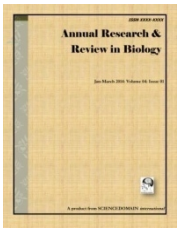

Annual Research & Review in Biology
4(9): 1461-1470, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Molecular Detection of *Entamoeba histolytica* in Different Water Sources of District Peshawar, Pakistan

Noor ul Akbar[1], Sultan Ayaz[1], Shafiqur Rahman[2], Sanaullah Khan[1], Shahid Niaz Khan[1], Aga Asad Noor[3], Bibi Ibtesam Shagufta[1], Farzana Raza[1] and Muhammad Waqar[3,4*]

[1]Department of Zoology, Kohat University of Science and Technology, Kohat 26000, Pakistan.
[2]Department of Plant Sciences, Kohat University of Science and Technology, Kohat 26000, Pakistan.
[3]Institute of Microbiology University of Sindh Jamshoro, Sindh, Pakistan.
[4]Genome Center for Molecular Diagnostics & Research (GCMD) Lahore, Pakistan.

Authors' contributions

This work was carried out in collaboration between all authors. Author NUA collected the samples, designed the study and performed Molecular analysis. Authors SA, SR, SUK, SNK, AAN, BIS, FR and MW help in literature search and wrote the manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 18th November 2013
Accepted 9th January 2014
Published 27th January 2014

ABSTRACT

Many species of protozoa present in the alimentary canal of human beings causing diseases. Due to water-borne diseases about 3.5 million people including 3 million children die throughout the world as well as about 98% deaths occur through extensive water-borne outbreaks in the emerging republics Only the diarrheal diseases cause greater than 1.5 million deaths per year. To investigate the presence of *E. histolytica* in different water sources of district Peshawar. The study was designed for molecular detection of *E. histolytica* in water sources. A total of 300 water samples were collected from different water sources of district Peshawar from May, 2011 to April, 2012. And for further process the samples were brought to the Department of Zoology Kohat University of Science and Technology, Kohat on time. Water samples (n=300) were collected from different water

**Corresponding author: Email: waqarkhan96@gmail.com;*

sources (Tube Well, Bore Well, Tap and Drain) in six different areas of District Peshawar (Pakistan). The water was filtered thorough whattman filter paper and the residue was subjected to DNA extraction and PCR was conducted for detection of *E. histolytica.* To increase the sensitivity of the test a small region (125-bp) of the SSU rRNA was targeted for the PCR amplification. *p*H of the water were also tested, mean value for over all *p*H was 8.21 (±0.06), including 8.16 (±0.20) of tube well water, 8.30 (±0.32) of bore well water, 8.26 (±0.24) of tap water and the *p*H of drain water was 8.11 (±0.48). Overall prevalence of *E. histolytica* in drinking water of district Peshawar was 11.33% (34/300) followed by 3.57% (2/56) in tube well, 2.74% (2/73) in bore well, 14.41% (16/111) in tap water and 23.33% (14/60) in drain water. The highest prevalence of *E. histolytica* was 65% recorded in tap water of Faisal Colony and *P*<.05 was considered significant. It was revealed from the current study that *E. histolytica* is present in water sources of some areas in Peshawar which may be due to flood and improper management of water scheme. The study also revealed that a proper treatment of water for human consumption is required especially in Faisal Colony in district Peshawar.

Key words: E. histolytica; PCR; water-borne; protozoa and faisal colony.

1. INTRODUCTION

Water for human life on the Earth is very important [1] and it is one of the greatest significant essentials for life after air [2]. A large number of infectious diseases are transmitted through water, contaminated with human and animal excreta [3]. Safe and healthy water has been defined as “water that is free from pathogenic agents free from harmful chemical substances, pleasant to taste and smell [4]. In developing countries, such as Pakistan, 60% of the population has no access to pure drinking water [5]. In many areas of Pakistan safe and fresh water supplies are at risk. Pakistan is in “high water stress condition”, which occurs when the ratio of use to availability exceeds 40 percent [6]. Due to water-borne diseases about 3.5 million people including 3 million children die throughout the world. About 98% deaths occur in the emerging republics where there water-born outbreaks are extensive. Only the diarrheal diseases cause greater than 1.5 million deaths per year [2]. Pacific Institute published a report in 2002, according to their prediction that in 2020 about 135 million people will die throughout the world from related illness, if no attention is given to the safe drinking water [7]. About 325 water related outbreaks of parasitic protozoan illness including *Entamoeba histolytica* (*E. histolytica)* have been recognized throughout the world [8].

The detection methods of *E. histolytica* in water is difficult economically because it takes much time and needs outclass microscope experts for identification [9]. Amebiasis is caused by *Entamoeba histolytica,* which is considered an important parasitic causative agent for the death of human beings throughout the world. The medical features and symptoms of amebiasis are amebic dysentery and liver abscesses and so about 15 million people have a violent disease which causes 0.1 million deaths in each year [10]. *E. histolytica* does not attack in the tissue of the mucosa and organs [11]. We are facing a water crisis due to increasing world population and growing contamination of usual resources. It is assessed by World Health Organization (WHO) that one billion people absence access to clean water for drinking [12]. *Entamoeba spp* was detected 14.4% through microscope in different water sources of Khyber Pukhtunkhwa [12].

So for the molecular detection of the parasites, PCR method is more sensitive than Microscopy and keeping in view the importance of the water-borne parasites, the present study is designed to assess the prevalence of *E. histolytica* in different water sources of district Peshawar.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out in District Peshawar, Khyber Pakhtunkhwa, which lies between 33º44′ and 34°15′ North latitude and 71º22′ and 71º42′ East longitudes. The total area of this district is 1,257 square km. The total population is 2.019 million bearing 1.061 million Male population and 0.958 million of female population. The occupation is mostly agriculture but few people are merchants, businesspersons and professional like doctors, engineers etc. The sites from where the water samples were collected were Saddar, Shaheen Town, University Town, Hayatabad, Khwaja Town and Faisal Colony. The samples were collected from 1st May, 2011 to 30th April 2012.

2.2 Sample Collection

A total of 300 water samples were collected from six different areas for the detection of *E. histolytica* including 56, tube well water, 73 bore well water, 111 tap water and 45 samples were of drain water. One liter of each water sample was collected in sterilized bottles, labeled (date of collection, name of the area and type of water) and was transported to the Molecular Parasitology and Virology Laboratory, Department of Zoology KUST, Kohat, for further experimental analysis through Microscopy for confirmation of some sample and Polymerase Chain Reaction (PCR). During sample collection some observation were noted like socioeconomic condition of the people of the collection sites. Ethical approval for this study was obtained from Ethical Committee of Kohat University of Science and Technology, Kohat.

2.3 Sample Processing

The water samples were filtered through Whatman filter paper in water filtration assembly. The filtered residue was further centrifuge at 6000 rpm for 10 minutes the supernatant was discarded and the residue was obtained in eppentdorf tubes were centrifuged at 10000 rpm for 8 min. 10µl of the residue were placed on the slides and made a thin film on it through wooden stick and stained with Hematoxyline observed under Tri ocular microscope at 10X, 40X and 100X magnification and the pictures were captured for record as shown in Fig. 1. The positive samples, which were confirmed microscopically by Dr. Sultan Ayaz, Chairman Department of Zoology KUST and identified by the different parameters showed in the methodology adopted by Colmer-Hamood [13] and were mixed to make a cocktail for positive control.

2.4 DNA Extraction and DNA Amplification (PCR)

DNA was extracted from the filtered residue containing 200µl by GF-1 Nucleic Acid Extraction Kits (Vivantis) with prescribed protocol. After DNA extraction, in a thermal cycler (NyxTechnix, USA) the PCR reaction performed along with Taq DNA polymerase (Fermentas, USA). The PCR product was amplified by mixing of 5µL of

extracted DNA with Taq Buffer 2.2 µL, $MgCl_2$, 2.4 µL, 1.0 µL dNTPs, followed by dH_2O (Medicated) 7.1 µL, Taq DNA polymerase enzyme 0.3 µL and 1.0 µL of each 10 Pico moles of forward and reverse primers. Primers used in PCR mixture were, Forward EH1 (3'-GTACAAAATGGCCAATTCATTCAATG-5') and Reversed EHD2 (5'-TACAAAGTGGCCAATTTATGTAAGTA-3') and making 135-bp of the amplicon size for the detection of the small region of SSU rRNA [14,15,16] as shown in Fig. 2. For each reaction 30 cycles applied in PCR initiated by 94ºC for 10 minutes as denaturation. Each cycle was consisted of 3 steps denaturation for 30 seconds at 94ºC, annealing for 60 seconds at 51ºC followed by elongation at 72ºC for 40ºC. The final elongation was for 5 min at 72ºC with some modification in [14,15,16] with some modification for standardization.

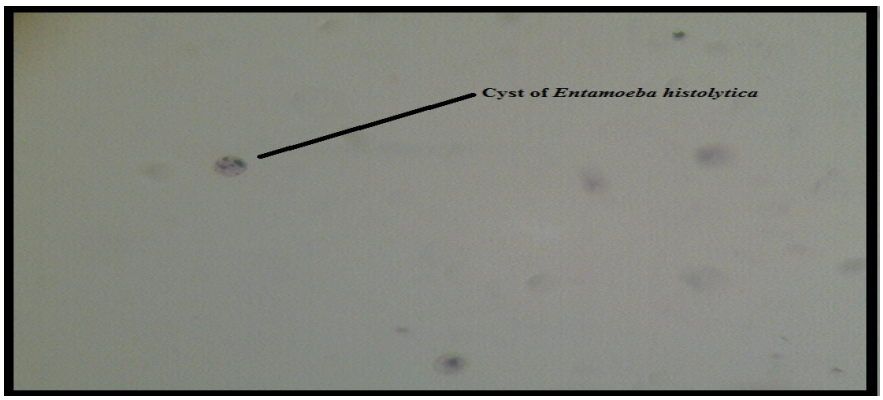

Fig. 1. Microscopy at 40X of *E. histolytica* in the positive control sample of drain water of Faisal Colony.

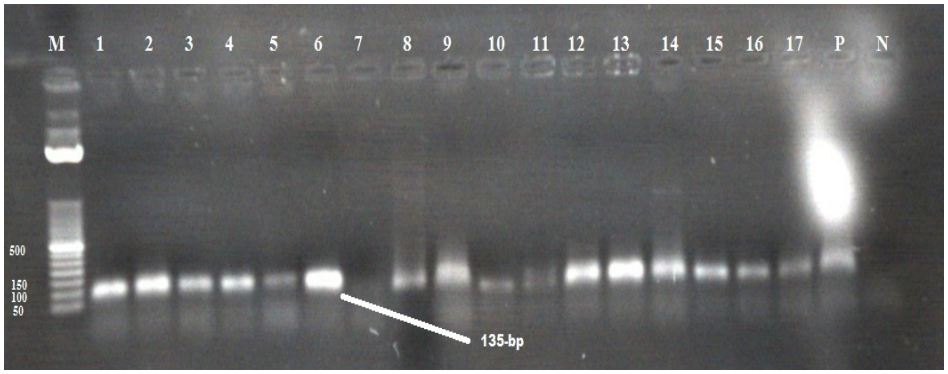

Fig. 2. PCR Gel photograph, M= Marker 50-bp, P=Positive control, N=Negative control, All lanes indicate positive (135-bp) except lane 7. Lane 1,2,3,4, are drain water and Lane 6 is tap water of Faisal Colony Lane. Lane 5, 7 to 11 are tap water of Khwaja Town and Saddar City, Lane 12, 13, 14 drain water of Khwaja Town, Lane 15 tap water of Faisal Colony, Lane 17 and 16 are bore well water of Khwaja Town [16].

2.5 Gel Electrophoresis

12µL sample containing 10µL of PCR product mixture and 2µL loading dye loaded in 2% agarose gel along with 12µl of DNA Ladder (100bp). The gel was run for 25 min at a voltage

of 120 volts and 500 mA current. Gel was then examined by UV transilluminator. The specific DNA amplified product of each sample was determined by identifying 135-bp bands for *E. histolytica* comparing with 100-bp DNA Ladder (Fermantas Germany), used as a size marker [14,15,16].

2.6 *p*H Values Determining

The *p*H values of different water sources were determined by means of digital *p*H meter and *p*H 7 solutions on the spot during collection.

2.7 Prevalence Rate

The prevalence rate was determined by the following formula [12].

Prevalence Rate = (No. of parasite detected in water sample/Total no. of water samples examined) ×100

2.8 Data Analysis

Statistical analysis was performed by using "STATISTIX", version 9.0, Korean made software. Variables included for evaluation were Tube well, bore well, tap and drain water and $P<.05$ values were considered significant.

3. RESULTS

Water samples (n=300) were collected from 6 different areas of molecular detection of *E. histolytica* including 56, tube well, 73 bore well, 111 tap water and 45 samples were collected from drain water. After PCR examined *E. histolytica* showed unlike results in different sources of dissimilar areas.

After DNA amplification through PCR the result showed variation in different areas of Peshawar. In all sources collected from sadder the results showed 0% out of 15 and 13 samples of tube well and bore water, Tap water 5% (1/20) and drain water 16% (2/12) results. Shaheen Town showed 33.33% (1/3) in tube well, 5% (1/20) in bore well, 30% (3/10) in tap and drain water results. University Town showed also different results; 0% out of 13, 12 and 21 samples from tube well, bore well and tap water respectively and 21.43% (1/12) from drain water. While in Hayatabad 0% in out of 17, 8 and 23 samples from tube well, bore well and tap water respectively. Drain water showed 8.33% (1/12) positive result. Khwaja Town showed also same result of Saddar and University Town in tube well water and bore well water which was 0% in 2 and 25 samples respectively. The result of tap water was 14.29% (1/7) and drain water was 33.33% (2/6). In Faisal Colony tube well water showed 50% (1/2), bore well water was 8.33% (1/12), tap water containing 65% (13/20) and drain water was 50% (3/6) positive results for *E. histolytica*.

Overall area-wise prevalence of *E. histolytica* in all sources collected from different areas from district Peshawar was 5% (3/60) from Saddar, 15% (6/40) from Shaheen Town, 5% (3/60) from University Town, 1.66% (1/60) from Hayatabad, 7.5% (3/40) from Khwaja Town and the Faisal Colony showed 45% (18/40) positive results for *E. histolytica*a as shown in (Table 1).

Water collected from four different water sources in which Tube well water showed 0% result collected from Saddar, University Town, Hayatabad and Khwaja Town in out of 15, 13, 17 and 2 samples respectively. While in Shaheen Town and Faisal Colony tube well water showed 14.29% (1/7) and 50% (1/2) positive results respectively for *E. histolytica*.

Similarly bore well water showed 0% result collected from sadder, University Town, Hayatabad and Khwaja Town in the total collected samples of 13, 12, 8 and 25 respectively. Though Shaheen Town and Faisal Colony showed 33.33% (1/3) and 8.33% (1/12) respectively, positive results for *E. histolytica*.

E. histolytica was absent in tap water of University Town and Hayatabad. The tap water of Sadar and Shaheen Town showed 5% in out of 20 samples, while in Khwaja Town 14.29% (1/7) and in Faisal Colony 65% (13/20) positive results were shown.

The drain water of Saddar showed 16% (2/12), Shaheen Town 30% (3/10), University Town 21.43% (3/14), Hayatabad 8.33% (1/12), Khwaja Town 33.33% (2/6) and Faisal Colony 50% (3/6) positive results for *E. histolytica.*

Overall source-wise prevalence of *E. histolytic*a was 3.57% (2\56) in Tube well water, 2.74% (2\73) in Bore well water, 14.41% (16\111) in Tap water while 23.33% (14\60) in Drain water. While overall prevalence of *E. histolytic*a was 11.33% recorded; 34 out of 300 different water samples were positive for *E. histolytica* as shown in (Table 1).

The result showed that drain water was more contaminated with *E. histolytica* than other sources. The negative result may be due to problem in handling or it was also possible that these samples may not contain *E. histolytica*.

The *p*H of water was also conducted which showed as the following categories.

In tube well water of Saddar, Shaheen Town, University Town, Hayatabad, Khwaja Town and Faisal Colony showed 7.92 (±0.13), 8.03, (±0.29), 8.06 (±0.29), 8.4 (±0.23), 8.2 (±0.05) and 8.4 (±0.05) respectively. Similarly Bore well water of Saddar, Shaheen Town, University Town, Hayatabad, Khwaja Town and Faisal Colony showed 8.2 (±0.13), 8.03 (±0.35), 8 (±0.07), 8.8 (±0.08), 8.2 (±0.05) and 8.6 (±0.06) respectively.

Tap water of Saddar, Shaheen Town, University Town, Hayatabad, Khwaja Town and Faisal Colony showed 8.1 (±0.24), 7.9 (±0.34), 8.5 (±0.26), 8.5 (±0.39), 8.2 (±0.18) and 8.4 (±0.04) respectively.

Similarly in drain water of Saddar, Shaheen Town, University Town, Hayatabad, Khwaja Town and Faisal Colony showed 8.3 (±0.15), 8.2 (±0.35), 8 (±0.35), 8.2 (±0.07), 7.2 (±0.08) and 8.5 (±0.05) respectively (Table 2)

Table 1. Prevalence of *E. histolytica* in different areas of district Peshawar

Area (n)	Tube well water Positive\total (%)	Bore water Positive\total (%)	Tap Water Positive\total (%)	Drain Water Positive\total (%)	Overall Samples Positive\total (%)
Sadar (60)	(0)	(0)	(5)	(16)	(5)
Shaheen Town (40)	(14.29)	(33.33)	(5)	(30)	(15)
University Town (60)	(0)	(0%)	(0%)	(21.43)	(5)
Hayatabad (60)	(0)	(0)	(0)	(8.33)	(1.66)
Khwaja Town (40)	(0)	(0)	(14.29)	(33.33)	(7.5)
Faisal Colony (40)	(50)	(8.33)	(65)	(50)	(45)
Total (300)	(3.57)	(2.74)	(14.41)	(23.33)	(11.33)

(%) = Percentage, n=total number, P =.05, significant

Table 2. *p*H mean values of different water sources collected from different areas of district Peshawar

Source of water	Area (n)	pH	±SE	Source of water	Area (n)	pH	±SE
Tube Well Water, n=40	Saddar (60)	7.92	±0.13	**Tap Water, n=60**	Saddar (60)	8.1	±0.24
	Shaheen Town (40)	8.03	±0.29		Shaheen Town (40)	7.9	±0.34
	University Town (60)	8.06	±0.29		University Town (60)	8.5	±0.26
	Hayatabad (60)	8.4	±0.23		Hayatabad (60)	8.5	±0.39
	Khwaja Town (40)	8.2	±0.05		Khwaja Town (40)	8.2	±0.18
	Faisal Colony (40)	8.4	±0.05		Faisal Colony (40)	8.4	±0.04
Bore Well Water, n=100	Saddar (60)	8.2	±0.13	**Drain Water, n=100**	Saddar (60)	8.3	±0.15
	Shaheen Town (40)	8.03	±0.35		Shaheen Town (40)	8	±0.35
	University Town (60)	8	±0.07		University Town (60)	8.2	±0.07
	Hayatabad (60)	8.8	±0.08		Hayatabad (60)	7.2	±0.08
	Khwaja Town (40)	8.2	±0.05		Khwaja Town (40)	8.5	±0.05
	Faisal Colony (40)	8.6	±0.06		Faisal Colony (40)	8.5	±0.06

(n) Total collected samples, (±SE) Standard errors of Mean

Overall *p*H values in tube well water of different areas of district Peshawar showed 8.16 (±0.20), bore well water 8.30 (±0.32), tap water 8.26 (±0.24) and drain water 8.11 (±0.48) *p*H values. And overall *p*H value was 8.21 (±0.06) as shown in (Table 3).

Table 3. Over all pH mean values of different water sources in district Peshawar

Source of water	pH (Mean)	±SE
Tube well water	8.16	±0.20
Bore well water	8.30	±0.32
Tap water	8.26	±0.24
Drain water	8.11	±0.48
Over all	8.21	±0.06

±SE (Standerd Error of Mean)

4. DISCUSSION

The current study was compared with different studies, which were carried out by different people in Pakistan or in the District Peshawar.

E. histolytica was present 10.4% in out of 230 samples collected from school children located in Hayatabad Peshawar [17] which was too high from our results because they were directly related to the stool samples of the School children. Overall prevalence of *E. histolytica* was 11.33% (34/300) which was too dissimilar to Karanis et al. (18.1%) [18]. The type and frequency of various parasites vary from region to region [17]. Microscopic examination was not enough to detect low level of infection but amplification of DNA can reveal it. So this study is entirely based on the detection of *E. histolytica* through the PCR diagnosis.

Similarly, the present study was correlated with the similar work carried out microscopically by Ayaz, et al. 2011, where in all of the three sources of water was contaminated with cysts of the Entamoeba parasite. The results indicate overall prevalence of 65.5% (295/450) of protozoa, including 14.4% (65/450) *Entamoeba spp* [8]. In other studies, *E. histolytica* and *E. coli* was recovered from the dirty waters and stool [19].

There were 95 samples tested for the detection of *E. histolytica* in which 68 were positive through PCR from patients [12], which was higher than our results. In Iran 35 out of 116 (30.2%) water samples were contaminated in the rural areas through microscopy [20] which were also a higher prevalence than our results. Another study was conducted in urban and rural areas of Iran, out of 16,592 stool samples 226 were positive bearing 1.36% of the total samples for *E. histolytica* through microscopy [21].

As the *p*H values were nearly standard (6.5 to 8.5) according to WHO stated by Irshad et al. [22] so no effect was observed in the prevalence of *E. histolytica* in the current study. In the present study, the positive samples mostly belonged to rural areas having low socioeconomic conditions. There was no awareness regarding cleanliness, sterilization and disinfection.

The present results may help the people for their health in prevention and supervision for amebiasis especially in children. It may also help the people in molecular detection of the parasites in the local laboratories.

5. CONCLUSION

It was concluded from the current study that high level of contamination of water was found in the tap water sources especially of Faisal Colony (Peshawar) which needs of a suitable source of drinking water to identify the threshold of water sources contamination that requires treatment. Preventing waterborne disease and the health effects of water contamination is vital to our nation's public health due to the fact that access to harmless drinking water is required cornerstone of public health.

It was recommended that PCR is more sensitive and accurate for the detection of the cysts of *E. histolytica.* It was also recommended that filtered water should be used for drinking and cooking.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

1. Ambashta RD, Sillanpaa M. Water purification using magnetic assistance: A review. Journal of Hazardous Materials. 2010;180:38-49.
2. Prüss-Üstün A, Bos R, Gore F, Bartram J. Safer water, better health: costs, benefits and sustainability of interventions to protect and promote health. Geneva, Switzerland: World Health Organization; 2008.
3. Yousaf S, Chaudhry MA. Microbiological Quality of Bottled Water Available in Lahore City. JPMS. 2013;3(2):110-112.
4. Maxcy-Rosenau-Last: Public Health & Preventive Medicine, 14th edition: Appleton & Lange, Simon & Scuster Company. 1998:619.
5. Khan M, Ihsanullah TS, Fazal M, Abdus S. Occurrence of pathogenic micro-organisms in food and water supplies in different areas of Peshawar, Nowshera and Charsada. Pak J Food Sci. 2000;10:37-40.
6. Economic survey of Pakistan 2002-2003:231.
7. Gleick PH. Dirty Water: Estimated Deaths from Water-Related Disease 2000-2020. Pacific Institute for Studies in Development, Environment, and Security Research Report, Oakland, California; 2002.
8. Karanis P. A review of an emerging waterborne medical important parasitic protozoan. Jpn. J. Protozool. 2006;39:(1):5-19.
9. Stewart S, McClelland L, Maier J. A fast method for detecting Cryptosporidium parvum oocysts in real world samples. Proc. SPIE. 2005;5692:341–350.
10. Fotedar R, Stark D, Beebe N, Marriott D, Ellis J, Harkness J. Laboratory diagnostic techniques for Entamoeba species. Clin Microbiol Re. 2007;20(3):511-32.
11. Thompson RCA, Smith A. Zoonotic enteric protozoa. Veterinary Parasitology. 2011;182:70–78.
12. Ayaz S, Khan S, Khan SN, Bibi F, Shamas S, Akhtar M. Prevalence of Zoonotic Parasites in Drinking Water of Three Districts of Khyber Pakhtunkhwa Province, Pakistan. Pak. J. life soc. Sci. 2011;9(1):67–69.
13. Colmer-Hamood JA. Fecal Microscopy Artifacts Mimicking Ova and Parasites. Lab Medicine. 2001;32(2):80-84.

14. Gonin P, Trudel L. Detection and differentiation of Entam-oeba histolytica and Entamoeba dispar isolates in clinical samples by PCR and enzyme-linked immunosorbent assay. J Clin Microbiol. 2003;41(1):237-41.
15. Mukherjee AK, Chowdhury P, Bhattacharya MK, Ghosh M, Rajendran K, Ganguly S: Hospital-based surveillance of enteric parasites in Kolkata, BMC Research Notes. 2009;2:110.
16. Ben Ayed S, Ben Abdallah R, Mousli M, Aoun K, Thellier M, Bouratbine A. Molecular differentiation of *Entamoeba Histolytica* and *Entamoeba Dispar* from Tunisian food handlers with amoeba infection initially diagnosed by microscopy. Parasite. 2008; 15:65-68
17. Shah SA, Zai S, Muhammad A. Correlation of Haemoglobin level with Intestinal Parasites in School Children. JPMI. 2006:232-234.
18. Karanis P, Sotiriadou I, Kartashev V, Kourenti C, Tsvetkova N, Stoja-nova K. Occurrence of Giardia and Cryptosporidium in water supplies of Russia and Bulgaria. Env Res. 2006;102(3):260-271.
19. Hernandez CF, Avendendo L. Simple modification of the Baermann method for diagnosis of Strongyloidiasis. Mem Inst Oswaldo Cruz, Rio de Janeiro. 2001;96:805-807.
20. Sadeghi GH, Mohammadian M, Nourani M, Peyda M, Eslami A. Microbiological Quality Assessment of Rural Drinking Water Supplies in Iran. Journal of Agriculture & Social Sciences. 2007:1813-2235.
21. Hooshyar H, Rezaian M, Farnia S, Solaymani MS. A field study of the distribution of E. histolytica/ E. dispar cysts passers in Northern, Central and Southern Iran. Iranian Journal of Public Health. 2004;33(2):28-32.
22. Irshad M, Malik N, Khan T, Faridullah. Effect of Solid Waste on Heavy Metal Composition of Soil and Water at Nathiagali-Abbottabad. J. Chem. Soc. Pak. 2011;33(6):830-834.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=397&id=32&aid=3461