International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Prevalence of *Entamoeba histolytica* and *Giardia lamblia* in District Hangu of Khyber Pakhtunkhwa, Pakistan

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Abstract

Giardia lamblia and *Entamoeba* histolytica are two important parasites of the gastrointestinal tract causing gastroenteritis in human population. The present study is carried out to check the prevalence of these two parasites in the stream and open well water of district Hangu of Khyber Pakhtunkhwa.200/two hundred sample were examined through PCR which showed 26.5% (53/200) and 22.5%(45\200) positive results for *Giardia lamblia* and *E.histolytica* respectively. The PCR product for *Giardia lamblia was 163bp* HSP (heat shock protein) and for *Entamoeba histolytica* was135bp small fragment of DNA.

Keywords: *Giardia lamblia; E.histolytica;* HSP; PCR

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1. Introduction

It is a famous quote that water is life but what have to do when this water without whom we can't survive becomes a threat for life by getting contaminated with small and innocent marauder creatures of God and start spreading infectious diseases. It is estimated that more than 90% of the deaths occur from infectious diseases worldwide. The majority population of the world still lives without access to healthy water and millions of people in developing as well as in developed world facing with a serious threat like diarrheal diseases, caused by the protozoan parasites due to their contamination in drinking water [1] and about 4000 children die every day from water borne disease [2].

The etiological agents for infectious diseases include viruses, bacteria, and microscopic protozon parasites. Among protozon parasites most important are the *Entamoeba histolytica* and *Giardia lamblia. Giardia lamblia* (*syn G. intestinalis*, *G. duodenalis*) is one of the most common intestinal parasites in the world and is the source cause of Giardiasis [3-4]. The primary victims of the *Giardia* sp are school going children in both developed and developing countries [5]. In Asia, Africa and Latin America the World Health Organization (WHO) estimated that round about 280 million people are annually infected with *Giardia spp* [6].

Giardiasis may result in different intestinal symptoms including diarrhea, steatorrhea, abdominal cramps, bloating, and flatulence, pale greasy and malodorous stools, and weight loss; nausea or vomiting may also occur. Active infection also causes Lactose intolerance which may last for several months after clearance of the parasite [7].

The prevalence rate of *Giardia* in the developed countries among asymptomatic persons is unknown, but it may range from 1.5 to 20 per cent [8]. According to this study the age social and economic status of the group considered are very important. WHO report in 1997 shows that many million people have invasive disease annually resulting in100, 000 deaths per years [9].

If we consider the age groups then *Giardia lamblia*, and *Entamoeba histolytica* cause morbidity especially in children. These parasites are the serious cause of infection in children throughout the world [10].

Entamoeba histolytica is another important parasite of the human gut. The disease caused by *Entamoeba histolytica* is called amoebiasis. It is the infection of human intestinal and extra-intestinal organ. After Malaria and Schistosomiasis the amoebic infection is considered as the third most common cause of death among parasitic diseases [11], worldwide 40-50 million cases are recorded annually and 40,000-100,000 deaths occur per year [12]. About 90% of the cases are asymptomatic of which only 4-10% develop complications i.e. colitis or extraintestinal diseases, this can be assessed if these cases are studied for one year [13].

In other words if we want to describe the prevalence of Amoebiasis we may say that *E. histolytica* infected approximately 10% of the world's population ,of which 50 million people have invasive disease due to *E histolytica*. Infection is predominantly seen in the tropical and subtropical regions the reason for that is the socioeconomic condition of this region. The prevalence of amebiasis depends upon the population of individuals affected; it is different between countries and between areas with different social and economic conditions.

Social and economic circumstances of the people i.e. low levels of education, poor hygiene, poor drinking water, overcrowded conditions, poor sanitation and demographic condition are the important factors in the prevalence of intestinel parasites [14] .The parasite is worldwide in distribution, high prevalence rates is found in the developing countries which is about 10% of the population[15].

E. histolytica and *G. lamblia* are the most important parasites to be considered for study for our area because both have a low infectious dose and spread through feces contaminated food and water. They have similar clinical presentations, and are commonly found in areas that lack proper sanitation system and clean water. Sanitation is the main problem in the district Hangu of Khyber pakhtunkhwa. These parasites have a simple life cycles having a resistant, infectious cyst form and a fragile, disease-causing trophozoite, which are the diagnostic stages of these parasites[16].So the chance for contamination is greater.

Keeping in view the above facts a study is designed to access the prevalence of *Giardia lamblia* and *E histolytica* in the water sources of district Hangu of Khyber Pakhtunkhwa and determined the risk factor.

2. Materials and methods

2.1 Study Area

The study was carried out in District Hangu of Khyber Pakhtunkhwa for detection of *Giardia lamblia* and *Entamoeba histolytica* parasites in open well and stream water in different villages/localities in the project areas. The district Hangu is situated in the north of The Khyber Pakhtunkhwa 33°32″ N latitude 71°04′ E longitude. The total area of district Hangu is approximately 1,097square kilometers and total population is 314,529. The average elevation of Hangu,Pakistan is 839 meters.sites from where the water samples were collected were Saidan banda, Malakabad and pass kaly. The samples were collected from 1st January, 2012 to 30th September 2012.

2.2 Sample Collection

A total of 200 water samples were collected from 3 different areas for the detection of *Giardia lamblia* and *Entamoeba histolytica* including 75 open well water, 80 bore well water, and 45 samples were collected from stream water. One liter of each water sample was collected in sterilized bottle, labeled (date of collection, name of area and source of water) and was transported to the Laboratory of Zoology Department, KUST, Kohat for further experimental analysis through, Polymerase Chain Reaction.

2.3 Water process

The water samples were filtered through Whatt-man filter paper in water filtration assembly and the materials were collected from the filter paper. The filtered residue were further centrifuge at 6000 rpm for 10 minutes the supernatant was discarded and the pallet was obtained in an eppendorf tubes and kept at -20°C in refrigerator for further process.

2.4 DNA Extraction

DNA was extracted by Vivantis GF-1 Nucleic Acid Extraction Kits.

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 10 Pico moles of forward and reverse primers.

Table 1: Primer sets used for *Giardia lamblia* and *Entamoeba histolytica* to the prediction amplicon sizes.

Primer	**Primer sequence**	**Prediction amplicon size**	**Target Gene**
Primer Set 'A'	*Giardia*		
Forward gdf	AGGGCTCCGGCATAACTTTCC	163-bp	HSP
Reversed gdf	GTATCTGTGACCCGTCCGAG		
Primer Set 'B'	*Entamoeba histolytica*		Small Unit of RNA
Forward ED1	TACAAAGTGGCCAATTTATGTAAGTA	135-bp	
Reversed EH1	GTACAAAATGGCCAATTCATTCAATG		

The cycle conditions for PCR is given below.

Table.2 PCR Cycle setup for *Giardia*

Stage	**Cycle**	**Step**	**Temperature**	**Time**
1	1	1	94 °C	5:00 min
2	35	1	94 °C	30 sec
		2	57 °C	30 sec
		3	72 °C	45 sec
3	1	1	72 °C	7:00 min
		2	4 °C	Hold

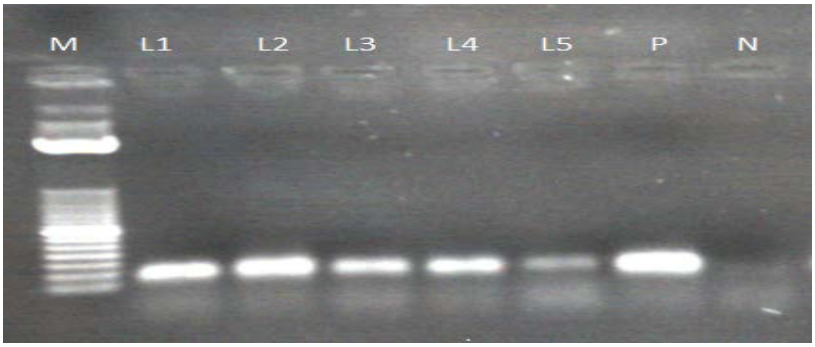

Figure 1: PCR gel Result for *Entamoeba histolytica*

L1 L2 L3 L4 L5 L6 L7 L8 L9 N L10 L11 LI2 M

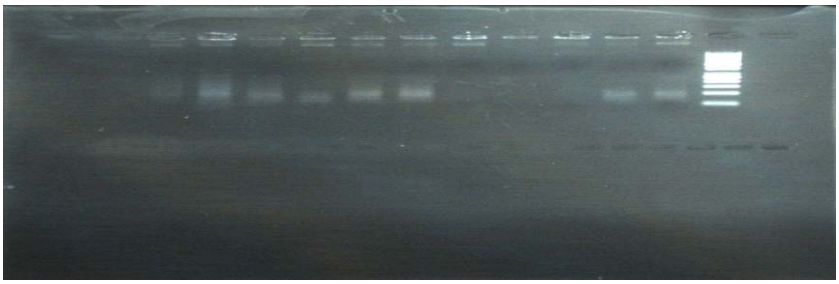

Figure 2: *PCR Gel* result for *Giardia lamblia*

2.4.1 Results

L4, L5, L6, L7, L8, L11, L12, are positive samples.

L9 and L10 are negative samples.

N is negative control

M is DNA Leader marker (100bp)

Table 3: PCR Cycle setup for *Entamoeba histolytica.*

Stage	Cycle	Step	Temperature	Time
1	1	1	94 °C	10:00 min
2	30	1	94 °C	30 sec
		2	51 °C	1:00 min
		3	72 °C	40 sec
3	1	1	72 °C	5:00 min
		2	4 °C	Hold

2.4.2 Results

L1, L2, L3, L4, L5, are positive samples.

N is negative control

M is DNA Leader marker (100bp)

2.5 Gel electrophoresis

The gel was run for 25 min at voltage of 120 volts and 500 mA current. Gel was then examined by UV transilluminator and Gel documentation for Picture.The specific DNA amplified product of each sample was determined by identifying the 163-bp and 135-bp bands for *Giardia lamblia* and *Entamoeba histolytica* respectively comparing with 100-bp DNA Ladder (Fermantas Germany), used as size marker.

2.6 Prevalence Rate

The prevalence rate was determined by the following formula [28].

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

2.7 Data Analysis

Statistical analysis was performed by using "STATISTIX", version 9.0, Korean made software. Variables included for evaluation were open well, bore well, stream, and $P<0.05$ values were considered the significant.

3. Results

The availability of clean water is very much essential for health and healthy environment in our poor community. The contaminated water is threat for transmission of water borne parasitic diseases as well as other bacteria and non-essential elements.

3.1 Prevalence of Giardia and Entamoeba spp in water sources in district Hangu.

A total of 200 water samples were collected from three different areas for the detection of *Giardia lamblia* and *Entamoeba histolytica* including 75 open well water, 80 bore well water, and 45 samples were collected from drain water (table 1). In the present study a total of 200 water samples were examined by means of PCR, which indicated 26.5% prevalence of *Giardia lamblia* and 22.5% was of *Entamoeba histolytica (*Table.2). Among these samples the prevalence of *Giardia lamblia was* 25.3% in open well water 21.25% in bore well water, and drain water observed 37.7 %. Similarly *Entamoeba histolytica* was detected 17.3% in open well water, 23.7 % bore well water and 28.8% in stream water.

3.1.1: By areas Prevalence of Giardia lamblia in District Hangu

After DNA amplification through PCR result showed variation in different areas of Hangu. The overall prevalence of *Giardia sp* was 26.5%(table. 3).

In all samples collected from *pass kaly* 21.8 % were positive for *Giardia lamblia*.20 % (8\40) samples were positive for *Giardia lamblia* out of 40 samples of open well, bore water showed 18% (2/11) and stream water 30.7% (4/13) positive results.

Malakabad showed 19.3% positive results for *Giardia lamblia*. Out of 15 samples of open well 13.33% (2\15) were positive. The bore water were consist of 15.6 % (5/32) positive samples, while stream water show33.3 %v (5\15) positive results .

Saidan banda showed 45 % (9\20) results for the *Giardia lamblia* in out of 20 samples from open well while the bore water showed 27% (10/37) and stream water was 47 % (8/17).

3.1.2: Prevalence of Entamoeba histolytica in different areas of District Hangu

The prevalence of *E. histolytica* was 22.5% (45\200) (table. 4) in Hangu while the source incidence is summarized as under:

In all sources of water collected from *pass kaly* 12.5% positive result for *Entamoeba histolytica* 12.5% samples were positive for *Entamoeba histolytica* out of 40 samples of open well, bore water showed o% out of 11 samples, and stream water showed 23.7% (3/13) results.

Malakabad showed 20.9% positive results for *Entamoeba histolytica* out of15 samples of open well 7.14% (1\15) were positive. The bore water were consist of 28% (9\22) positive samples, while stream water show20% (3\15) positive results.

Saidan banda showed 35(7\20) % results for *Entamoeba histolytica* in out of 20 samples from open well. While the bore water showed 27% (10/37) and stream water was 47% (7/17).

3.2.1: Prevalence of Giardia in different water sources of District Hangu

Source wise prevalence of *Giardia lamblia* can be elaborated as shown in table 5. *Open well* water showed over all prevalence of 25.3% from all areas. 20 % results were found in samples collected from pass kaly in out of 40 samples While, Malakabad and Saidan banda showed positive results for *Giardia lamblia*, (13.3%) 2\15 and (45%) 9\20 respectively. Similarly *bore well* water showed over all prevalence of 21.25%.Samples collected from pass kaly showed 18% (2\11) result, from Malakabad showed 15.6 (5\32) and from Saidan banda showed 27% (10\37).

The *stream water* showed over all prevalence of 37.7%), Samples collected from pass kaly showed 30.7 (4\13) result, from Malakabad showed 33.3% (5\15) and from Saidan banda showed 47% (8\17), see Table 5.

3.2.2: Prevalence of Entamoeba Histolytica in different water sources of District Hangu

Source wise prevalence of *Entamoeba histolytic* was as follows (table 5). *Open well* water showed over all prevalence of 17.3% (13\75) 12.5% results were found in samples collected from pass kaly in out of 40 samples While, Malakabad and Saidan banda showed positive results for *Entamoeba histolytic*, (6.6%)1\15and (35%) 7\20 respectively.

Similarly *bore well* water showed over all prevalence of 23.7%. Samples collected from pass kaly showed 0% (0\11) results, from Malakabad showed 28% (9\32) and from Saidan banda showed 27% (10\37), see table 5. The *stream water* showed over all prevalence of 28.8% , Samples collected from pass kaly showed 23.7% (3\13) result, from Malakabad showed 20% (3\15) and from Saidan banda showed 41% (7\17).

Table 1: Water samples collected from different areas of District Hangu

Location	Open Well	Bore Water	Stream Water	Total Samples
Pass kaly	40	11	17	68
Malakabad	15	32	15	62
Saidan banda	20	37	13	70
Total	75	80	45	200

Table 2: Overall prevalence of *Giardia sp* and *Entamoeba sp* in District Hangu

Area	*Giardia*	*Entamoeba*
Pass kaly	14\64 (21.8%)	8\16 (12.5%)
Malakabad	12\62 (19.3%)	13\62 (20.9)
Saidan banda	27\74 (36.5%)	24\74 (32%)
Total	53/200 (26.5%)	45\200 (22.5%)

Table 3: Prevalence of *Giardia lamblia* in different areas of District Hangu

Area (n)	Open well water Positive\total (%)	Bore water Positive\ total (%)	Stream Water Positive\total (%)	Over All Samples Positive\ total (%)
Pass kaly	8\40 (20%)	2\11 (18%)	4\13 (30.7%)	14\64 (21.8%)
Malakabad	2\15 (13.3%)	5\32 (15.6%)	5\15 (33.3%)	12\62 (19.3%)
Saidan banda	9\20 (45%)	10\37 (27%)	8\17 (47%)	27\74 (36.5%)
Total	19\75 (25.3%)	17\80 (21.25%)	17\45 (37.7%)	53/200 (26.5%)

Table 4: Prevalence of *Entamoeba Histolytica* in different areas of District Hangu.

Area (n)	Open well water Positive \total (%)	Bore water Positive\total (%)	Stream Water Positive\total (%)	Over All Samples Positive\total (%)
Passs kaly	5\40 (12.5%)	0\11 (0%)	3\13 (23.7%)	8\64 (12.5%)
Malakabad	1\15 (7.14 %)	9\32 (28%)	3\15 (20%)	13\62 (20.9%)
Saidan banda	7\20 (35%)	10\37 (27%)	7\17 (41%)	24\74 (32%)
Total	13\75 (17.3%)	19\80 (23.7%)	13\45 (28.8%)	45\200 (22.5%)

Table 5: Prevalence of *Giardia lamblia* and *Entamoeba histolytica* in different water sources of District Hangu.

Source of water	Area	*G.lamblia*	*E.histolytica*
Open Well Water	Pass kaly	8\40 (20%)	5\40 (5%)
	Malakabad	2\15 (13.3%)	1\15 (7.14 %)
n=75	Saidan banda	9\20 (45%)	7\20 (35%)
	Total	19\75 (25.3%)	13\75 (17.3%)
Bore Well Water	Pass kaly	2\11 (18%)	0\11 (0%)
	Malakabad	5\32 (15.6%)	9\32 (28%)
n=80	Saidan banda	10\37 (27%)	10\37 (27%)
	Total	17\80 (21.25%)	19\80 (23.7%)
Stream Water,	Pass kaly	4\13 (30.7%)	3\13 (23.7%)
	Malakabad	5\15 (33.3%)	3\15 (20%)
n=45	Saidan banda	8\17 (47%)	7\17 (41%)
	Total	17\45 (37.7%)	13\45 (28.8%)

4. Discussion

The prevalence of E.*histolytica* and *G.lamblia* which cause gastroenteritis in Hangu, Khyber Pakhtunkhwa, was reported in the present study .The study was purely based on molecular detection techniques. The investigations were carried out for two important parasites *E.histolytica* and *G.lamblia* an area where the sanitation system is too much poor and the land condition is such that allows contamination to underground water, because the aquifer is high.

The detection of *Entamoeba histolytica and G.lamblia* was made through the PCR diagnosis.PCR was the most accurate diagnostic assay and was recognized as a most reliable method in the diagnosis of *G.lamblia* and

Entamoeba histolytica. Gonin and Trudel (2003) found that PCR is the more sensitivity and specific method in differentiating *E. histolytic and E. dispar* in stool samples as compared to ELISA and microscopy [17].

Our study revealed that *G.lamblia and Entamoeba histolytica* cause Giardiasis and amoebiasis in the endemic region in Hangu. The present study has shown the prevalence of *G.lamblia and Entamoeba histolytica* parasites in drinking water of district Hangu.

A total of 200 samples were collected, among these samples 75 were of open well water, 80 bore well water and 45stream water were examined through PCR. The prevalence (%) of *Giardia*, and *Entamoeba histolytica* in each category of water samples were determined. Overall prevalence of *G. lamblia* was 26.5% (53/200) in which prevalence in open well water was 25.3 % (19/75), in bore well water it was 21.25 % (17/80), and in stream water 37.7 % (17/45). Similarly overall prevalence for Entamoeba histolytica was 22.5 % (45/200) among these *Entamoeba histolytica* was detected 17.3 % (10/75) in open well water, 23.7 % (19/80) in bore well water, and 28.8 % (13/45) in stream water.

There are some many factors affecting the prevalence of *G.lamblia* consisting of sanitary condition and environmental conditions such as location of sampling, animal diversity in the areas, climatic condition, season, volume of sample, rainy seasons and population of the animals in the areas etc. That's how the propagation of *G.lamblia* is high in developing countries than that of developed countries [18].

The DNA of *G.lamblia* and *Entamoeba histolytica* was detected in 53 and 45 water samples respectively. The result showed that the contamination of stream water was more with *G.lamblia* and *Entamoeba histolytica* than other sources. The reason for this may be the direct connection of the sewerage system to the streams.

Similarly the water containing different samples of open well bore well, and streams were processed through molecular detection for *Entamoeba histolytica* which showed the following results, 12.25 % (8/64) from pass kaly, 20.9 % (13/62) from Malakabad, and the Saidan banda showed 32 % (24/74) positive results for *Entamoeba Histolytica.*

The results of the present study confirm the findings of clinical studies conducted by [25] that had shown the presence of *G.lamblia* alongside with *Cryptosporidium* parasites in the human population [19]

According to one of the report which had shown *Entamoeba histolytica*, *Giardia lamblia*, are two of the major causes of diarrheal disease of protpzones origin [20]. *E. histolytica* causes approximately 100,000 deaths worldwide each year, which makes it second only to malaria as a cause of mortality due to a protozoan parasite [21].

The present work was compared with the similarly work carried out microscopically by [28] where in the all the three sources of water was contaminated with eggs or cysts or of the *G.lamblia* and *Entamoeba* parasite. The results indicate overall prevalence of 65.5% (295/450) of protozoa, including *G.lamblia* 18.5% (61/450) and *Entamoeba* spp. 14.4% (65/450) [22]. In other studies recovered *E. histolytica and E. Coli from* the sewage water and stool [23].

A prevalence of 11% for *E. histolytica* was recorded in children of Delhi, India by Kaur [24]. Aza *et al*. reported 21.0% prevalence of intestinal parasites in seven villages of Malaysia [25]. Prevalence of *E. histolytica* from preschool children in Bangladesh was 15.6% [26]. A study was conducted by Heidari and Rokni in day-care centers in Damghan city, Semnan province, Iran and recorded the overall prevalence of *E. histolytica* as 2.4% [27]. Barnawi reported 2.7% prevalence of E. *histolytica* was found in patients attending three hospitals in Jeddah, Saudi Arabia [28].

Our recent study results are comparable to the studies, carried out five or six decades ago in developed countries, indicating Pakistan is still on risk and needs a lot to limit the spread. In U.S.A., Fchaust and Headlee [29] revealed a prevalence of 12.4% in 1936 was recorded which reduced to 10% in 1988, revealed by Hubbard *et al* . This decrease in the prevalence of giardiasis in U.S.A reflects the effects of improved environmental and personal hygiene [30].

The present results may help the people for their health in prevention and supervision for Giardiasis and 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 present study that the *Giardia lamblia* and *Entamoeba histolytica* are present in Hangu region and was detected having size of 163 bp and 135bp through PCR. It has also been determined that PCR is more sensitive and specific than microscopy examination. The following are the recommendations.

1. It is recommended that further genomic study may be carried out to determine the genomic assemblages of both the parasites in the region and work should be stream line to develop the recombinant DNA vaccine against these two parasites.

2. Measures should be taken to provide safe drinking water to these communities, Standard of personal hygiene must be improved through public awareness campaign.

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

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