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Molecular detection of *Cryptosporidium parvum* in different water sources of Tehsil Takhte Nasrati, District Karak

Ihsan Ullah*[*], *Munawar Saleem Ahmad*[*,**], *Sultan Ayaz*[,****], *Noor ul Akbar*[***], *Muhammad Naeem*[*], *Abdussalam*[*****], *Muhammad Ismail*[***], *Asmat Ullah*[***]**

**Department of Zoology, Hazara University, Garden Campus Mansehra. Khyber Pakhtunkhwa, Pakistan*
***Department of Zoology, University of SWABI. Khyber Pakhtunkhwa, Pakistan*
**** Department of Zoology, Kohat University of Science and Technology, Kohat. Khyber Pakhtunkhwa, Pakistan*
*****College of veterinary sciences, Abdul Wali Khan University Khyber Pakhtunkhwa, Pakistan*
******Department of Microbiology, Abasyn University Peshawar, Khyber Pakhtunkhwa, Pakistan*

(Corresponding author: Munawar Saleem Ahmad)
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ABSTRACT: *C. parvum* is an obligate parasite, causing human Cryptosporidiosis which is the most common waterborne diseases found throughout the world. It's outbreaks are linked with contaminations of drinking water. The current study was designed to check out the prevalence of *C. parvum* (*C. parvum*) in different water sources of Tehsil Takhte Nasrati District Karak through PCR. Three hundred samples were collected from dam water, well water, pond water and spring water, including 75 samples from each source, which were filtered and followed by DNA extraction. The extracted DNA was amplified through PCR. The overall water-borne parasitic prevalence of *C. parvum* was 14.7% (11/75) positive. Among pond water 24% (18/75) samples were positive for *C. parvum.* Similarly, in well water 2.7% (2/75) samples were positive for *C. parvum* and in spring water 9.4% (7/75) samples were positive for *C. parvum.* The results showed that pond and dam water source was more contaminated with *C. parvum* as compared to other sources of water.

Key words: *C. parvum*, diarrhea, water, PCR, Pakistan

INTRODUCTION

There are several water-borne protozoan parasites which cause present in the alimentary canal of a human being different types of diseases that affect about 3.5 million people throughout the world including three million deaths of children per year (Akbar *et al*., 2014). Humans, domestic animals and wildlife are also affected by these parasites. Three hundred and twenty five water associated parasitic protozoan disease outbreaks are reported by worldwide (Kramer *et al*., 2001).

C. parvum is an obligate parasite, which requires a proper host to produce and release oocysts for infections. The main cause of human Cryptosporidiosis is *C. parvum* (Appelbee *et al.,* 2005). Cryptosporidiosis has global distribution and 50.8% of water-borne diseases are caused due to these parasites. In developing countries 8-19% of diarrheal diseases can be credited to *Cryptosporidium* (Gatei *et al.,* 2006) *Cryptosporidium* was first known as a cause of gastrointestinal disease in humans, and a main cause of diarrhea in both children and adults globally in 1976 (Yoder *et al*., 2009).

C. parvum is spread through insufficiently treated water supplies and contaminated municipal water system. Cryptosporidiosis is the most common waterborne diseases found worldwide. Human infection occurred by ingestion of *Cryptosporidium* oocysts. Oocysts of *Cryptosporidium* survive for long periods showing high resistance to disinfectants which enables them to and still remain infective. *Cryptosporidium* is considered cause of childhood diarrheal cases about 20% in developing countries and is potentially fatal complication of AIDS (Karanis *et al*., 2006). Infection by *Cryptosporidium* can be transmitted from food and water which is fecaly unhygienic also transmitted from animal to person and person to person (Dupont *et al.,* 1995). Cryptosporidiosis Outbreaks have also been linked with contaminations of drinking water, water park wave pools and Swimming pools. The untreated ground or well water and supply system which supply water for community for drinking purposes can also be sources of contamination (Juranek, 1995). Polymerase chain reaction technology provides higher sensitivity in specific diagnosis to species level.

Polymerase chain reaction has the ability to analyze a large number of samples with fast, repeatable and highly accurate examination (Fayer *et al.,* 2000).

MATERIALS AND METHODS

A. Study Area

The study was carried out in tehsil Takhti Nasrati District Karak, Khyber Pakhtunkhwa. The occupation is mostly agriculture, but few people are merchants, businesspersons and professional like doctors, engineers and teachers, mostly the people of the current area have low economic condition ranges from about R.s 10000 to R.s 50000 per month, while their water sources are mainly composed of well and water supply systems connected with different dams and springs, where there the water stored from rain water.

B. Study Area Sites

The sites from where the water samples were collected were TakhteNasrati City, Khadda Banda, Siraj Khel, Serki Nasrati, Mianki Banda, Jehangeri Banda, Inzer Banda, Alwar Banda, Zeera banda and Shnawa GudiKhe. The samples were collected from 1st July, 2013 to 31st October, 2013 after approved by the Ethical Committee of Hazara University, Garden Campus Mansehra. Khyber Pakhtunkhwa, Pakistan.

C. Sample Collection

A total of 300 water samples were collected in the current study for the detection of *C. parvum* in different water sources in different villages/localities of Tehsil Takhte Nasrati, including 75 samples each from dam water, well water, pond water and spring water sources, containing one liter water sample from each source in sterilized and properly labeled bottle with collection date, area name and water type on the spot. All of the samples were transferred to the Department of Zoology laboratory, Kohat University of Science and Technology Kohat for further process through Polymerase Chain Reaction (PCR) (Akbar *et al.,* 2014; Ayaz *et al.,* 2013; Alam *et al.,* 2013).

D. Samples Processing

The samples of water were filtered through Whatman filter paper (Cat No. 1442). A sample pellet was obtained and mixed with 1 ml buffer phosphate solution in an Eppendorf tube and kept in -20ºC in refrigerator for further processing (Akbar *et al.,* 2014; Ayaz *et al.,* 2013; Alam *et al.,* 2013).

DNA Extraction. The DNA was extracted from the above solution by using a Genomic DNA purification kit (#k0512, Thermoscientific) which is a complete, ready-to use reagent for the easy and simultaneous isolation of total DNA from liquid samples with minor modification.

DNA was extracted according to the manufacturer's instructions which follow step by step. Three hundred µl of 100% cold ethanol was added to the pellet after discarding the supernatant and centrifuge at 10000 RPM for 3-4 minutes. Discard the supernatant. Again Wash the pellet once with 150 µl 70% cold ethanol. The DNA wash step was repeated and the tubes were stored vertically to dry for 10 minutes. 40 µl of distilled water was added to the pellet and incubated at 55°C for 10 minutes in hot plates, and were kept on -40°C for further use (Akbar *et al*., 2014; Alam *et al.,* 2013).

DNA Amplification (PCR). PCR reaction was carried out in the Amplitronyx thermal cycler (NyxTechnik Model No. A-6, Inc, San Diego, CA, USA) with Taq DNA polymerase (Fermentas USA) The amplification was performed with 5 µl of extracted DNA by using 10 pm of forward and Reverse Primers used for *C. parvum* were AWA72F (AGTGCTTAAAGCAGGCAACTG), and AWA1235R (CGTTAACGGAATTAACCAGAC*)* targeting the 18S rRNA (Ayaz *et al.,* 2013). For each reaction 35 cycles were applied in PCR initiated by 94ºC for 5 minutes as denaturation. Each cycle was consisted of 3 steps denaturation for 30 seconds at 94ºC, annealing for 30 seconds at 60ºC followed by elongation at 72ºC for 45 Sec. The final elongation was for 7 min at 72ºC (Ayaz *et al.,* 2013) with some modification for standardization.

Gel Electrophoresis. A PCR product mixture of 10 µL was mixed with 2 µL loading dye and then loaded into the wells on agarose gel, which was prepared by dissolving 1.5 grams of affairs in 100 ml of 0.5X TBE buffer in reaction bottle (Akbar *et al*., 2014; Alam *et al.,* 2013).

The specific DNA amplified product of each sample was determined by identifying 556-bp bands for *C. parvum* (Fig.1).

Prevalence Rate. The prevalence rate of parasite was determined by using the following formula Prevalence Rate = (Positive samples/Total no. of water samples examined) ×100.

E. Statistical Analysis

The data were analyzed by using the Univariate ANOVA (Statistix 9) and $P \leq 0.05$ values were considered significant (Alam *et al.,* 2013; Ayaz *et al.,* 2011).

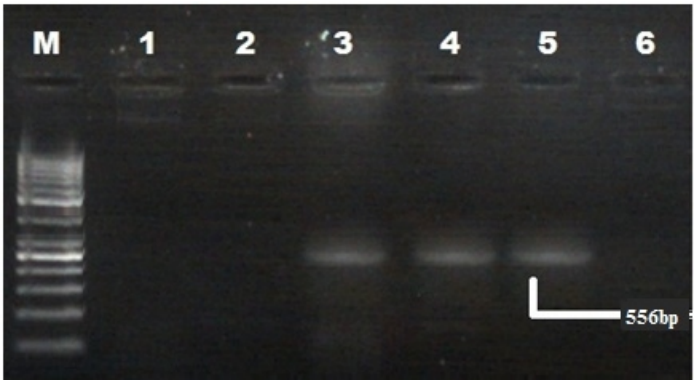

Fig. 1. Agarose gel electrophoresis photograph of PCR amplified product of *C. parvum* in environmental water samples. **[**M: Gene marker 50 bp, Lane 1 and 2: show negative samples.
Lane 3 and 4: Positive samples showing 556bp bands, Lane 5: Positive control.
Lane 6: Negative control.]

RESULTS AND DISCUSSION

Cryptosporidium is considered clinically significant human pathogen. In the last 20 years *Cryptosporidium* gained a lot of attention being a coccidian protozoan parasite (Keusch *et al.,* 1995). *Cryptosporidium* is considered cause of childhood diarrheal cases about 20% in developing countries and is a potentially fatal complication of AIDS (Karanis *et al.,* 2007). The methodology was the same as used by other people (Akbar *et al.,* 2014; Ayaz *et al.,* 2013; Alam *et al.,* 2013; Ayaz *et al.,* 2011) along with few differences. In the current study detection of zoonotic parasite *C. parvum* in different water sources of Tehsil Takhte Nasrati District Karak were Identified through PCR.

Table 1: Area wise prevalence of *C. parvum* in different water sources of Tehsil Takhte Nasrati District Karak.

Location(n)	**Pond Water Positive/total (%)**	**Dam Water Positive/total (%)**	**Well Water Positive/total (%)**	**Spring Water Positive/total (%)**
TakhteNasrati City(25)	-	-	-	3/25 (12%)
Khadda Banda (25)	-	-	-	1/25 (4%)
Siraj Khel(25)	-	-	-	3/25 (12%)
Serki Nasrati(75)	-	11/75 (14.7%)	-	-
Mianki Banda(25)	11/25 (44%)	-	-	-
Jehangeri Banda(25)	5/25 (20%)	-	-	-
Inzer Banda(25)	2/25 (8%)	-	-	-
Alwar Banda(25)	-	-	0/25 (0%)	-
Zeera banda(25)	-	-	1/25 (4%)	-
Shnawa GudiKhe(25)	-	-	1/25 (4%)	-

(-) is denoted for non-collected samples (%) = Percentage, n=total number, P =.05, significant

The overall prevalence for *C. parvum* in different sources of water is 12.7% (38/300). The prevalence of *C. parvum* in pond water is 18/75 (24%), in dam water 11/75 (14.7%), similarly in well water sources 2/75 (2.7%) and in spring water 7/75 (9.4%) (Table 1). The overall prevalence of *C. parvum* in different areas of Tehsil Takthe Nasrati District Karak was 38/300 (12.7%), among them the prevalence of *C. parvum* in Takhte Nasrati city was 3/25 (12%) and in Khadda Banda was 1/25 (4%), in Siraj Khail Gerang 3/25 (12%), In Serki Nasrati 11/75 (14.7%), In Alwar Banda 0/25 (0%), In Zeera bBanda 1/25 (4%), in Shnawa Gudi Khel 1/25 (4%), in Mianki Banda 11/25 (44%), in Jehangeri 5/25 (20%), in Inzer Banda 2/25 (8%) (Table 1).

As compared to other area of Khyber Pakhtunkhwa (Ayaz *et al.,* 2013; Alam *et al.,* 2013; Ayaz *et al.,* 2011) who conducted the similar study in the different area of the province our findings were slightly different. In Kohat the prevalence rate of *C. parvum* was 6.66% (10/150) (Ayaz *et al.,* 2013). While in Different Water Sources of District Bannu, Khyber Pakhtunkhwa the overall prevalence of *C. parvum* was 16% (Alam *et al.,* 2013) which was also slightly different from our results. The reasons may be different, the life style of the people, the area, sample size and the source of contaminations like the cattle which are living near the water sources in the study area. The similar study was also conducted in three different districts of Khyber Pakhtunkhwa, which were Kohat, Karak and Hangu. The overall prevalence rate of *C. parvum* was 19.5% (Ayaz *et al.,* 2011).

In the current study the overall prevalence of *Cryptosporidium* in different sources of water was 12.7% (38/300). The overall prevalence of *C. parvum* source wise in different areas of Tehsil Takhte Nasrati District Karak is like that in Pond water of the village Mianki Band is 11/25 (44%), in Village Jehangeri 5/25 (20%), in Inzer Banda 2/25 (8%). The prevalence of *Giardia* in Dam water was 11/75 (14.7%) in Serki Nasrati. *Giardia* prevalence shows in Well water sources was 0/25 (0%) in Alwar Banda, 1/25 (4%) in Zeera Banda, in Shnawa Gudi Khel 1/25 (4%). Similarly *C. parvum* prevalence in spring water was 3/25 (12%) in the Tehsil Takhte Nasrati city, 1/25 (4%) in Khadda Banda and 3/25 (12%) in Siraj Khel.

In the current study *C. parvum* was identified by using PCR. A similar study was conducted in Russia and Bulgaria for the detection *C. parvum in* drinking water samples of different origin were collected from Rostov (southern Russia), Sofia and Varna (Bulgaria). 18.1% samples were positive for *C. parvum*. The parasite was detected from tap, river, well and waste water (Nannini *et al.,* 2002). In similar studies C. *parvum* was reported by different scholars in several parts of the world. For instance *Cryptosporidium* were detected in different water sources of Lege Dini (Ethiopia). The total prevalence of different parasites was 47.5% in which *Cryptosporidium* prevalence was 12.2% (Nannini *et al.,* 2002). 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* species. 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 species* (Appelbee *et al.,* 2005).

Table 2: Prevalence of *C. parvum* in different water sources.

Serial No.	Water Source	Total Samples	Positive	%Prevalence	P Value
1	**Dam water**	75	11	14.7	
2	**Pond water**	75	18	24	
3	**Well water**	75	2	2.7	0.0000
4	**Spring water**	75	7	9.4	
	Grand Total	300	38	12.7	

In our finding the most prevalence rate of *C. Pravaum* was observed in Pond water (24%) followed by Dam water (14.7%) and Spring water (9.4%). While the lowest prevalence rate of *C. Pravaum* was obsereved in well water (2.7%) (Table 2). The protozoan parasite was found in a reservoir, pond and river/canal water (Appelbee *et al.,* 2005). While in the present study the prevalence for *C. parvum* is 12.7%. The differences may be due to the sample size, mode of life of the people as well as of the animals like cattle, which live near the water sources (ponds, dams and springs) can contaminate the sources of water and help in spreading of the parasite. The present results may help the people for their health in prevention and supervision for cryptosporidiosis.

CONCLUSION

From the above study it was concluded that all the water sources in the given areas were contaminated due to *C. parvum* especially the pond water. The lack of authorities' attention to such problems, the unimportance of protecting drinking water sources from contamination and use of better water treatment to avoid infections in these areas was found in findings and conclusions.

It was recommended for the above study that the people should be aware about the *C. parvum* infection. Usage of Clean and boiled water is very useful to prevent the infection of this parasite. The competent authorities should give proper attention to protecting the different water sources from contamination.

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COMPETING INTEREST

The authors have declared that they have no competing interests exist.

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