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Investigation on *Cryptosporidium* spp. in water samples collected from River Melet in Ordu by Loop Mediated Isothermal Amplification (LAMP)

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Abstract
Cryptosporidium spp. were identified in water samples collected from River Melet in Ordu Province by Loop Mediated Isothermal Amplification (LAMP). The water samples are regularly taken every month from River Melet where mix the sea point (M-1), near solid waste storage area (M-2), Ordu-Giresun on the highway bridge location (M-3), near the city's industry (M-4) and in the city out (M-5) from December, 2010 to November, 2011. 60 water samples were flocculated by Aluminum Sulfate and they were used for the counting of *Cryptosporidium* sp. oocysts by Modified Acid-Fast (MAF). The highest number of samples were purified by sucrose-gradient and DNA were extracted for LAMP assay. The number of oocycsts decreased in the form of M-3, M-4, M-2, M-1 ve M-5, respectively in five stations of River Melet. The six water samples from per site, which is the highest numbers of oocysts by MAF, were analyzed by LAMP and they were found positive. Ordu has rich water resources, but, the availability of them have been reduced because of the waste waters discharge of river and sea directly without processing. Parasites move to rivers, lakes and streams by rainfall in this area. Thereby, the drinking, agriculture and recreational use of water is important to identify potential risk factors for the protection of the public health.

Keywords: *Cryptosporidium*, LAMP, River Melet

INTRODUCTION

Cryptosporidium spp. and *Giardia duodenalis* are important causes of protozoan waterborne diseases. These parasites are transmitted by both zoonotic and anthroponotic cycles and they are cause of widespread gastrointestinal illnesses. These pathogenic protozoan are transmitted through contaminated water and food and ninety percent of the reported outbreaks of these protozoan come through water and ten percent of are through food [1,2,3,4]

LAMP assay have been developed for DNA amplification and there is no need of heat denaturation of double-stranded DNA products to initiate as the polymerase chain reaction (PCR) [5]. It is possible to have a great number of copies from the targeted DNA in a short period in constant temperature without the contribution of technical skills and professional equipment [6]. There are numerous reports regarding as the successful use of LAMP in the biomedical field including the detection of viruses, bacteria, fungi and parasites [7,8]. LAMP is a recently developed technology for the diagnosis of parasites especially *Cryptosporidium* spp. [4,9].

In this study we investigated *Cryptosporidium spp.* in water samples by LAMP from River Melet of Ordu Province of Turkey in the Black Sea area. The LAMP method is useful for the detection of waterborne cryptosporidiosis outbreaks.

MATERIALS AND METHODS

Sampling Sites

All samples were obtained in the period between December, 2010 to November 2011. Five sampling sites in River Melet of Ordu were selected for detection of *Cryptosporidium* spp. The site 1 was River Melet where mix the sea point (M-1); The site 2 was River Melet near solid waste storage area (M-2), The site 3 was Ordu-Giresun on the highway bridge location (M-3), The site 4 was River Melet near the city's industry (M-4); The site 5 was River Melet in the city out (M-5) (Fig 1).

River Melet is a natural boundary between the Middle Black Sea and the Eastern Black Sea region. All canyon of River Melet is rich with various streams. Due to the rapid change in the direction of East of Ordu, River Melet is now discharging through the city to the sea. The rivers of Ordu which is taking resources from the mountains parallel to the beach reaches the shore from the deep and steep valley. Because of the geological structure of the provincial land, It give rise to a lot of erosion. Melet River area features has a typical Black Sea climate. Thereby, summers are hot, winters are relatively mild, cold and rainy in all seasons.

Water sample collection and Microscopic detection

Water samples were collected as previously described by [4]. Briefly, 10 L of per samples were flocculate with

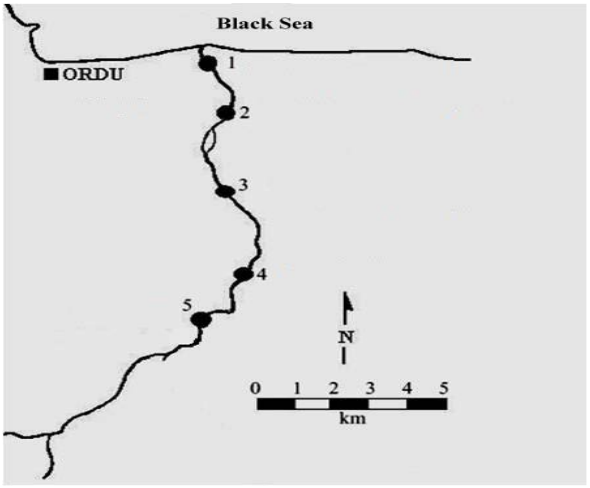

Figure 1. The map of sampling sites.

M-1: River Melet where mix the sea point; M-2: River Melet near solid waste storage area; M-3: River Melet in Ordu-Giresun on the highway bridge location; M-4: River Melet near the city's industry; M-5: River Melet in the city out.

$Al_2(SO_4)_3$ and then concentrated by sucrose-gradient and centrifugation. The wet preparations in water pellet smear were prepared and examined for oocysts by Acid-Fast staining method as described by [6].

DNA Extraction

Genomic DNA was extracted from the sucrose pellets by the QIAamp DNA Mini Kit (Qiagen, Germany) by modifiying with following the addition of 15 freeze-thaw cycles as prior to described [4,10]. DNA was eluted in 50 µL TE buffer and kept at -20°C until used in LAMP reactions.

LAMP assays for Cryptosporidium

LAMP assay was performed in water samples to [9] and [11] targeting the SAM gene. Briefly, LAMP were set up in a final volume of 25 µL containing 12.5 µL 2X reaction buffer (40 mM Tris–HCl, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M betaine, 2.8 mM each deoxynucleoside triphosphate), 8 U *Bst* DNA polymerase (New England Biolabs, Japan), 1.3 µL primer mixture (40 pmol each of the FIP and BIP primers, 20 pmol each of the LF and LB primers, and 5 pmol each of the F3 and B3 primers), 2 µL DNA and 8.2 µL distilled water. The samples were left at 63°C for 60 min and then heated at 85°C for 5 min to final the reaction. The LAMP products were analyzed by agarose gel electrophoresis and visualized under UV light after ethidium bromide staining as mentioned before by [4].

LAMP in spiked water pellets

10 oocysts from the stock solution were added in 10% aliquots of 2 tap and 7 river water concentrated sample pellets. After the DNA extraction, LAMP have been applied for all 9 spiked sediments as demonstrated in Fig 2.

Sensitivity of the LAMP Assay

The sensitivity of the LAMP assay was evaluated with genomic DNA of *C. parvum* (Iowa) control sample. The diluted to known amount of DNA (a range of 10 ng to 100 fg) were used for the sensitivity of LAMP.

Statistical analysis

Descriptive data analyses were done using microsoft excel and hypothesis testing using SPSSv.18. Statistical analysis was performed using one-way analysis of variance (ANOVA). The comparisons between groups were done using a post hoc test, Tukey test. The odds ratio, 95% confidence interval and $P < 0.05$ value was statistically significant or nearly statistically significant (i.e. near a value of 0.05). In addition, a multiple comparison regression model that included all variables was calculated [12,13,14].

RESULTS

Occurence of Cryptosporidium spp. by MAF

A total of 60 river water samples collected from River Melet of Ordu city were examined by MAF. *Cryptosporidium* sp.oocysts were detected in 60 samples from River Melet of Ordu city throughout the year. The results from the prevalence of *Cryptosporidium* oocysts in the river water samples collected December, 2010 to November, 2011 were demonstrated in Table 1. Of the 12 river samples collected at site 1 from River Melet, river samples taken from March was highest number with 38 *Cryptosporidium* sp. oocysts per 0.5 L. River samples from site 2 of Melet were highly positive with 51 *Cryptosporidium* sp. oocysts per 0.5 L in March. At site 3 in River Melet, the maximum presence of *Cryptosporidium* spp. were detected with 50 oocysts per 0.5 L in February. The highly positive river water samples for the occurrence of *Cryptosporidium* spp. were found in site 4 of River Melet with 53 oocysts per 0.5 L in March. The river samples from site 5 of Melet were found positive with 27 *Cryptosporidium* sp. oocysts per 0.5 L in April.

Table 1. The number of *Cryptosporidium* sp. oocysts by MAF in water samples from River Melet of Ordu

Investigation Time			Sampling Sites		
	M-1	**M-2**	**M-3**	**M-4**	**M-5**
	10	**13**	20	14	2
	22	**11**	**39**	**32**	3
	19	8	**50**	**36**	4
December, 2010-November, 2011	**38**	**51**	**24**	**53**	**23**
	22	**37**	**47**	12	**27**
	19	11	7	9	4
	3	1	**21**	**13**	5
	2	1	3	12	**9**
	3	**21**	**35**	11	**8**
	9	**14**	13	**21**	**19**
	14	12	10	**52**	**9**
	3	5	6	7	5
Total	164	185	275	272	118

Molecular detection of Cryptosporidium spp.

The selected pellets of 9 (2 tap and 7 river water samples) spiked water samples were tested by LAMP as positive controls. LAMP were detected (100%) for *Cryptosporidium* DNA in all selected samples (Fig 2).

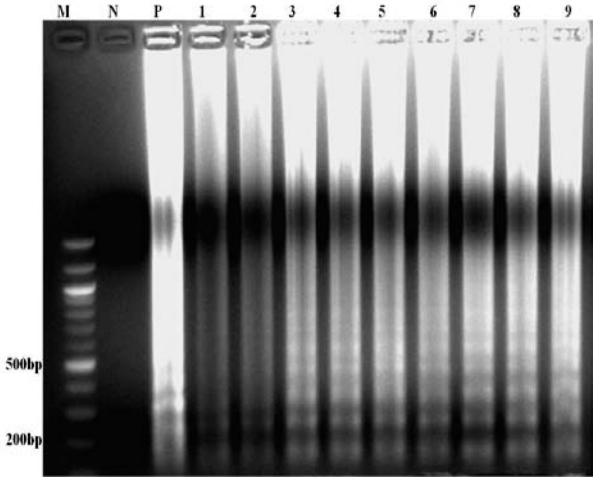

Figure 2. Detection of *Cryptosporidium* spp. by SAM-1 LAMP in spiked water pellets. M: 100 bp ladder

N: negative control (distilled water); P: positive control (*C. parvum* Iowa DNA); lanes 1-4: spiked tap water; lanes 5-9: spiked river water samples.

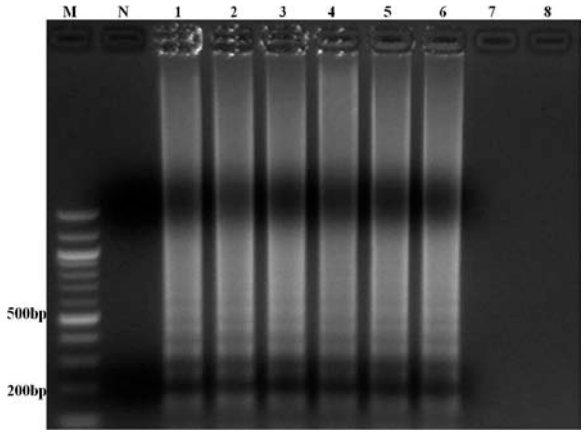

Figure 3. Sensitivity of the LAMP. M: 100 bp ladder
N: negative control (distilled water); lane 1: 10 ng; lane 2:1ng; lane 3: 100 pg; lane 4:10 pg; lane 5: 1 pg; lane 6: 100 fg; lane 7:10 fg.

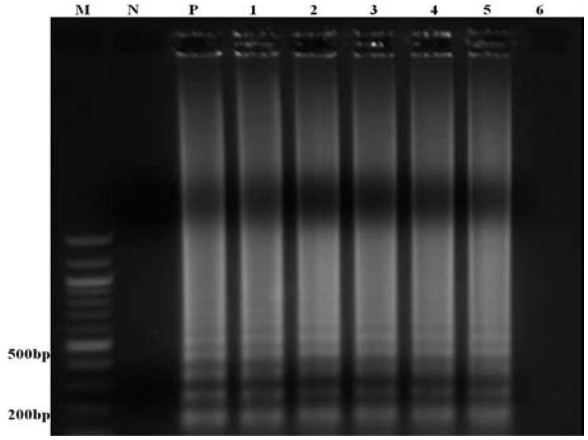

Figure 4. *Cryptosporidium* spp. in selected highly positive samples from River Melet of Ordu by LAMP

M: 100 bp ladder; N: negative control (distilled water); P: positive control (*C. parvum* Iowa DNA); lane 1: M-1; lane2: M-2; lane 3: M-3; lane 4: M-4; lane 5: M-5;

As previously explained by [4], the serial dilutions of *C. parvum* DNA (Iowa) were tested for sensitivity test of LAMP. Briefly, the detection limit for the SAM-1 LAMP was found to be 100 fg, while was 1 pg by nested PCR assays (Fig 3).

All six samples, which illustrated the highest number of *Cryptosporidium* sp. oocysts, from all sites of River Melet were used for LAMP assays. *Cryptosporidium* DNA were detected in all selected river water samples from River Melet by SAM-1 LAMP assay (Fig 4).

The highest numbers of *Cryptosporidium* spp. were found in all of the river water samples at site 1, 2, 4 and 5 in spring than the other seasons. But for site 3, winter had more rainy than the others. The river water contamination with *Cryptosporidium* sp. oocysts in spring was typically peaked during the March at site 1, 2 and 4 and April for site 5. River samples from site 3 were highly positive in February than the other months of winter (Fig 5 and 6).

In our study, when we checked total rainfall (mm) in Ordu, winter was more rainy than the other seasons. That is why we did not find any correlation between the number of oocysts and season as well. The average monthly rainfall and temperature in Ordu were illustrated in (Fig 7).

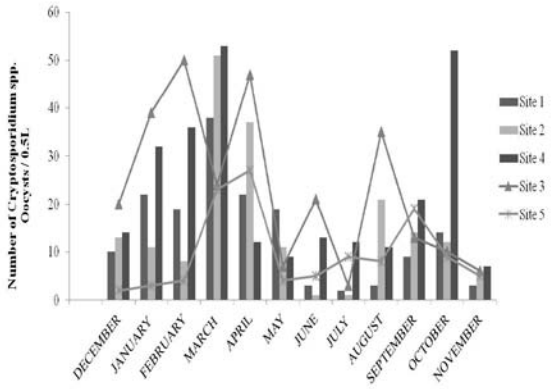

Figure 5. The numbers of *Cryptosporidium* sp.oocysts in River Melet of Ordu in each of the months.

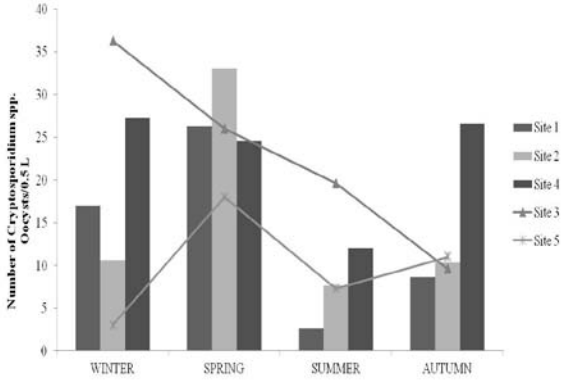

Figure 6. The numbers of *Cryptosporidium* sp.oocysts in River Melet of Ordu in each of the seasons.

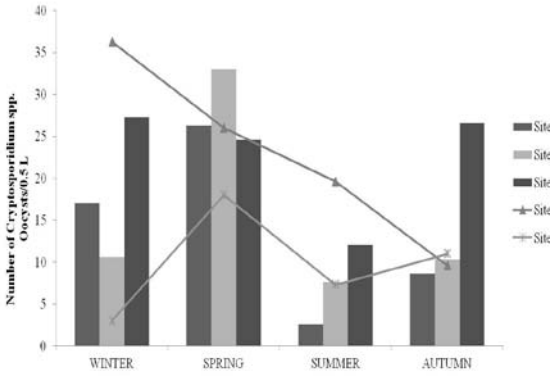

Figure 7. The average monthly rainfall and temperature in Ordu

The similar seasonal patterns were presented in previously study by finding the number of *Cryptosporidium* spp. and *G. duedonalis* in all of the wastewater more frequent in spring and summer than in the other seasons as described by [15,16,17,18,19,20]. But unlike summer, we found the number of *Cryptosporidium* more frequent in spring than in the other seasons.

The contamination of *Cryptosporidium* spp. are not just depending on seasonality. Fertilization of pastures with animal manure, lambing, cattle farming, calving could be reason for contamination as it was demonstrated by [20] and in addition agricultural practices and dysfunction of sewage treatment plants on study time might be reason for contamination as described by [21].

The results of unvariate analysis showed that difference in probability of failure with months between March and December, May, June, July, November was a significant (Table 2). The number of *Cryptosporidium* spp. oocysts per 0.5 L at site 1 are just as likely to be contaminated as site 2, 3, 4 and 5 in River Melet of Ordu. The similar results were obtained between autumn and the other seasons. Thereby, there was no any a significant variables for the all seasons (Table 3 and 4).

Molecular methods must to be used to determine the distinctions between the species of some protozoan instead of classical methods which are not adequate for the identification as described by [6]. In recent years, LAMP has frequently been used to detect waterborne protozoan by illustrating the advantages of this method in several publication [4,11].

The SAM-1 LAMP assay which is targeting sadenosyl-methionine synthetase (SAM) gene were used for detection of three Cryptosporidium species: *C. parvum*, *Cryptosporidium hominis* and *Cryptosporidium meleagridis* according to [11]. In our study, we performed SAM-1 LAMP assay to present the levels of contamination by *Cryptosporidium* in river water samples as well.

As previously reported by [14], the occurence of *Cryptosporidium* spp. in sea and drinking water samples from the Sinop Province of Black Sea in Turkey by MAF. According to autors this data can just be able to support the molecular results. They have to use molecular analysis in order to identify whether the parasites asset in this samples are human pathogenic or not. In our study, we presented similarly that how common *Cryptosporidium* spp. are in this area by MAF as a classical method. Subsequently, we selected highly contaminated six samples from per site to determine the species of these parasites by LAMP. All of the samples from investigated area were found positive by LAMP. In addition, in here, the spiked samples were used as internal positive controls in LAMP assay and LAMP was positive (100%) for *Cryptosporidium* DNA in 9 spiked water samples pellets.

CONCLUSION

Cryptosporidium spp. were identified in water samples collected from River Melet in Ordu Province by molecular method. It will help our understanding of the levels of contamination by *Cryptosporidium* in the investigated areas. This contamination should be followed to standardize river water from the study areas at least as a seasonal to protect

Table 2. Multiple comparisons between months for *Cryptosporidium* sp. oocysts detection in water samples from River Melet of Ordu

Measured *Cryptosporidium* spp. oocysts			
Investigation months		**(95% Confidence interval)**	**p value***
March	December	**26.0*** (0.39-51.60);	p=0.044
	January	16.4 (9.20- 42.00);	p=0.558
	February	14.4 (11.20-40.00);	p=0.734
	April	8.8 (16.80-34.40);	p=0.988
	May	**27.8*** (2.19-53.40);	p=0.023
	June	**29.2*** (3.59-54.80);	p=0.013
	July	**32.4*** (6.79-58.00);	p=0.004
	August	22.2 (3.40-47.80);	p=0.147
	September	22.6 (3.00-48.20);	p=0.131
	October	18.4 (7.20-44.00);	p=0.384
	November	**32.6*** (6.99-58.20);	p=0.003

public h ealth. It i s r ecommended t hat the m unicipalities should build advanced purified sewage systems o r sewage discharge to remote locations in the drinking water sources, since *Cryptosporidium* spp. transmitted by fecal-oral route.

Table 3. Multiple c omparisons be tween s ites for *Cryptosporidium* sp. o ocysts detection in w ater s amples from River Melet of Ordu

Number of *Cryptosporidium* spp. oocysts/0.5L in River Melet of Ordu		
Seasons		***Cryptosporium* spp.** **(max-min) p value ***
Autumn	Winter	5.06 (29.01-17.81); p=0.868
	Spring	12.33 (35.74-11.08); p=0.389
	Summer	3.04 (20.01-26.81); p=0.965

Table 4. Multiple c omparisons be tween s easons for *Cryptosporidium* sp. o ocysts detection in w ater s amples from River Melet of Ordu

Number of *Cryptosporidium* spp. oocysts/0.5L in River Melet of Ordu			
Study Sites		***Cryptosporium* spp.** **(max-min)**	**p value ***
Site 1	Site 2	1.75 (17.60- 4.10);	p=0.998
	Site 3	9.25 (25.10- 6.60);	p=0.476
	Site 4	9.00 (24.85- 6.85);	p=0.503
	Site 5	3.83 (12.01-19.68);	p=0.960

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