# Evaluation of immunomagnetic separation and the sucrose flotation methods coupled with immunofluorescence or PCR for detection of Cryptosporidium and Giardia (oo)cysts in water samples

Mohammad Reza Mahmoudi[1*], Asad Mirzaei[2]

1. Department of Parasitology, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran
2. Dapartment of Parasitology, Faculty of Medicine, Ilam University of medical sciences, Ilam, Iran

## Abstract

**Introduction:** Detection of Cryptosporidium and Giardia parasites in water samples is usually performed by US Environmental Protection Agency 1623 method. Nevertheless, the USEPA1623 method still need improvement, to prevent and control the water borne parasitic disease. Therefore, we undertook the present study.

**Materials and methods:** Totally 48 surface water samples were collected. Four samples from 12 sites and samples of each site were evaluated by IMS-IFA, SF-IFA, IMS-PCR and SF-PCR. These typically involve sample filtration by membrane filter, separation by Sucrose flotation or immunomagnetic separation (IMS) methods and detection of (oo)cysts by PCR or immunofluorescent staining.

**Results:** Same samples were evaluated by the different techniques at the same time showing a rate of Cryptosporidium oocysts detection of 8 (66.6%) by IMS-IFA, 7 (58.3%) by SF-IFA, 10 (83%) by IMS-PCR and 0% by SF-PCR. Giardia cysts detected in, 5 (41.7%) by IMS-IFA, 3(25%) by SF-IFA, 7 (58.4%) by IMS-PCR and 2 (17%) by SF-PCR.

**Conclusion:** Data analysis showed a higher sensitivity of IMS-PCR for the detection of Giardia and Cryptosporidium (oo)cysts respectively in comparison with others techniques used in this study. IMS prior to DNA extraction showed a higher sensitivity to eliminate or reduce PCR inhibitors that presence in water samples.

**Keywords:** Giardia, Cryptosporidium, IMS, Flotation methods, PCR, IFA, Water

## Introduction

Immunological and molecular methods used to assess the prevalence and sources of waterborne protozoa. The recovery yield of Cryptosporidium and Giardia (oo)cysts from water depends on the purification and identification methods used. Recently, immunomagnetic separation (IMS) purification method recommended by US Environmental Protection Agency  (US-EPA) is widely used, but this method still has some limitations and need improvement (1-4).

Although IMS are standard procedures for purification of Cryptosporidium oocysts and Giardia cysts in water samples but it has some limits. Apart from IMS for purifying oocysts from water samples, some other methods, such as sucrose floatation and Percoll-sucrose centrifugation were also applied (5-7).

Plus purification methods, the recovery yield of Cryptosporidium and Giardia (oo)cysts from water depends on the detection methods too.

Nested PCR appears to be more sensitive than IFA for detecting of oocysts in water concentrates (4, 8, 9). Compared to method 1622/1623, the PCR methods have the ability to differentiate Cryptosporidium species that are infective to humans from those that are not infective to humans (10). But, PCR inhibitors present in water samples are major problem in the molecular detection of microorganisms in environmental samples (11-15).

The presence of Giardia and Cryptosporidium in different water sources in Iran makes it imperative to develop Standard methods to maximise public health surveillance of waterborne protozoa like Giardia and Cryptosporidium .

To authors Knowledge, no comparison of IMS or SF coupled with IFA or PCR for recovering the Cryptosporidium and Giardia (oo)cysts in same surface water samples was reported .

Therefore, in this paper, we compared the efficiencies of different methods for purification and detection of Cryptosporidium and Giardia (oo)cysts, in field river water samples.

## Materials and methods

Totally 48 surface water samples were collected from 12 sites. Four samples from each site that were evaluated by IMS-IFA, SF-IFA, IMS-PCR and SF-PCR. For each sample, five liters of environmental water samples from river water were filtered through a 142 mm diameter membrane filter with a pore size of 1.2 μm. The filter was rinsed two times by 50 ml of 0.1% PBS-Tween 80. Then, the entire sample was transferred into a 50-ml Falcon tube and concentrated by centrifugation in at 3000 g for 10 min. The supernatant was discarded, and the pellet with the (oo)cysts was subjected to different purification and detection methods. These typically involve separation by Sucrose flotation or immunomagnetic separation (IMS) methods and PCR or immunofluorescent staining, for detection of oocysts .

**Sucrose floatation & Immunomagnetic separation (IMS):** All samples were treated with sucrose flotation (SF) method according previous study (16) and IMS methods. All IMS kits do not perform equally well , since, great recoveries have been obtained previously with the Dynal IMS procedure (17) so, in present study this kit use for imounomagnetic procedure. IMS procedure was performed according to the manufacturer's instructions (Dynabeads G/C combo IMS kit; Dynal A.S., Oslo, Norway), as performed in our previous study (16).

**DNA extraction and PCR methods:** The DNA was extracted with the QIAamp DNA minikit as recommended Jiang et al. (2005) (15).

A nested–PCR was used to amplify a 825-bp fragment of Cryptosporidium oocyst 18s RNA (18). PCRs reaction were performed as described in our previously published paper (19). A Semi-nested PCR assay, using the primers to amplify a 432-bp fragment of the Giardia glutamate dehydrogenase gene (GDH) (20). The PCR reactions performed as described in our previously paper (16).

**IFA methods:** A previously published IFA protocol was performed to detect Cryptosporidium and Giardia (oo)cysts (21, 22). Cryptosporidium and Giardia (oo)cysts were identified on the basis of their size, shape, and structure, according to guideline described in method 1623.

## Results

Totally 25/42 samples were positive for Cryptosporidium oocysts and 17/25 samples for Giardia cysts. Same samples were evaluated by the different techniques at the same time showing a rate of Cryptosporidium oocysts detection of 8 (66.6%) by IMS-IFA, 7 (58.3%) by SF-IFA, 10 (83%) by IMS-PCR and 0% by SF-PCR.

Giardia cysts detected in, 5 (41.7%) by IMS-IFA, 3(25%) by SF-IFA, 7 (58.4%) by IMS-PCR and 2 (17%) by SF-PCR.

**Table 1.** Results of different purification and detection of (oo)cyst in water samples.

| | Giardia | | Cryptosporidium | |
|---|---|---|---|---|
| Methods | Negative | Positive | Negative | Positive |
| IMS-PCR | 5/12 (41.6%) | 7/12 (58.4%) | 2/12 (17%) | 10/12 (83%) |
| SF-PCR | 10/12 (83%) | 2/12 (17%) | 12/12 (100%) | 0/12 |
| IMS-IFA | 7/12 (58.3%) | 5/12 (41.7%) | 4/12 (33.4%) | 8/12 (66.6%) |
| SF-IFA | 9/12 (75%) | 3/12 (25%) | 5/12 (41.7%) | 7/12 (58.3%) |
| Total | 31/48 | 17/48 | 23/48 | 25/48 |

Data are shown as ratio or percent.

## Discussion

The recovery yield of Cryptosporidium and Giardia (oo)cysts from water depends on the purification and identification methods used. Purification by IMS, and detection by IFAUSEPA method 1623 has been widely used with recovery rates varying from 40.0 to 100% (23-26).

However for some organisms there are no IMS procedures, and IFA detection method is unable in identification of Cryptosporidium or Giardia species.

Therefore some others purification methods, such as sucrose floatation and Percoll-sucrose centrifugation (5-7) and detection methods like PCR (4, 8, 9) were also applied.

In the present study, we found that IMS method enhanced with PCR assay (IMS-PCR) showed slightly higher positive results than IMS-FA, SF-IFA and SF-PCR. Also others studies have shown, that nested PCR is more sensitive than microscopy (4, 8, 9).

As shown in this study, mainly DNA extracted from (oo)cysts purified by IMS from water samples produced the most PCR amplification and SF-PCR gives less positive results than IMS-PCR so PCR inhibitors were more present in oocyst purified by SF method.

Although, our investigations showed that IMS appears to be more sensitive than flotation procedures but that not all IMS procedures yield the same results (27).

However, IMS had some advantages, such as rapidity in processing and less personnel skill required than sucrose floatation technique.

Although our data show, sucrose floatation technique gives less positive results than IMS, but SF was cost-effective and easier to perform as the IMS technique so sucrose floatation is an alternative way when IMS method is not suitable. As in a study, Koompapong et al 2009 suggest using SF-IFA technique for detecting oocysts in water samples especially in water with high turbidity, low or high pH, and high iron particle in water samples. (28). In present study, SF technique enhanced with FA give more positive results than IMS-IFA and SF-PCR methods that may due PCR inhibitor because SF method couldn't eliminate or considerably reduce substances that might be inhibitory to DNA amplification by PCR.

As mention above, Plus purification methods, the recovery yield of Cryptosporidium and Giardia (oo)cysts from water depends on the identification methods too.

In IFA detection method, some object cross react with commercial antibodies resulting false positives (29). IFA cannot differentiate Cryptosporidium species or strains from humans and animals (10). Although PCR has some advantages over IFA but PCR is susceptible to many inhibitors present in samples (11-15). Also empty oocysts cannot be detected by PCR methods, So IFA adds significant value to PCR-negative results (30, 31).

As mention above, must keep in mind, each method has each own advantages and disadvantages, so dependent to aim and the design of the study, a combination of techniques should be used to make sure that water samples is or is not contaminated and infectious. High

efficiency, reasonable cost and Aim of study are important items in the selection of the method. However, each of these methods has some limits thus, the

development of other purification and detection technique is necessary for the assessments of these waterborne pathogens in environmental samples.

## References

1. Feng YY, Ong SL, Hu JY, Song LF, Tan XL, Ng WJ. Effect of particles on the recovery of cryptosporidium oocysts from source water samples of various turbidities. Appl Environ Microbiol. 2003;69(4):1898-903.
2. Kuczynska E, Boyer DG, Shelton DR. Comparison of immunofluorescence assay and immunomagnetic electrochemiluminescence in detection of Cryptosporidium parvum oocysts in karst water samples. J Microbiol Methods. 2003;53(1):17-26.
3. Fujino T, Matsuo T, Okada M, Matsui T. Detection of a small number of Cryptosporidium parvum oocysts by sugar flotation and sugar centrifugation methods. J Vet Med Sci. 2006;68(11):1191-3.
4. LeChevallier MW, Di Giovanni GD, Clancy JL, Bukhari Z, Bukhari S, Rosen JS, et al. Comparison of method 1623 and cell culture-PCR for detection of Cryptosporidium spp. in source waters. Appl Environ Microbiol. 2003;69(2):971-9.
5. Chesnot T, Schwartzbrod J. Quantitative and qualitative comparison of density-based purification methods for detection of Cryptosporidium oocysts in turbid environmental matrices. J Microbiol Methods. 2004;58(3):375-86.
6. Bonadonna L, Briancesco R, Ottaviani M, Veschetti E. Occurrence of Cryptosporidium oocysts in sewage effluents and correlation with microbial, chemical and physical water variables. Environ Monit Assess. 2002;75(3):241-52.
7. Muchiri JM, Ascolillo L, Mugambi M, Mutwiri T, Ward HD, Naumova EN, et al. Seasonality of Cryptosporidium oocyst detection in surface waters of Meru, Kenya as determined by two isolation methods followed by PCR. J Water Health. 2009;7(1):67-75.
8. Nichols RA, Campbell BM, Smith HV. Identification of Cryptosporidium spp. oocysts in United Kingdom noncarbonated natural mineral waters and drinking waters by using a modified nested PCR-restriction fragment length polymorphism assay. Appl Environ Microbiol. 2003;69(7):4183-9.
9. Hanninen ML, Horman A, Rimhanen-Finne R, Vahtera H, Malmberg S, Herve S, et al. Monitoring of Cryptosporidium and Giardia in the Vantaa river basin, southern Finland. Int J Hyg Environ Health. 2005;208(3):163-71.
10. Xiao L, Alderisio KA, Jiang J. Detection of Cryptosporidium oocysts in water: effect of the number of samples and analytic replicates on test results. Appl Environ Microbiol. 2006;72(9):5942-7.
11. Monis PT, Saint CP. Development of a nested-PCR assay for the detection of Cryptosporidium parvum in finished water. Water Res. 2001;35(7):1641-8.
12. Sluter SD, Tzipori S, Widmer G. Parameters affecting polymerase chain reaction detection of waterborne Cryptosporidium parvum oocysts. Appl Microbiol Biotechnol. 1997;48(3):325-30.
13. Stinear T, Matusan A, Hines K, Sandery M. Detection of a single viable Cryptosporidium parvum oocyst in environmental water concentrates by reverse transcription-PCR. Appl Environ Microbiol. 1996;62(9):3385-90.
14. Widmer G. Genetic heterogeneity and PCR detection of Cryptosporidium

parvum. Adv Parasitol. 1998;40:223-39.

15. Jiang J, Alderisio KA, Singh A, Xiao L. Development of procedures for direct extraction of Cryptosporidium DNA from water concentrates and for relief of PCR inhibitors. Appl Environ Microbiol. 2005;71(3):1135-41.

16. Mahmoudi MR, Kazemi B, Mohammadiha A, Mirzaei A, Karanis P. Detection of Cryptosporidium and Giardia (oo)cysts by IFA, PCR and LAMP in surface water from Rasht, Iran. Trans R Soc Trop Med Hyg. 2013;107(8):511-7.

17. Campbell AT, Grøn B, Johnson SE. Immunomagnetic separation of Cryptosporidium oocysts from high turbidity water sample concentrates. In: Fricker C R, Clancy J L, Rochelle P A, editors; Fricker C R, Clancy J L, Rochelle P A, editors. International symposium on waterborne Cryptosporidium. Denver, Colo: American Water Works Association; 1997. pp. 91–96. 1997.

18. Xiao L, Alderisio K, al. LJe. Identification of species and sources of Cryptosporidium oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. Appl Environ Microbiol. 2000;66(12):5492-8.

19. Mahmoudi MR, Bahram Kazemi B, Mohammadiha A, Mirzaei A, Karanis P. Detection of Cryptosporidium and Giardia (oo)cysts by IFA, PCR and LAMP in surface water from Rasht, Iran. Trans R Soc Trop Med Hyg. 2013;107(8):511-7.

20. Read CM, Monis PT, RC. T. Discrimination of all genotypes of Giardia duodenalis at the glutamate dehydrogenase locus using PCR-RFLP. Infect Genet Evol. 2004;4(2):125-30.

21. Mahmoudi M. R, Kazemi B, Mohammadiha A, Mirzaei A, Karanis P. Detection of Cryptosporidium and Giardia (oo)cysts by IFA, PCR and LAMP in surface water from Rasht, Iran. Trans R Soc Trop Med Hyg. 2013;107(8):511-7.

22. Mahmoudi M, Ashrafi K, Abedinzadeh H, Tahvildar-Bideruni F, Haghighi A, Bandehpour M, et al. Development of sensitive detection of cryptosporidium and giardia from surface water in iran. Iran J Parasitol. 2011;6(3):43-51.

23. Miller WA, Lewis DJ, Pereira MD, Lennox M, Conrad PA, Tate KW, et al. Farm factors associated with reducing Cryptosporidium loading in storm runoff from dairies. J Environ Qual. 2008;37(5):1875-82.

24. Iacovski RB, Barardi CR, Simoes CM. Detection and enumeration of Cryptosporidium sp. oocysts in sewage sludge samples from the city of Florianopolis (Brazil) by using immunomagnetic separation combined with indirect immunofluorescence assay. Waste Manag Res. 2004;22(3):171-6.

25. Downey AS, Graczyk TK. Maximizing recovery and detection of Cryptosporidium parvum oocysts from spiked eastern oyster (Crassostrea virginica) tissue samples. Appl Environ Microbiol. 2007;73(21):6910-5.

26. Ware MW, Wymer L, Lindquist HD, Schaefer FW, 3rd. Evaluation of an alternative IMS dissociation procedure for use with Method 1622: detection of Cryptosporidium in water. J Microbiol Methods. 2003;55(3):575-83.

27. Bukhari Z, McCuin RM, Fricker CR, Clancy JL. Immunomagnetic separation of Cryptosporidium parvum from source water samples of various turbidities. Appl Environ Microbiol. 1998;64(11):4495-9.

28. Koompapong K, Sutthikornchai C, Sukthana Y. Cryptosporidium oocyst detection in water samples: floatation technique enhanced with immunofluorescence is as effective as immunomagnetic separation method. Korean J Parasitol. 2009;47(4):353-7.

29. Rodgers MR, Flanigan DJ, Jakubowski W. Identification of algae which interfere with the detection of Giardia cysts and Cryptosporidium oocysts and a method for alleviating this interference. Appl Environ Microbiol. 1995;61(10):3759-63.

30. Smith HV, Nichols RA. Cryptosporidium: Detection in water and food. Exp Parasitol. 2010.

31. Karanis P, Sotiriadou I, Kartashev V, Kourenti C, Tsvetkova N, Stojanova K. Occurrence of Giardia and Cryptosporidium in water supplies of Russia and Bulgaria. Environ Res 2006;102:260-71.