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Molecular Detection of *Giardia lamblia* and *Cryptosporidium parvum* in Different Water Sources of District Bannu, Khyber Pakhtunkhwa, Province of Pakistan

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Authors' contributions

This work was carried out in collaboration between all authors. Author MSA collected the samples and perform molecular detection. Authors SUK, SA, NUA, MS, IA, MI and MW help literature search and manuscript writing. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Background: *Cryptosporidium parvum* and *Giardia lamblia* are intestinal parasites that predominantly causes "waterborne" infections that are transmitted through consumption of contaminated water. Both parasites typically cause an acute short-term infections with self-limiting diarrhea as the main symptom in people with intact immune systems. However, in immunocompromised individuals, the symptoms are particularly severe and might be fatal.
Methods: The study was carried out in District Bannu Khyber Pukhtunkhwa, Pakistan for the detection of *G. lamblia* and *C. parvum* parasites in drinking water in different villages/localities (Kakki, Jamon Road, Kotka Juma Khan, Sokari, Mandan and Bannu City). Water samples n=75 were collected from different water sources between 1st August 2011 to 30th January 2012. These samples included tap, pond, borewell and hand pump water that were filtrated and residue was subjected to amplify by PCR.
Results: Overall prevalence of parasites was 36% (25/75), containing tap 17.64% (9/51)

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and pond water 75% (6/8), bore well water 41.66% (5/12) and hand pump water 50% (2/4). Similarly over all prevalence rate of tap water for *C. parvum* was 7.84% (4/51) while for *G. lamblia* was 9.80% (5/51) positive. The present study revealed that the people of the area should use the cleaned and filtered water.

Conclusion: Contamination of water with *G. lamblia* and *C. parvum* was found in water sources especially the drinking ones, of District Bannu which need proper water treatment to decontaminate and large scale studies are needed.

Keywords: G. lambli; C. parvum; diarrhea; water; PCR.

1. INTRODUCTION

Cryptosporidium parvum and *Giardia lamblia* cause human Giardiasis and Cryptosporidiosis respectively. The main symptom of both is a self-limiting diarrhea in people with intact immune systems. Infections in children and HIV positive individuals may lead to considerable morbidity and/or mortality. *Cryptosporidium* is an obligate parasite, which requires a host to produce and release oocysts for infectious [1]. *C. parvum* spread is through insufficiently treated water supplies and contaminated municipal water system. It is one of the most common waterborne diseases and is found worldwide. A human infection occurs by ingesting the oocysts. The high resistance of *Cryptosporidium* oocysts to disinfectants such as chlorine bleach enables them to survive for long periods and still remain infective.

Cryptosporidium is considered as cause of about 20% of childhood diarrheal cases in developing countries and is a potentially fatal complication of AIDS [2].

Giardiasis occurs with prevalence of 2 to 5 percent in developed countries and 20% to 30% in developing regions of Asia, Africa and Latin America [3]. Zoonotic infections are serious threat to global health and the financial system that transmitted by contaminated water [4]. Waterborne transmission is believed to be the main pathway of disease concerned in a number of great outbreaks [5]. *Cryptosporidium* and *Giardia* are the main frequent enteric parasites of domestic animals and humans and are being gradually more detected as parasites of a varied variety of wildlife species [6-9].

Giardia describes a genus of flagellate protozoan parasites of the small intestine that infects a wide range of vertebrates. There are three main species described: *Giardia angilis, Giardia muris*, and *Giardia intestinalis* [10,11]. *Giardia intestinalis*, also is known as *Giardia duodenalis* and *Giardia lamblia*, is the species known to infect humans [11,12].

Giardia intestinalis is one of the most ubiquitous enteric parasites in the world, capable of infecting virtually all mammals and even some other vertebrates, such as birds [6]. The contamination of aquatic ecosystems with *G. intestinalis* from untreated or improperly treated waste water and agricultural overflow is an increasing concern [13,14,15].

The present study was designed to determine the prevalence of *C. parvum* and *G. lamblia* in different water sources of district Bannu using molecular techniques such as PCR.

2. MATERIALS AND METHODS

2.1 Study Area and Sample Collection

The study was carried out in District Bannu Khyber Pukhtunkhwa, which is northernmost province of Pakistan, and one of 24 districts that make up the Khyber Pakhtunkhwa province of *Pakistan.* The district forms a basin drained by the Kurram river and the Gambila or Tochi river, which run down from the hills of Waziristan. It aimed at for the detection of *G. lamblia* and *C. parvum* parasites in drinking and drainage water in different villages/localities (Kakki, Jamon Road, Kotka Juma Khan, Sokari, Mandan and Bannu City) collected from 1st August 2011 to 30th January 2012. A total of 75 samples including 51 tap water, 12 bore well water, 8 pond water and 4 hand pump water were collected. One liter was collected for each sample in sterilized and labeled (date of collection, name of area and type of water) bottle, and was transported to the laboratory of Department of Zoology, Kohat University of Science and Technology (KUST) for further process.

2.2 Water Samples Processing

The water samples were filtered through Whatman filter paper Grade 6: 3 µm ?and the residue was obtained and mixed with 1 ml buffer phosphate solution in an eppendorf tube and kept at -20ºC in refrigerator for further process.

2.3 DNA Extraction and Detection of *Giardia lamblia* and *Cryptosporidium parvum* by PCR

The DNA was extracted from the above solution by Trizol (Invitrogen Inc., Carlsbad, CA, USA) which is a complete, ready-to use reagent for easy and simultaneous isolation of total RNA, DNA and proteins from liquid samples with minor modification. PCR reaction was carried out in a Amplitronyx thermal cycler (NyxTechnik, Inc, San Diego, CA, USA). The reaction mixture consisted of 5µL Taq DNA polymerase (Fermentas USA) Taq Buffer, 4.2 µL, $MgCl_2$, 2.4 µL, dNTPs, 1.0 µL, Primers used to detect *G. lamblia,* were Forward GDF (**3'-**AGGGCTCCGGCATAACTTTCC**-**5') and Reverse GDR, (5**'-**GTATCTGTGACCCGTCCGAG-3') designed for heat shock protein gene, containing 163-bp length for *G. lamblia* [16]. While for primers used to detect *C. parvum* were forward, CSF; (3'-AGTGCTTAAAGCAGGCAACTG**-**5') and Reverse CSR; (5'-CGTTAACGGAATTAACCA GAC-3'), which were designed to amplify 556-bp fragment of the 18S rRNA gene specific to *C. parvum* [17]. A 1.0 µL of 10 Pm? concentration of each, dH_2O 5.3 µL and genomic DNA, 5.0 µL were mixed properly in PCR tubes separately, for *G. lamblia* having *G. lamblia* primers and the other mixture consisted of *C. parvum* primers with final volumes 20µL and run for 35 cycles after completing initial denaturation for 5 minutes at 94ºC. Each cycle consisted of 3 steps; denaturation at 94ºC for 30 seconds, annealing for *G. lamblia* was 57ºC for 30 seconds and for *C. parvum* was 65ºC for 30 seconds. Elongation was 72ºC for 45 seconds. The final elongation step was 72ºC for 7 minutes.

2.4 Gel Electrophoresis

PCR product mixture of 12 µL including 2 µL loading dye was loaded in 2% agarose gel and DNA bands were compared with 50bp of DNA ladder marker, {Thermo Scientific Life Science/Fermentas USA (#SM0373)} was run parallel to samples for 25 min and gels were examined under UV Transilluminator (Cleaver Scientific USA). The specific DNA amplified

product of each sample was determined by identifying 163-bp (Figure 1) bands for *G. lamblia* and 556-bp bands for *C. parvum.*

2.5 Prevalence Rate

The prevalence rate was determined by using the following formula [18]:

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

2.6 Statistical Analysis

The data was analyzed by using the Univariate ANOVA (Statistix 9) and P≤0.05 values were considered significant.

3. RESULTS AND DISCUSSION

Giardia lamblia and *Cryptosporidium parvum* are the most common enteric parasites of humans and domestic animals [6-9] *G. lamblia* is a common cause of diarrheal illness in humans, mainly in children [8]. The implication of *Cryptosporidium* was firstly recognized to be one of an opportunistic pathogen in AIDS patients [19]. Among the different water sources, children consuming water from tap and tube well water 9.5 and tap and well were significantly (23.8%) infected of *G. lamblia* (P<0.05) [20].

In the present study total water samples were 75 consisted of 51 tap water, 8 ponds water, 12 borewell water and 4 hand pump water that were tested by PCR. Overall prevalence of parasite both? in these samples was 36% (25/75), among these the prevalence in tap water was 17.64% (9/75) and in pond water it was 75% (6/8), borewell water 41.66% (5/12) and hand pump water 50% (2/4). Similarly among the overall water born parasitic prevalence, the rate of contamination of tap water with *C. parvum* was 7.84% (4/51) while for *G. lamblia* it was 9.80% (5/51) positive. In pond water, 62.5% (5/8) samples were positive for *C. parvum* compared with 75% (6/8) that were positive for *G. lamblia*. Similarly among borewell water 41.66% (5/12) samples were contaminated with one of the parasites, 16.66% (2/12) were contaminated with *C. parvum* and 25% (3/12) were contaminated with *G. lamblia.* As for in hand pump water, 50% (2/4) samples were contaminated with either parasite, *C. parvum* was detected in 25% (1/4), and *G.lamblia* was detected in 25% (1/4) (Table 1 & Table 2). These results were marginally greater for *G. lamblia* than the study conducted in Nepal (12.0%) [21]. In a previous study of drinking water from tap water (effluent), open well water, stream water (influent) in Kohat, Pakistan, over all contamination with both parasites was 12%, among these 5.33% were contaminated with *G. lamblia* and 6.66% were contaminated with *C. parvum* [22].

The same study reported contamination with *Giardia* in spring water (8%), tap water (6%), and well water (2%) compared with *Cryptosporidium* in spring water (14%), tap water (6%), and well water (0%).

In the present study overall water contamination was 36%, of which 16% with *C. parvum* and 20% with *G. lamblia* which is slightly greater than other studies in Khyber Pakhtunkhwa (Pakistan) reporting overall prevalence of 33.6%, of which *C. parvum* was detected in 19.5% and *G. lamblia* was detected in 14.1% [18,22].

In similar studies, *G. lamblia* and *C. parvum* were reported in drinking water from several countries. For instance in a study of drinking water in Shanghai (China) contamination was detected in 50% of the water samples, where *C. parvum* was detected in 32% of the samples, and *G. lamblia* was detected in 18% [23], which is higher than our study. Another study conducted in Hungaria, over all water contamination rate was 40% in which 13.3% was contaminated with *C. parvum* and 26.7% was contaminated with *G. lamblia* [24].

The present study reported on the prevalence of *G. lamblia* in different areas of district Bannu in which the overall prevalence in Bannu city was (1/15) 6.67% and in Kotka Juma Khan was (4/13) 30.77%, in Jamon Road it was (1/10) 10%, in Sokari (3/18) 20%, in Mandon (2/10) 20% and in Kakki (4/9) 44.5% (Table 1). Overall prevalence by source of water, *G. lamblia* was detected in tap water in different areas of District Bannu was highest in Kotka Juma Khan 2/9 (22%), the contamination rate in the other areas were as follows: In Bannu city was 0/13 (0%), in Jamon Road 1/10 (10%), in Sokari 1/10 (10%), in Mandon 1/09 (11%), and in Kakki 0/0 (0%). The prevalence of *G. lamblia* in borewell water was highest in Kotka Juma Khan with 1/3 (33%) contamination, the rest were 0/1 (0%) in Bannu City, 0/0 (0%) in Jamon Road, 2/8 (25%) in Sokari, and 0/0 (0%) in Kakki. *G. lamblia* contamination in ponds was as follows: 1/1 (100%) in Bannu City, 1/1 (100%) in Kotka Juma Khan, 0/0 (0%) in Jamon Road, 0/0 (0%) in Sokari, 1/1 (100) in Mandan and in Kakki 3/5 (60%). No contamination with *G. lamblia* was detected in hand pumps in Bannu City, Kotka Juma Khan, Jamon Road, Sokari, and Mandon, while in Kakki it was detected in 1/4 (25%) (Table 1).

Overall contamination of samples with *C.parvum* in different areas of district Bannu was highest in Kotka Juma Khan with and (3/13) 23.07% contamination, the rest were as follows: (1/15) 6.67% in Bannu city, (1/10) 10% in Jamon Road, (2/18) 11.2%, in Sokari in Mandon (2/10) 20%, and in Kakki (3/9) 33.34% (Table 2).

Overall source wise prevalence of *C. parvum* in different areas of Bannu was also same that in tap water of Bannu city, which was higher in Mandon 1/09 (11%) than other areas reported as follows: 0/13 (0%), in Kotka Juma Khan 1/9 (11%), in Jamon Road 1/10 (10%), in Sokari 1/10 (10%), and in Kakki 0/0 (0%). The contamination rate of *C. parvum* in borewell water was highest in in Kotka Juma Khan with 1/3 (33%) contamination, while the other areas were as follows: 0/1 (0%) in Bannu City, 0/0 (0%) in Kakki and Jamon Road, and 1/8 (13%) in Sokari. Contamination of pond water with *Cryptosporidium* was highest in Kakki 2/5 (40%), contamination in the other areas was same in Bannu City, Mandan and in Kotka Juma Khan with 1/1 (100%) in each city, while none was detected in Jamon Road and Sokari. No contamination of hand pump water with *C. parvum* was detected in Bannu City, in Kotka Juma Khan, Jamon Road, Sokari or in Mandon, while it was detected in 1/4 (25%) in Kakki (Table, 2).

In a study conducted in Russia and Bulgaria for the detection of *G. lamblia* and *C. parvum* in drinking water samples of different origin (surface, tap, bottled, well, spring and waste water) were collected from Rostov (southern Russia), Sofia and Varna (Bulgaria) reported (9.6%) of the samples were positive for *G. lamblia* and (18.1%) were positive for *C. parvum*. Both parasites were detected in tap, river, well and waste water [25]. In the present study the contamination rates with *G. lamblia* (20%) and (for *C. parvum*16%) were higher than in the above study.

In an earlier study conducted in Thailand, water samples of different origin were collected from six Tsunami affected southern provinces of Thailand in early 2005, in which (12.7%) were positive for *Cryptosporidium spp*. and nine (7.6%) were positive for *Giardia spp.*

Additional water samples from two of the same areas were examined 3 years later, in the early 2008, (11.9%) samples were positive for *Cryptosporidium spp*., and (7.1%) were positive for *Giardia spp*. Both protozoans were found in reservoir, pond and river/canal water [1]. Our study found greater contamination of water sources with *G. lamblia* and *C. parvum which is* due to hygienic and pipes leaking problems in the study area.

A similar study was also conducted at Kohat, Karak and Hangu districts of Khyber Pakhtunkhwa province, Pakistan. In which water samples were collected from tap, pond and drain water [18]. The contamination rates of *G. lamblia* and *C. parvum* in each water source samples were reported as follows: 65.5% of the samples, contained protozoa, among which *G. lamblia* and *C. parvum* were 18.5% and 19.5 % respectively. The prevalence of *C. parvum* (16%), and *G. lamblia* (20%) were slightly different in the present study, these differences might be due to differences in climatic conditions, in addition to various other factors like environmental, geographical distribution, hygienic conditions, socio economics status of the society, less awareness and also less education of the people in that area.

Table 1. Prevalence of *G. lamblia* in water samples of different areas of district Bannu

Location (n)	Tap water Positive\total (%)	Borewell water Positive\total (%)	Ponds water Positive\total (%)	Hand pumps Positive\total (%)
Bannu City (15)	0\13 (0%)	0\1 (0%)	1\1 (100%)	0\0 (0%)
Kotka Juma khan (13)	2\9 (22%)	1\3 (33%)	1\1 (100%)	0\0 (0%)
Jamon Road (10)	1\10 (10%)	0\0 (0%)	0\0 (0%)	0\0 (0%)
Sokari (18)	1\10 (10%)	2\8 (25%)|	0\0 (0%)	0\0 (0%)
Mandan (10)	1\09 (11%)	0\0 (0%)	1\1 (100%)	0\0 (0%)
Kakki (9)	0\0 (0%)	0\0 (0%)	3\5 (60%)	1\04 (25%)

(%) denoted for percentage, n= total number, ($P \leq 0.05$), *Significant? There is no statistical analysis*

Table. 2 Prevalence of *C. parvum* in water samples of different areas of district Bannu

Location (n)	Tap water Positive\total (%)	Borewell water Positive\total (%)	Ponds water Positive\total (%)	Hand pumps Positive\total (%)
Bannu City (15)	0\13 (0%)	0\1 (0%)	1\1 (100%)	0\0 (0%)
Kotka Juma khan (13)	1\9 (11%)	1\3 (33%)	1\1 (100%)	0\0 (0%)
Jamon Road (10)	1\10 (10%)	0\0 (0%)	0\0 (0%)	0\0 (0%)
Sokari (18)	1\10 (10%)	1\8 (13%)|	0\0 (0%)	0\0 (0%)
Mandan (10)	1\09 (11%)	0\0 (0%)	1\1 (100%)	0\0 (0%)
Kakki (9)	0\0 (0%)	0\0 (0%)	2\5 (40%)	1\4 (25%)

(%) denoted for percentage, n= total number, ($P \leq 0.05$), *Significant? There is no statistical analysis*

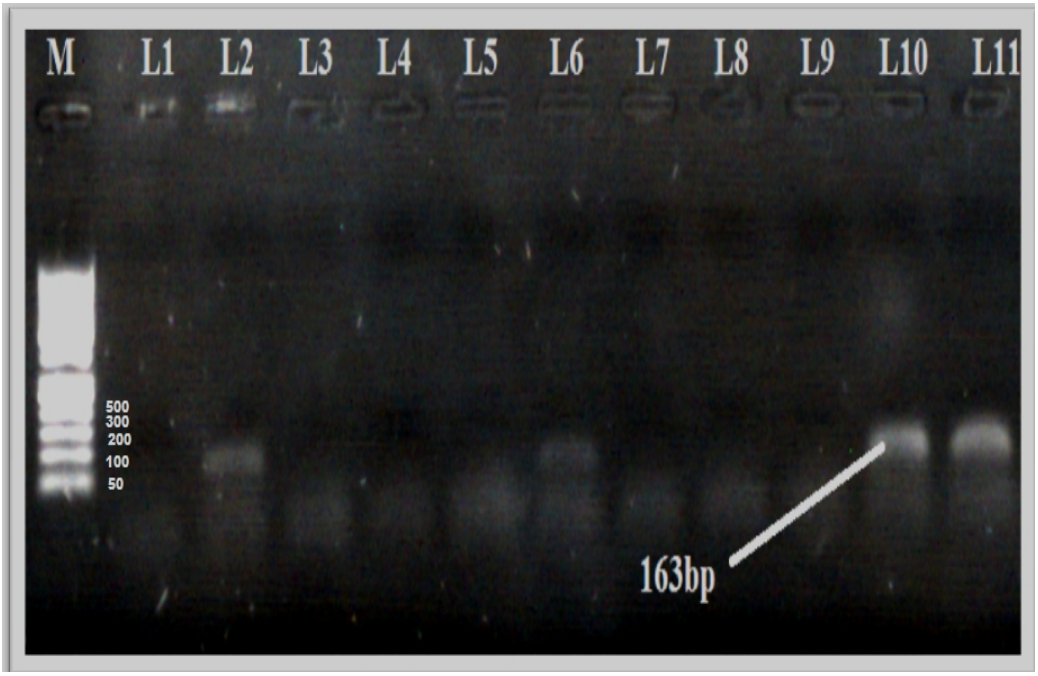

Figure 1. *Giardia lamblia* DNA amplification by PCR, M = Marker 100 bp, L1= Negative control

L2: Positive control (163bp), L6, L10 and L11 are positive samples, L3, L4, L5, L7, L8 and L9 are negative samples

4. CONCLUSION

Contamination of District Bannu water with *G. lamblia* and *C. parvum* was found in water sources especially the drinking ones, which needs proper water treatment to decontaminate and large scale studies are needed. Frequent drinking water testing should be tested each year particularly in the study area.

The use of PCR assay used in the current study proved to be helpful in the diagnosis of *G. lamblia* and *C. parvum* in water samples. It could be used as a tool for epidemiological investigation of different water sources in developing countries especially in flood infected areas of Pakistan like Peshawar, Nowshera, Dir, Swat and Some areas of Punjab, where water sources are contaminated with drain water. Such studies would bring the authorities attention to such problems and the importance of protecting drinking water sources from contamination and use of better water treatment to avoid infections in these areas.

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COMPETING INTERESTS

The authors declare that they have no competing interests

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