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Multiplex PCR assay for simultaneous detection and differentiation of *Entamoeba histolytica*, *Giardia lamblia*, and *Salmonella* spp. in the municipality-supplied drinking water

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Abstract:

BACKGROUND: The contamination with *Entamoeba histolytica*, *Giardia lamblia*, and *Salmonella* spp. in drinking water is the most prevalent in Indian subcontinent, but often difficult to detect all these pathogens from the drinking water.

MATERIALS AND METHODS: A multiplex polymerase chain reaction (mPCR) method was developed to detect contamination of municipality-supplied drinking water with *E. histolytica*, *G. lamblia*, and *Salmonella* spp. The primers were designed to target small subunit of 16S rRNA type gene of *E. histolytica* and *G. lamblia*, and invasive A gene of *Salmonella typhimurium*. The optimized mPCR assay was applied on 158 municipality-supplied drinking water samples collected from Delhi.

RESULTS: Out of total 158 water samples, 89 (56.32%) were found positive for the targeted pathogens by mPCR while conventional methods could be detected only in 11 (6.96%) samples. The mPCR assay showed 100% sensitivity and specificity for these pathogens in comparison with culture and microscopic detection. Of the 89 mPCR-positive samples, *G. lamblia*, *E. histolytica*, and *Salmonella* spp. were present in 35 (22.15%), 26 (16.45%), and 28 (17.72%), respectively. Nine (5.69%) samples were positive for both *E. histolytica* and *G. lamblia*, 10 (6.32%) were positive for *G. lamblia* and *Salmonella* spp., and 8 (5.06%) had *Salmonella* spp. and *E. histolytica*. Nonetheless, 3 (1.89%) samples were positive for all three pathogens.

CONCLUSIONS: The present assay is an alternative to conventional methods to serve as highly sensitive, specific, and economical means for water quality surveillance to detect the outbreak caused by *E. histolytica*, *G. lamblia*, and *Salmonella* spp. pathogens.

Key words:

Drinking water, *Entamoeba histolytica*, *Giardia lamblia*, multiplex polymerase chain reaction, *Salmonella* species

Introduction

Over the last 100 years, provision of the safe and pure drinking water is one of the most important advances for public health regulatory bodies. However, still >1 billion people do not have the access of pure water and >2.4 billion population

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lacks adequate sanitation. The unhygienic conditions and contaminated water accounts for >1 billion cases of waterborne infection annually and contributing about 10.25 million deaths worldwide, 95% of the diseases are in children of <5 years of age.^[1] The etiological agents for these infections are viruses (Norwalk-like viruses, rotaviruses,

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and enteric adenoviruses), bacteria (*Campylobacter jejuni*, *Shigella*, *Salmonella*, enterotoxigenic *Escherichia coli*, and cytotoxigenic *Clostridium difficile*), and parasites (*Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum*).^[2-4] The presence of *E. coli* and free-living amoeba indicates highly sensitive indicator of fecal contamination and signifies the presence of other pathogenic bacteria, virus, and protozoa in drinking water.^[5] The presence of fecal contaminants like *E. histolytica*, *G. lamblia*, and *Salmonella* spp. in drinking water is important because these are most common causative agent of gastrointestinal illness to the humans.

E. histolytica is causative organism for amoebic liver abscess disease. Worldwide, the disease manifestation causes 100,000 deaths annually.^[6] The presence of *E. histolytica* is found in >90% of the water-borne outbreaks caused by pathogenic protozoan and rest 10% are associated with the food.^[7]

G. lamblia is also common intestinal pathogenic organism and has been associated with numerous outbreaks of water-borne diseases.^[8-10] It is endemic in many countries, predominantly in the third world, with 30%–50% of children harboring parasite continuously or intermittently.^[11] *Giardia duodenalis* synonym of *G. intestinalis* and *G. lamblia* is found in wide range of mammalian hosts.^[12] Presence of 5 or more *Giardia* cyst in 100 liters of drinking water is sufficient to cause Giardiasis disease.^[13] *Giardia* is the common cause of parasitic gastroenteritis in human and is a major health problem worldwide.^[14] *Giardia* survives for months in water without nutrients and exists as a hardy, highly infectious cyst, and oral ingestion causes the infection. The common symptoms persist for 2–6 weeks, which includes diarrheal, nausea, stomach cramps with gas, poor nutrient absorption, and weight loss.

Salmonella is Gram-negative, rod-shaped, and nonspore-forming *Enterobacteriaceae*.^[15] It is important bacterial pathogen implicated in water and food-borne outbreaks.^[16] Based on lipopolysaccharide (O), flagellar protein (H) and sometimes, the capsular (Vi) antigen, the family is classified into serovars (serotypes) and based on virulence factors. The *Salmonella* spp. is responsible for typhoid fever to 16 million persons, gastroenteritis illness to 1.3 billion individual and causes 3 million deaths yearly worldwide.^[17] Microscopic observation, culture growth on specific media, and biochemical tests are the used methods for detection of these in water. However, these methods are labor-intensive, time-consuming and costly. Therefore, the use of molecular techniques, i.e., polymerase chain reaction (PCR) is an alternate choice. PCR assay can detect the small quantity of DNA of pathogens present in the water and waste-water samples. As it is considered the better tool and has a potential to

overcome many of the limitations posed by conventional methods. It will be more economical and practical if assay detects the multiple pathogenic species in a single PCR reaction tube. The use of multiplex PCR (mPCR) for pathogen detection in clinical and environmental samples is well known.^[18,19] The mPCR provides a smart choice against monoplex PCR. Therefore, the purpose of the present study was to develop and evaluate the mPCR assay to detect the prevalence of two different etiological agents, i.e., parasite and bacteria particularly *E. histolytica*, *G. lamblia*, and *salmonella* spp. in drinking water which is a novelty of this study.

Materials and Methods

Study Site and Consent

This study was carried out in Division of Clinical Microbiology and Molecular Medicine, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India. Written consents duly signed were taken from the owner of the house or sample collection site before collecting the samples.

Assay controls

The DNA of *E. histolytica* (HM-1 strain) and *G. lamblia* (Portland-I strain) and *Salmonella* Spp. were used as a control for mPCR. The *E. histolytica* and *G. lamblia* DNA was kind gift of Dr. Alok Bhattacharya, Jawaharlal Nehru University, New Delhi, and Dr. Rakesh Sehgal, Post Graduate Institute of Medical Education and Research, Chandigarh, India. For *Salmonella* spp. DNA, a single typical colony was picked from known culture agar plate and inoculated in 5 ml of nutrient broth and was incubated for overnight at 37°C. The bacterial cells harvested by centrifuging at 6000 × g for 10 min at 4°C. DNA was isolated by phenol–chloroform extraction method,^[20] and isolated DNA was dissolved in 30 µL of Tris-EDTA (TE) buffer.

Design of primers

The primers were designed by using primer-BLAST online tool at NCBI to target small ribosomal subunit 16S RNA gene of *E. histolytica* (accession no. X64142), *G. lamblia* (accession no. M54878), and invasive A gene of *Salmonella typhimurium* (accession no. U3237). The primers were custom synthesized by MWG Biotech Private Limited, Bengaluru, India [Table 1].

Standardization of multiplex polymerase chain reaction assay

The DNA of all three pathogens was used for mPCR standardization. The 3 µL DNA, (100 ng) of each pathogen was spiked in 10 µL of nuclease free water to serve as positive control, and nontargeted enteric pathogens' DNA was used as negative control. The PCR reaction mixture was prepared by adding 3 µL (100 ng)

Table 1: Details of primer used in the study

Primer	Sequence (5'-3')	Oligomers	Targeted gene	Amplicon (bp)
<i>E. histolytica</i>				
E-F	AGG TTT CAG TCT CGT TCG TTA C	22	Small subunit 16S rRNA gene	477
E-R	ATC CAT GAT CGC TAT AAG ATG CAC	24		
<i>G. lamblia</i>				
G-F	GTC CAA CCG TTG TCC TGA G	19	Small subunit 16S rRNA gene	74
G-R	ACG CTC TCC CCA AGG AC	17		
<i>Salmonella</i> spp.				
S-F	GTT GTC TTC TCT ATT GTC ACC GT	23	Invasive-A gene	293
S-R	CGA AAT ACC GCC AAT AAA GTT CAG	24		

E. histolytica=*Entamoeba histolytica*, *G. lamblia*=*Giardia lamblia*

of template DNA, 2.5 µL *Taq* polymerase buffer with 1.5 mM MgCl₂ (×10) (Fermentas, Canada), 0.5 µL dNTPs (10 mM) (Fermentas, Canada), 1.5 µL forward and reverse primers each (10 pmol/µL), 0.5 µL *Taq* DNA polymerase (3U/µL) (Fermentas, Canada); final volume was made up to 25 µL with nuclease-free sterile water.

The reaction was carried at temperature of 95°C for 5 min before initial PCR amplification cycle. The temperatures cycle used for amplification were 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s for 35 cycles followed by final extension at 72°C for 7 min in thermal cycler (Bioer's XP thermal cycler). The amplified gene products were resolved in 1.8% agarose gel.

Evaluation of multiplex polymerase chain reaction assay on municipality-supplied drinking water samples

Collection, filtration, and concentration of water

A total of 158 municipality-supplied drinking water samples (20 L volume) were collected from different residential area of Delhi between periods of April, 2007 and May, 2010 in 25 L polypropylene container and carried to laboratory in minimal period of time. The samples were filtered and concentrated through 0.22 µm polyethersulfone pellicon-2 minifilter (Millipore Corporation, Billerica, MA01821) in a masterflex pellicon-2 miniholder using a Millipore vacuum pressure pump at 115V and 3 ψ. The filtration and concentration unit was rinsed with double-distilled water followed by 0.1N NaOH before and after each use to prevent cross contamination between two samples. Finally, 20–25 ml of sample was further centrifuged at 12,000 rpm for 20 min to concentrate it up to 0.5 ml volume.

DNA isolation from the water retentate for *Entamoeba histolytica*, *Giardia lamblia*, and *Salmonella* spp.

From the concentrated water retentate, 200 µL volume was used for *E. histolytica*, *G. lamblia* and *Salmonella* spp. DNA isolation. The DNA extraction was carried out by QIAmini stool DNA extraction kit (Qiagen, USA) as per the manufacturer's instructions. The extracted DNA was used for the presence of pathogen detection by standardized mPCR assay. The amplified gene products

specific to targeted nucleotide sequences were visualized by agarose gel electrophoresis.

Microscopy and culture methods for *Entamoeba histolytica*, *Giardia lamblia*, and *Salmonella* spp detection

The presence of targeted pathogens in concentrated water retentate was detected by direct microscopy and culture techniques. The *E. histolytica* *G. lamblia* parasites (ova and cysts) detected by microscopically and *Salmonella* spp. by culture techniques followed by biochemical methods. Lugol's iodine and direct saline wet mount of concentrated water was used to visualize cyst and trophozoites of *G. lamblia* and *E. histolytica*. For *Salmonella* spp., a loopful retentate was streaked on MacConkey agar plate and was incubated overnight at 37°C for 18–24 h. The presence of typical single colony obtained was subculture for biochemical analysis and agglutination assay with *Salmonella* specific anti-sera (Serobac, BioRad) for confirmation.

Results

Optimization of the multiplex polymerase chain reaction assay

The mPCR for simultaneous detection of *E. histolytica*, *G. lamblia*, and *Salmonella* spp. were standardized with DNA of control strains. Developed mPCR assay precisely amplified the targeted genes of *E. histolytica*, *G. lamblia*, and *S. typhimurium* as 477 bp, 74 bp and 293 bp respectively [Figure 1]. The mPCR was evaluated with DNA of other nontargeted enteric pathogens such as *E. coli*, *Iodamoeba buestchili*, and *Endolimax nana*. No amplification was seen in the spiked or in the neat specimens (data not shown), giving us 100% specificity.

Specificity and sensitivity of multiplex polymerase chain reaction assays primer pairs

The primer specificity was tested with DNA of targeted control pathogens and nontargeted but related organisms, i.e., *E. coli*, *I. buestchili*, and *E. nana*. There was no amplification in the DNA of nontargeted pathogens while desired amplicons were seen in all

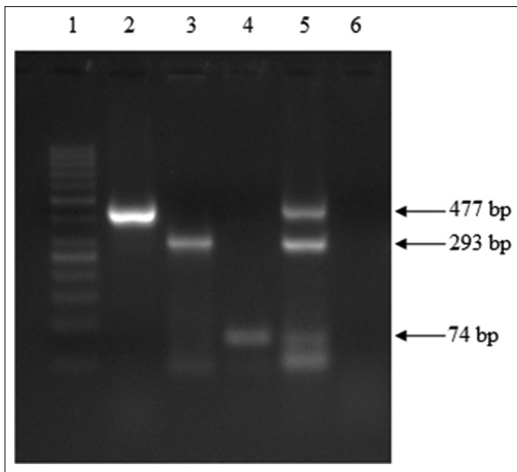


Figure 1: Standardization of multiplex polymerase chain reaction assay; Lane 1: DNA ladder 50 bp; Lane 2: *Entamoeba histolytica* (477 bp); Lane 3: *Salmonella typhimurium* (293 bp); Lane 4: *Giardia lamblia* (74 bp); Lane 5: Positive control; *Entamoeba histolytica* + *Salmonella typhimurium* + *Giardia lamblia*; Lane 6: Negative control DNA

targeted pathogens. A total of ten amplified products were randomly selected and sequenced. The assembled sequences were compared with reference sequences by BLAST analysis, which showed 98%–100% homology indicating high specificity of our mPCR. The minimum concentration detectable by species-specific PCR varied in sensitivity according to the species. As determined by agarose gel electrophoresis, the optimal sensitivity for detection of *E. histolytica*, *G. lamblia*, and *Salmonella* spp. were 0.0651 ng/ μ L, 0.0138 ng/ μ L, and 0.0762 ng/ μ L, respectively [Figure 2a-c].

Detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Salmonella* spp. by multiplex polymerase chain reaction

The mPCR-based detection of *E. histolytica*, *G. lamblia*, and *Salmonella* spp. in 158 municipality-supplied drinking water samples was significant finding of the study. These samples included 05 (3.16%) samples microscopically positive for *G. lamblia*, 02 (1.26%) for *E. histolytica* and 05 (3.16%) were culture positive for *Salmonella* species. One sample (0.63%) was positive for *G. lamblia* and *E. histolytica*; but, none of these samples were found positive for *Salmonella* spp. and *E. histolytica*. The positivity rate of in-house mPCR was significantly high as compared to standard methods [Table 2]. From total samples, 89 (56.32%) were found positive. Of these, 35 (22.15%) were positive for *G. lamblia*, 26 (16.45%) for *E. histolytica*, and 28 (17.72%) for *Salmonella* spp. [Table 3]. There were 9 (5.69%) samples positive for both *E. histolytica* and *G. lamblia* whereas 10 (6.32%) were positive for *G. lamblia* and *Salmonella* spp. and 08 (5.06%) for *Salmonella* spp. and *E. histolytica*. Only 03 (1.89%) samples were positive for all three pathogens [Figure 3].

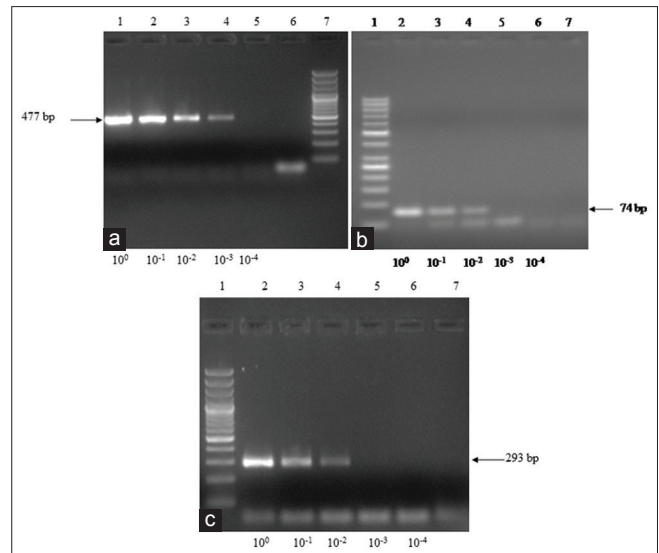


Figure 2: Sensitivity analysis of multiplex polymerase chain reaction on the DNA isolated from *Entamoeba histolytica*, *Giardia lamblia*, and *Salmonella typhimurium*. The DNA concentration was measured by spectrophotometer at 260 nm. (a) Amplification from serial four-fold dilution of *Entamoeba histolytica* DNA; Lane 1: 10^0 ; 2: 10^{-1} ; 3: 10^{-2} ; 4: 10^{-3} ; 5: 10^{-4} ; 6: Negative DNA; 7: DNA ladder-50 bp. (b) Amplification from serial four-fold dilution of *Giardia lamblia* DNA; Lane 1: DNA ladder-50 bp; 2: 10^0 ; 3: 10^{-1} ; 4: 10^{-2} ; 5: 10^{-3} ; 6: 10^{-4} ; 7: Negative control DNA. (c) Amplification from serial four-fold dilution of *Salmonella typhimurium* DNA; Lane 1: DNA ladder-50 bp; 2: 10^0 ; 3: 10^{-1} ; 4: 10^{-2} ; 5: 10^{-3} ; 6: 10^{-4} dilution; 7: Negative control DNA

Discussion

The mPCR assays are technically more superior to conventional methods such as microscopic and culture technique due to their accuracy and reliability. PCR-based detection of different water-borne pathogen in environment samples has been done, and it can be applied as promising substitute for the existing conventional methods.^[21-23] Although the monoplex PCR is well accepted for initial standardization, it becomes cumbersome and costly for identifying multiple pathogens in the tested samples. Because in the monoplex PCR, the laboratory has to perform the test individually for each suspected pathogen. In this process, very often, the copathogens are missed. However, mPCR can screen numerous microbial organisms simultaneously in a single tube setup. Desouky *et al.*, (2003) developed an mPCR assay to monitor water quality for pathogenic bacteria presence.^[24] Whereas Rai *et al.*^[25] used immunofluorescence assay (IFA) combined with polymerase chain reaction for the detection of microbial load mainly *Giardia*, *Entamoeba*, and *Cryptosporidium*.^[25] The mPCR assays have been developed against enteric pathogens also but none of these assays detect parasites (*G. lamblia* and *E. histolytica*) and bacteria (*Salmonella* species) simultaneously in this combination. These pathogens are most commonly found in contaminated water and are responsible for various gastro illness of human. The detection of these pathogens

Table 2: Detection of *Entamoeba histolytica*, *Giardia lamblia* and *Salmonella* spp. by multiplex polymerase chain reaction assay and conventional (microscopy and culture) methods

	Conventional method			mPCR assay		
	Microscopy		Culture	<i>Salmonella</i> spp.	<i>G. lamblia</i>	<i>E. histolytica</i>
	<i>E. histolytica</i>	<i>G. lamblia</i>	<i>Salmonella</i> spp.			
Positive (%)	2 (1.26)	5 (3.16)	4 (2.53)	28 (17.72)	35 (22.15)	26 (16.45)
Negative (%)	156 (98.73)	153 (96.83)	154 (97.46)	130 (82.27)	123 (77.84)	132 (83.54)
Total (%)	158 (100)					

E. histolytica=*Entamoeba histolytica*, *G. lamblia*=*Giardia lamblia*, mPCR=Multiplex PCR, PCR=Polymerase chain reaction

Table 3: Comparison of standard methods and multiplex polymerase chain reaction assay for presence of pathogens in collected samples

Targeted pathogens (alone or in combination)	By standard method (%)	By mPCR assay (%)
<i>E. histolytica</i> only	2 (1.26%)	26 (16.45%)
<i>G. lamblia</i> only	5 (3.16%)	35 (22.15%)
<i>Salmonella</i> spp. only	4 (2.53%)	28 (17.72%)
<i>E. histolytica</i> and <i>G. lamblia</i> both	1 (0.63%)	9 (5.69%)
<i>G. lamblia</i> and <i>Salmonella</i> spp. both	1 (0.63%)	10 (6.32%)
<i>E. histolytica</i> and <i>Salmonella</i> spp. both	0 (0.0%)	8 (5.06%)
<i>E. histolytica</i> , <i>G. lamblia</i> and <i>Salmonella</i> spp. all three pathogen	0 (0.0%)	3 (1.89%)

E. histolytica=*Entamoeba histolytica*, *G. lamblia*=*Giardia lamblia*, mPCR=Multiplex PCR, PCR=Polymerase chain reaction

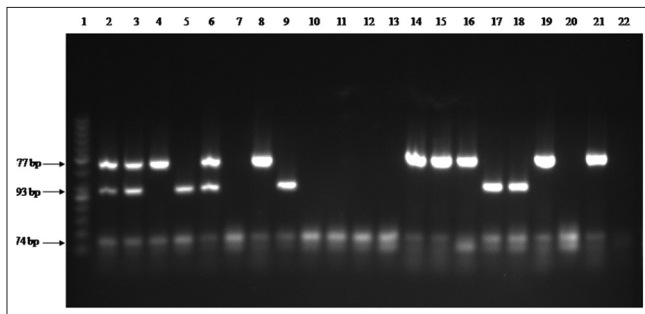


Figure 3: Multiplex polymerase chain reaction amplification of *Entamoeba histolytica*, *Giardia lamblia* and *Salmonella* spp. in collected water. Lane 1: DNA ladder-50 bp; Lane 2: Positive control (*Entamoeba histolytica* + *Salmonella typhimurium* + *Giardia lamblia*); Lane 3–21: Water samples; Lane 22: Negative control DNA

by PCR is more sensitive than microscopic method^[26,27] and is also shown in our result. Out of 158 samples, only 11 (6.96%) samples were positive by conventional techniques while 89 (56.32%) samples were positive with mPCR.

The control of water-borne outbreak needs efficient and sensitive detection of disease causing pathogens. However, in spite of the various advantages of the mPCR assay, the initial standardization requires skill manpower and good laboratory setup. Furthermore, even after preliminary evaluation, depending on the nature of sampling, it can also be affected by inhibitory substances being present in the environmental samples. This can impact the sensitivity of the assay. In the present study, the inhibitors present in the municipality-supplied

drinking water were removed by use of inhibit-X tablets supplied along with the DNA extraction kit. Therefore, the scope of our finding provides a new detection method for easy surveillance of such pathogens.

Conclusions

The mPCR assay is highly sensitive and specific in comparison to conventional microscopic and culture techniques. The developed novel assay is suitable for routine screening of drinking water and ruling out the contamination with *E. histolytica*, *G. lamblia*, and *Salmonella* spp. All three pathogens can be detected in one reaction and in a single reaction tube.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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