Denmark), 15 ρM of each primer and 10 ng of extracted DNA.	Amplification was carried out in a thermocycler (Techne Ltd., Cambridge, UK) at 95˚C for 5 min; followed by 30 cycles at 94˚C for 30 s, at 58˚C for 30 s, at 72˚C for 30 s; and a final extension at 72˚C for 5 min	[66]
E. histolytica	Forward: 5'-AAGCATTGTTTCTAGATCTGAG-3' Reverse: 5'-AAGAGGTCTAACCGAAATTAG-3' Target genome: 18 S rRNA Amplifying product size: 439 bp	A final volume of 30 µL, containing 15 µl of 2× PCR master mix, 15 ρM of each primer and 10 ng of the PCR product described above	35 cycles at 94˚C for 30 s, at 55˚C for 30 s and at 72˚C for 30 s under identical conditions for the initial denaturation and final extension as that of the primary reaction	
E. dispar	Forward: 5'-TCTAATTTCGATTAGAAC TCT-3' Reverse: 5'-TCCCTACCTATTAGACATAGC-3' Target genome: 18 S rRNA Amplifying product size: 174 bp			
E. histolytica & *E. dispar*	Forward: 5'-TAA GAT GCA GAG CGA AA-3' Reverse: 5'-GTA CAA AGG GCA GGG ACG TA-3' Target genome: 16 S rRNA Amplifying product size: 800 bp	12.5 µL master mix, 200 nM from each primer, and 3 µL of the template DNA.	Same as mentioned in Ngui *et al.* [53] with some modification in annealing temperature (56˚C)	
E. histolytica	Forward: 5'-AAG CAT TGT TTC TAG ATC TGA G-3' Reverse: 5'-AAG AGG TCT AAC CGA AAT TAG-3' Target genome: 16 S rRNA Amplifying product size: 439 bp	12.5 µL master mix, 200 nM from each primer, and 1 µL of the template DNA.	Identical as mentioned for the primary reaction with a modified annealing temperature (48˚C).	[67]
E. dispar	Forward: 5'-TCT AAT TTC GAT TAG AAC TCT-3' Reverse: 5'-TCC CTA CCTATT AGA CAT AGC-3' Target genome: 16 S rRNA Amplifying product size: 174 bp			

Continued

E. histolytica & E. dispar	Forward: 5'-TAAGATGCACGAGAGCGAAA-3' Reverse: 5'-GTACAAAGGGCAGGGACGTA-3' Target genome: Small subunit rRNA			
E. histolytica	Forward: 5'-AAG CAT TGT TTC TAG ATC TGA G-3' Reverse: 5'-AAG CAT TGT TTC TAG ATC TGA G-3' Target genome: Small subunit rRNA Amplifying product size: 439 bp	Final sample volume was 20 µL with 10 µL multiplex master mix, 3 µL forward and reverse primers, 4 µL ddH_2O and 3 µL 5 - 10 ng DNA	Initial denaturation was occurred at 94˚C for 5 minutes followed by 35 cycles with denaturation at 94˚C for 30 sec, annealing at 58˚C for 90 sec with extended heating at 72˚C for 90 sec.	[37]
E. dispar	Forward: 5'-TCT AAT TTC GAT TAG AAC TCT-3' Reverse: 5'-TCC CTA CCTATT AGA CAT AGC-3' Target genome: Small subunit rRNA Amplifying product size: 174 bp			
E. histolytica	Forward: 5'-ATGCACGAGAGCGAAAGCAT-3' Reverse: 5'-GATCTAGAAACAATGCTTCTCT-3' Target genome: 18 S rRNA Amplifying product size: 166 bp	Final sample volume was 20 µL with 10 µL multiplex master mix, 3 µL forward and reverse primers, 4 µL ddH_2O and 3 µL 5 - 10 ng DNA	Initial denaturation at 94˚C for 5 minutes followed by 35 cycles with denaturation at 94˚C for 30 sec, annealing at 58˚C for 90 sec with extended heating at 72˚C for 90 sec.	[68]
E. dispar	Forward: 5'-ATGCACGAGAGCGAAAGCAT-3' Reverse: 5'-CACCACTTACTATCCCTACC-3' Target genome: 18 S rRNA Amplifying product size: 752 bp			
E. histolytica & E. dispar	Forward: 5'-TTTGTATTAGTACAAA-3' Reverse: 5'-GTA[A/G]TATTGATATACT-3' Target genome: 16 S like rRNA	Final sample volume was 25 µL comprising 2.5 µL of 10 × PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 1.4 µL of deoxynucleoside triphosphate mix or 0.75 µL of deoxynucleoside triphosphate mix (10 mM each dNTP, Biogene, Kimbolton), 0.3 µL (5 IU/µL) of Taq polymerase, 0.3 µM of each primer, and 2.5 µL of template DNA	Initial denaturation at 96˚C for 2 min, followed by 30 cycles—each consisting of 92˚C for 60 s (denaturation), 43˚C for 60 s (annealing), and 72˚C for 90 s (extension). Finally, one cycle of extension at 72˚C for 5 min was performed	[69]
E. histolytica	Forward: 5'-AATGGCCAATTCATTCAATG-3' Reverse: 5'-TTTAGAAACAATGCTTCTCT-3' Target genome: 16 S like rRNA	Same as the genus specific reaction	Same as the genus specific reaction but at a higher annealing temperature of 62˚C.	
E. dispar	Forward: 5'-AGTGGCCAATTTATGTAAGT-3' Reverse: 5'-TTTAGAAACAATGTTTTTC-3' Target genome: 16 S like rRNA			

PCR is 100 times more sensitive than ELISA [1]. Gonin and Trudel [55] found that microscopic analysis possessed higher sensitivity than that of the ELISA particularly when few numbers of the microorganism was present. However, it is less expensive and relatively easier to detect the protozoan species with ELISA compared to PCR analysis [74].

5. Treatment to Inactivate *Entamoeba* Species

5.1. Physical Treatment Processes

Since *Entamoeba* species are highly resistant to common disinfectants and both trophozoites and cysts are relatively larger than most of the other common waterborne microorganisms, physical treatment processes can effectively remove the protozoan species. In the case of primary sedimentation, removal of protozoa (*Giardia lamblia*) was 0.11 $\log_{10}$ [75]. The removal rates of the *Entamoeba* cysts were reported as 0.49 $\log_{10}$ using an advanced primary treatment process [76]. Rapid sand filtration was found to remove 1.0 $\log_{10}$ at filtration rates less than 2.4 m/hr [19]. In addition, Bitton, Jimenez *et al.* and Leong reported [76] [77] [78] that further inactivation (2.0 $\log_{10}$) can be accomplished by the addition of a coagulant. However, it is recommended to conduct flocculation before primary treatment [75]. A report published by the US Army [79] cited by Schaefer *et al.* [80] revealed 98.5% and 99.8% removal of *Entamoeba histolytica* by coagulation with alum and soda ash, respectively followed by sedimentation. Jimenez *et al.* [81] used a Microsand (0.1 - 1.0 mm DIA) filter along with aluminum sulfate ($Al_2(SO_4)_3$) (40 - 60 mg/L) as a coagulant.

Shukla *et al.* [82] used chitosan oligosaccharide-coated iron oxide nanoparticles to remove *E. histolytica* while employing an external magnetic field. The nanoparticles were synthesized and incubated in a contaminated water sample containing known numbers of protozoa cysts. The concentration of the nanoparticles and incubation period were varied at a pH of 7.0 and a temperature of 37˚C. The protozoan cysts were attached to the synthesized nanoparticle material during incubation and removed using a magnetic separator. The maximum removal percentage (86%) was obtained at a 4 mg/mL of nanoparticle concentration for an incubation time of 35 minutes.

5.2. Radiation

Mtapuri-Zinyowera *et al.* [83] investigated the use of solar radiation to inactivate *Entamoeba* species. Cysts were inactivated efficiently at 50˚C due to the synergistic effect of solar radiation and heat, with total eradication achieved at 56˚C. Mohamed *et al.* [84] also measured the efficiency of solar energy to destroy *Entamoeba* species with one-sided blackened bottles vertically exposed to the sun for 7 hours at an ambient temperature of 40˚C and allowing the temperature to rise to 50˚C - 60˚C. They found complete elimination at a temperature above 56˚C.

Maya *et al.* [85] investigated ultraviolet (UV) light to inactivate amphizoic amoebae present in water samples. The researchers used *Acanthamoeba culbertsoni* and *Acanthamoeba* species as the target microorganisms and found that a high UV dose of 173 mW·s/cm^2 is needed for an effective contact time period of 1200 sec to achieve complete inactivation of the tested species. In addition, the study concluded that dose of 60 mW·s/cm^2 of UV light is required to achieve a 2.0-log inactivation. However, further investigations are needed to reveal the dose and effective contact time to inactivate *Entamoeba* species.

Ryu *et al.* [86] found that using UV/TiO_2 enhanced inactivation by reducing the dosage required by 56% compared to UV alone for oocyst removal. Several studies [86] [87] [88] investigated UV/TiO_2 photocatalytic inactivation of protozoan species. A complete inactivation of *Giardia intestinalis* can be obtained after 30 minutes contact in a UV/TiO_2 system [87]. Inactivation rates for *Cryptosporidium parvum* oocysts were 1.3, 2.6 and 3.3 $\log_{10}$ at UV dosages of 2.7, 8.0 and 40.0 mJ/cm^2, respectively [86]. Therefore, this can also be an emerging technique to inactive the *Entamoeba* species.

5.3. Disinfection Processes

Although, chlorine is used worldwide for disinfection, *Entamoeba* species are particularly resistant to typical dosages [19]. However, there have been several studies (presented in **Table 6**) where the required CT values under different pH and temperatures were investigated. In general, chlorine disinfection is more efficient at an acidic pH (<7.0) when more of the chemical is in the hypochlorous acid (HOCl) form. The effectiveness of chlorine as a disinfectant is highly temperature and pH dependent. The most powerful disinfectant is gaseous Cl_2, followed by hypochlorous solution and chloramines, which are the least effective [89].

Bromine can be a more effective disinfectant against *Entamoeba* species compared to chlorine. Stringer *et al.* [92] cited by WHO [94] examined that a bromine dose of 1.5 - 4.0 mg/L with a contact time of 10 minutes can achieve

Table 6. Different concentration of Cl_2 dosages and suitable working temperatures and pH.

Protozoan species	Temperature (˚C)	pH	CT value (mg/L·min)	Inactivation ratio (%)	References
E. histolytica*/ *E. dispar	-	10	120	99	[19]
E. histolytica	30	7.0	20	99	[90] [91] [92]
	30	7.0	25	99.9	
	30	9.0	70	99.9	
	5	6.0	90	99	[80]
	23-26	7.5 - 8	60 - 80	99	[93]

3.0 $\log_{10}$ inactivation of *E. histolytica* at a pH and temperature of 4.0˚C - 10.0˚C and 4˚C - 10˚C, respectively. Like chlorine, bromine also works best at a pH of 6.0 - 7.0 since the chemical stays in the hypobromous acid (HOBr) form. Liquid bromine can also be used for disinfection. However, it is recommended to use bromine stick (an organic substance with a mixture of bromine, chlorine and dimethyl hydantoin which is available in tablets or cartridges) instead of liquid bromine because there are risks associated with metal reactivity, and it is corrosive. Bromine is not suitable to use for disinfection of drinking water as it imparts a medicine-like taste and should only be employed in emergency cases [92]. However, a bromine concentration higher than 0.5 mg/L causes eye and mucous membrane irritation [95].

Chang [96] cited by WHO [97] mentioned that elemental iodine (I_2) can be 2 to 3 times more effective for *Entamoeba* disinfection particularly at a pH range of 5 - 7. In addition, I_2 has greater penetration capability than that of HIO, which makes it an effective disinfectant to use against biofilms in distribution systems, deep injection wells and other facilities. However, further research is required to ensure its effectiveness to penetrate and inactivate *Entamoeba* colonies formed in hydraulic pipelines.

There have also been limited studies regarding the use of ozone to disinfect *Entamoeba* species. Due to the greater oxidant potential, ozone is considered highly toxic against waterborne microorganisms [98]. The effluent water samples from the Shahid Beheshti treatment plant in Iran where ozone was being used as the disinfectant were tested for the waterborne parasites, and no living protozoan species were found [98]. This indicates that ozone disinfection can be very effective against *E. histolytica* and *E. dispar*. The study of Newton and Jones [99] cited by National Research Council [89] stated that 98% to over 99% inactivation of *E. histolytica* suspended in water samples can be achieved by a CT value as low as 0.15 mg·min/L. The study also revealed that the disinfection technique provided similar removal efficiency for temperature and pH ranging from 10˚C - 30˚C and 6.5˚C - 8.0˚C, respectively.

Several other disinfection techniques to inactivate protozoa have been reported with mixed results including primary sedimentation, trickling filter with sludge digestion and drying, and oxidation ditch with sedimentation with removal rates of 0.05 to 0.3 $\log_{10}$ [19] [100]. These studies also stated that the waste stabilization pond technique can achieve removal higher than 2.0-$\log_{10}$ with a minimum retention time of 25 days.

6. Conclusions and Recommendations

Both *E. histolytica* and *E. dispar* are most commonly found in human feces and as a result, enter the environment via septic tanks and partially treated wastewater releases, especially in developing and tropical weather countries. Although the existence of *Entamoeba* species depends on temperature, the parasites start to become inactivate quickly over 40˚C and ultimately, become completely inac-

tivated at a temperature above 45˚C. The relationship of the protozoan species with its host and infection mechanisms has been well established. The primary media of transferring *Entamoeba* species within the hosts' body are via ingestion of fecal contaminated water and food.

For the detection of the *E. histolytica* and *E. dispar* in water and wastewater samples, three techniques were reviewed, namely microscopic analysis, ELISA test and PCR assay. The microscopic test has very low sensitivity, and it is also almost impossible to distinguish the cysts of *E. histolytica* and *E. dispar*, as they are morphologically similar. On the other hand, ELISA test is easier to conduct compared to PCR analysis and is also a cost-effective approach. Although it showed lower sensitivity than that of the microscopic analysis in one of the studies, researchers found it to be a more suitable option to detect and differentiate the species. However, there have been limited studies conducted on *Entamoeba* detection in water or wastewater using ELISA. On the other hand, WHO endorsed the PCR assay to be the most effective approach to detect and differentiate the protozoa species, and the test also possesses a high sensitivity.

A number of studies have already been conducted to propose a suitable and cost-effective approach to inactivate *Entamoeba* species. Since the parasite species are highly resistant to the normal dosages of chlorine and the size of its cysts is also comparatively larger, filtration can be effective. Currently, available filtration systems are capable of removing 98.5% to 100% of both species from water samples. Addition of coagulants such as alum and soda ash followed by sedimentation or filtration processes increase the removal efficiency. Solar radiation in conjunction with heat can also be a cost-effective approach to inactivate *Entamoeba*, particularly at temperatures above 50˚C.

Although normal chlorine dosages cannot penetrate and disinfect cysts of *E. histolytica* and *E. dispar*, previous studies showed that CT values ranging from 20 to 120 mg·min/L are capable of inactivating 99% to 99.9% of the protozoa present depending on temperature and pH. Several other investigations were conducted to reveal the effectiveness of using bromine as a disinfectant and found that it is more efficient than chlorine, especially at a low temperature ranging from 4˚C - 10˚C. However, bromine creates taste and odor issues while treating for drinking water and hence, it is recommended to use only in emergency cases. Besides chlorine and bromine, iodine can also be another effective disinfectant, but more investigations are required to reveal its suitability.

All the disinfection procedures presented in this review are mainly focused on the inactivation of *Entamoeba histolytica*. There have been very limited studies about disinfection techniques to specifically remove *Entamoeba dispar*. In addition, the effectiveness of electron beam technique has never been evaluated for the *Entamoeba* species. Rawat and Sarma [101] revealed that even 1 kGy of electron beam dose may result in complete inactivation of coliforms, *Salmonella-Shigella*, *E. coli* and almost 3.0 $\log_{10}$ removal of bacterial species. Hence, it can be an emerging and efficient technique to treat *Entamoeba* species. Moreover, investigations are needed to reveal the most suitable approaches to inactivate *E.*

dispar in formed biofilms in pipe networks, which is a case presented in the study of Meeroff *et al.* [16]. Although Meeroff *et al.* [16] mentioned several techniques and conditions to penetrate and inactivate biofilm produced by other microorganisms such as *Pseudomonas* and *Giardia lamblia*, the recommended inactivation conditions for *Entamoeba dispar* still remain to be investigated.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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