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An Attempt to Devise a Cost-effective Method to Detect *Oocysts* of *Cryptosporidium spp* from Commercially Retailed Coriander Leaves: A Pilot Study

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ABSTRACT

This study aimed at assessing the prevalence of *Cryptosporidium oocysts* in Coriander leaves (*Coriandrum sativum)* sold in open aired markets in Mumbai. In the first phase of the study, an attempt was made to devise a method to detect the oocysts cost effectively. After standardization, the recovery efficiency of oocysts from spiked coriander leaf rinsate was found to be 34%. In the second phase, this standardized method was used for screening commercial coriander samples. Results of the pilot study on marketed coriander leaves showed 8/74 (11%) samples harbouring oocysts of *Cryptosporidium spp* at a mean concentration of 16.66 ± 6.23 oocysts/10-gram coriander, which is notably high and needs to be further investigated using a larger sample size from different locations from food safety point of view.

Keywords *Cryptosporidium, coriander leaves, oocyst, filtration, pilot.*

About 3-5 billion people suffer from infectious diarrhea globally by consuming contaminated food and water and about 1.5 million succumb to it (NCDC, GOI 2017). However, very few of these cases are reported making it all the more challenging to determine the exact causative agent. Although the food safety and standards authority of India (FSSAI) has implemented various regulations to ensure food safety, non bacterial pathogens are seldom screened in foods. As of today, there are very few in-depth parasitological screenings undertaken in India, the latest being a comprehensive study conducted in Chandigarh (Utaaker, *et al.,* 2017). Most parasitic diseases being non-notifiable are under-reported in the developing countries due to the limited extent of detection by routine surveillance systems (Ryan, *et al.,* 2017). Secondly, there is no 'one method suits all' concept in food microbiology, thus screening parasites in foods involves varied approaches based on the type of food matrix and parasitic load. Furthermore, protozoa analysis especially in a much complicated food matrix is cost-driven and requires expertise; hence there are very few laboratories in the country having capability for such type of analysis.

Most common protozoa viz: *Cryptosporidium spp* and *Giardia spp* are capable of surviving in fresh produce for months without active multiplication (Utaaker, *et al.,* 2015). In terms of its significance as a foodborne pathogen, *Cryptosporidium* appears in the top five potential foodborne pathogens (WHO, 2014). Several genotypes of *Cryptosporidium* are associated with foodborne outbreaks of cryptosporidiosis globally (Ryan, *et al.,* 2017). Their salient features like low infectious dose, small size, and robustness make them a legitimate public health concern (Utaaker, *et al.,* 2017). *Cryptosporidium spp* is a major contaminant associated with leafy greens like winter grown Cabbage (Sim, *et al.,* 2017), Spinach and Lettuce (Maikai, *et al.,* 2012), Mint and Corrainder (Bahadori, *et al.,* 2013).

However, with time and increased awareness on health, the practice of undercooking food to retain its natural taste and preserve heat labile nutrients is slowly taking precedence (Rai, *et al.,* 2008). People all over the world are gradually moving towards consumption of salad vegetables as a major part of their diet. Salad vegetables are consumed raw or partially cooked and hence pose a significant health risk to the consumer. This is an alarming trend considering ability of oocysts to survive in them for increased periods of time.

Intent of this study was to investigate and draw attention to the prevalence of *Cryptosporidium spp* in leafy foods sold in open aired markets, devising a

cost-effective method. We chose coriander sample (*Coriandrum sativum)* essentially because is embellished on most salads, traditional Indian foods and street foods. The attempt was also to devise a cost-effective detection method that can be used in routine Microbiology laboratories with basic infrastructure. This paper presents the pilot scale data involving standardization and analysis of 74 coriander samples for oocysts of *Cryptosporidium spp*.

MATERIALS AND METHODS

The entire study was conducted in two phases:

PHASE I: Method development and standardization

Water (tap) and coriander leaf-rinsate were used as the two matrices to determine the recovery efficiency of *Cryptosporidium parvum* oocysts by the newly developed method.

Standardization of the Method by Matrix Spike:

a. Preparation of spike solution

Irradiated oocysts of *Cryptosporidium spp* containing $5X10^5$oocysts/ml were procured from Bhavan's Research Center; Mumbai originally sourced from Waterborne, Inc, USA. These oocysts were diluted using 0.1% polysorbate 20 (Himedia Labs Pvt Ltd) and 0.85% sodium chloride (Himedia Labs Pvt Ltd) to give a final concentration of 1000 oocysts/ml. The spike numbers were confirmed using hemocytometer.

b. Tap water spike

80 ml of the regular tap water from a clean tap was used without any pre-treatment and spiked with approximately 1000 oocysts/ml. The flask was manually swirled for 15 seconds.

c. Coriander spike

Coriander (100-120 grams) was purchased from the local market and was chopped in approximately 2-3 inch size using a knife separating the roots. 10 grams of coriander leaves were weighed on electronic balance (Contech CA 223) to ensure accuracy in weight measurement. The weighed sample was taken in a 500ml capacity Erlenmeyer flask and 80ml of distilled water was added in it. The flask was then kept on a shaker for 45 minutes to allow all the debris stuck to the leaves to enter the solution. Post shaker treatment, the coriander leaves were separated leaving behind the rinsate. 80ml of the rinsate was spiked with approximately 1000 oocysts and thoroughly mixed by manually shaking for 15 seconds to ensure uniform mixing of the oocysts.

Sample processing:

The rinsate solution was pre-filtered through a cellulose filter paper of >11ì pore size (Himedia Labs Pvt Ltd) into a 50ml capacity plastic centrifuge tube (Tarsons). The pre-filtered solution was further passed through a Whatmann filter paper No 1 of 11ì pore size (Himedia Labs Pvt Ltd) ensuring secondary filtration. This two-step filtration was done to remove any interfering particles. The filtered solution was placed in a swing arm centrifuge (Eppendorf 5804R) and centrifuged (1100g for 10 minutes). The centrifuged solution was aspirated using a vacuum aspirator, concentrating the sample to a 0.5ml pellet which was used for microscopic identification.

Staining and identification:

Each pellet was subjected to two staining protocols viz: fluorescent staining (ISO 15553:2006) and Ziehl Neelson Carbol Fuchsin staining (El Naga, *et al.* 2014). The only modification introduced in ZNCF was the use of 3% sulphuric acid instead of 1% to ensure appropriate decolourization for the identification of oocysts under 100 X. Spherical structures showing apple-green fluorescence (ISO 15553:2006) were counted as oocysts by the FITC method while pink colored, round to oval structures (Vohra *et al.* 2012) were counted as oocysts by ZNCF method.

The recovery efficiency was determined by both staining methods and reported as follows: %R = (N/T) X 100

where N was the number of oocysts counted in each slide with 50iL sample which was multiplied by 10 to get the count in the 0.5ml pellet, and T represented the total oocysts spiked (USEPA, 2005) .

PHASE II: Analysis of marketed coriander leaves

Sample collection and processing:

Mumbai, being one of the busiest cities in India, is densely populated with a large number of local

markets functional all day. The sampling sites were included the main market area of a particular municipal ward (mcgm.gov.in) that had a significant number of vendors catering to more than 100 people daily. Of all the wards in the sampling schedule, maximum numbers of samples (29) were collected from vendors selling coriander in the K-East ward. This was mainly due to ease in sample collection and transportation time to the laboratory for analysis. All the sampling sites were open-aired and easily accessible to the residents of the area.

A total of 74 coriander samples were collected and processed from January 2018 to April 2019. Since coriander is sold in the form of a bunch, one bunch of coriander weighing approximately 100-120 grams was considered as one sample. The samples were transported into the laboratory, stored at 4^{O}C prior and processed as mentioned above within 15 hours of sampling. Each coriander sample was processed as mentioned above except for the spiking step.

Reporting of oocysts:

The pellet was then subjected to FITC staining by the protocol as stated above. The stained slides were screened for the presence of *Cryptosporidium spp* oocysts and the count was reported as: *Number of oocysts present/10 grams of coriander leaves.*

Statistics

All the data generated was subjected to descriptive statistics using QI MARCOS 2018 software in Microsoft Excel 2007. Mean and standard deviation for both the staining methods was estimated separately. Efficiencies of both staining methods were compared by "two-tailed paired t-test" followed by a precision analysis to evaluate both the staining methods. The p-value of <0.05 was considered significant at 95% confidence level.

RESULTS AND DISCUSSION

Evaluation of the method:

The newly devised method was evaluated to check the recovery efficiency of oocysts in artificially contaminated water and rinsate matrices. There was a consistent loss of 2-4ml sample from both the matrices during the step of double filtration. Tap water gave a recovery of 100% of spiked oocysts (Table 1). However, same was not observed in case of coriander leaf rinsate where the recovery efficiency was 34.42%. The reduced recovery of oocysts in latter case could be attributed to the differences in the non-covalent interactions between the oocysts and the matrix (Cook, *et al*., 2007).

Comparing the two staining methods, the recovery efficiency of oocysts in coriander rinsate was 34.42% by FITC method and 27.57% by ZNCF method. FITC was more reliable due to clarity of observation, distinct oocyst identification, and adequate speed for enumeration of oocysts. With ZNCF method, there was ambiguity in identifying oocysts from the background artefacts (fig 1), inability to differentiate oocysts from other similar structures (artefacts) picking up the carbol fuchsin stain. This was in agreement with Khurana, *et al.* (2012) where similar problems were encountered differentiating *C.parvum* oocysts from the yeast-like structures. ZNCF method also proved to be time-consuming which was consistent with Vohra *et al.* (2012). Therefore, FITC method was employed for the enumeration of oocysts in a much-complicated coriander matrix.

Prevalence of *Cryptosporidium oocysts* in coriander:

Of the total 74 samples analyzed, the oocysts of *Cryptosporidium spp* were detected in eight coriander samples. The results indicate 11%

Table 1: Recovery Efficiency by FITC for detection of *Cryptosporidium* oocysts

Particulars	Tap Water matrix (n=3)		Coriander leaf rinsate matrix (n=7)	
	ZNCF	FITC	ZNCF	FITC
Mean Recovery (%)	44.6 (±19.3)	103.6 (±15.0)	27.57 (±9.6)	34.42 (±12.6)
P value ($\alpha = 0.05$)	0.063 Not Significant		0.291 Not Significant	

Figures in parentheses indicate standard deviation.

contamination of coriander with oocysts of *Cryptosporidium* at a mean concentration of 16.66 ± 6.23 oocysts/ 10 gram coriander (Table 2). The prevalence of *Cryptosporidium spp* in fresh produce has been a consistent finding in developing countries like Nigeria (Maikai, *et al.,* 2012), Bangladesh (Rahman, *et al.,* 2014), and Iran (Avazpoor, *et al.,* 2015) over the last decade. Our findings corroborated with a recent study conducted in Chandigarh (India) that reported a 10% prevalence of oocysts/cysts of *Cryptosporidium/ Giardia spp* in coriander leaves (Utaaker, *et al.,* 2017) following a modified ISO method (Utaaker, *et al.,* 2015). One plausible explanation for such high levels of contamination could be the use of faecally contaminated water to grow coriander or rinse the coriander prior with contaminated water to time of selling. All the local vendors across Mumbai sell the vegetables on the streets with no basic facilities to ensure cleanliness and sanitation of vegetables before selling. Besides, a common observation at the local market is sprinkling water on all the vegetables, including coriander to keep it fresh and appealing for the customers. Thus, the quality of water used for sprinkling could be another route through which the oocysts could enter the coriander leaves. The treated waste water, on the other hand which could be reused for irrigation purposes can also cross contaminate the leafy greens at source. A recent study has reported a count of 11/ L oocysts of *Cryptosporidium* in treated waste water (effluent) used to grow leafy green vegetables (Domenech, *et al.,* 2018). Another route of contamination could be "over-handling" of the vegetables by people. It is a common practice of storing vegetables on open cart, handling both by buyers and sellers which increases the chances of contaminating coriander leaves. Since there is no specific demarcation in selling different types of vegetables in the cart, one cannot deny the possibility of cross contamination of the parasites from other vegetables into coriander. As the detection limit of the method is quite high, the samples showing absence of *Cryptosporidium spp* by this method should also be treated with caution.

Table 2: Prevalence of *Cryptosporidium parvum* oocysts in Coriander leaves

No of samples collected	No of positives (Percentage positives)	No of oocysts/10 grams
74	8 (10.81)	16.66 ±6.23

Limitations of the study & Future Prospects

The above study is very preliminary, proof of concept with two objectives; one, if oocysts of *Cryptosporidium spp* could be recovered in water and coriander rinsate without IMS, which is the most expensive consumable in the IS 18744:2016 protocol, later being FITC stain which was used in this study after evaluating it with ZNCF stain. The later did not work out particularly in the rinsate as it gave considerable background interference. The second objective was to make a preliminary evaluation on the presence of oocysts in the commercially retailed coriander leaves. Though both the above objectives were met in this pilot study, the following limitations should be addressed by designing a more comprehensive and large scale study:-

a. This method needs to be validated against the existing Standard ISO Method (ISO 18744:2016) where IMS has been used and exact parameters like LOD, LOQ should be established using adequate experimental blanks.
b. Instead of using rinsate, the study should be conducted by using coriander leaves as the sample.
c. Possibility of losing oocysts during the filtration steps should be evaluated.

Future study should include evaluation of molecular detection method over microscopic. Once the method is validated, besides coriander, other leafy vegetables like mint, parsley and the like which too are consumed raw for garnishing and as salad should be evaluated for presence of protozoa.

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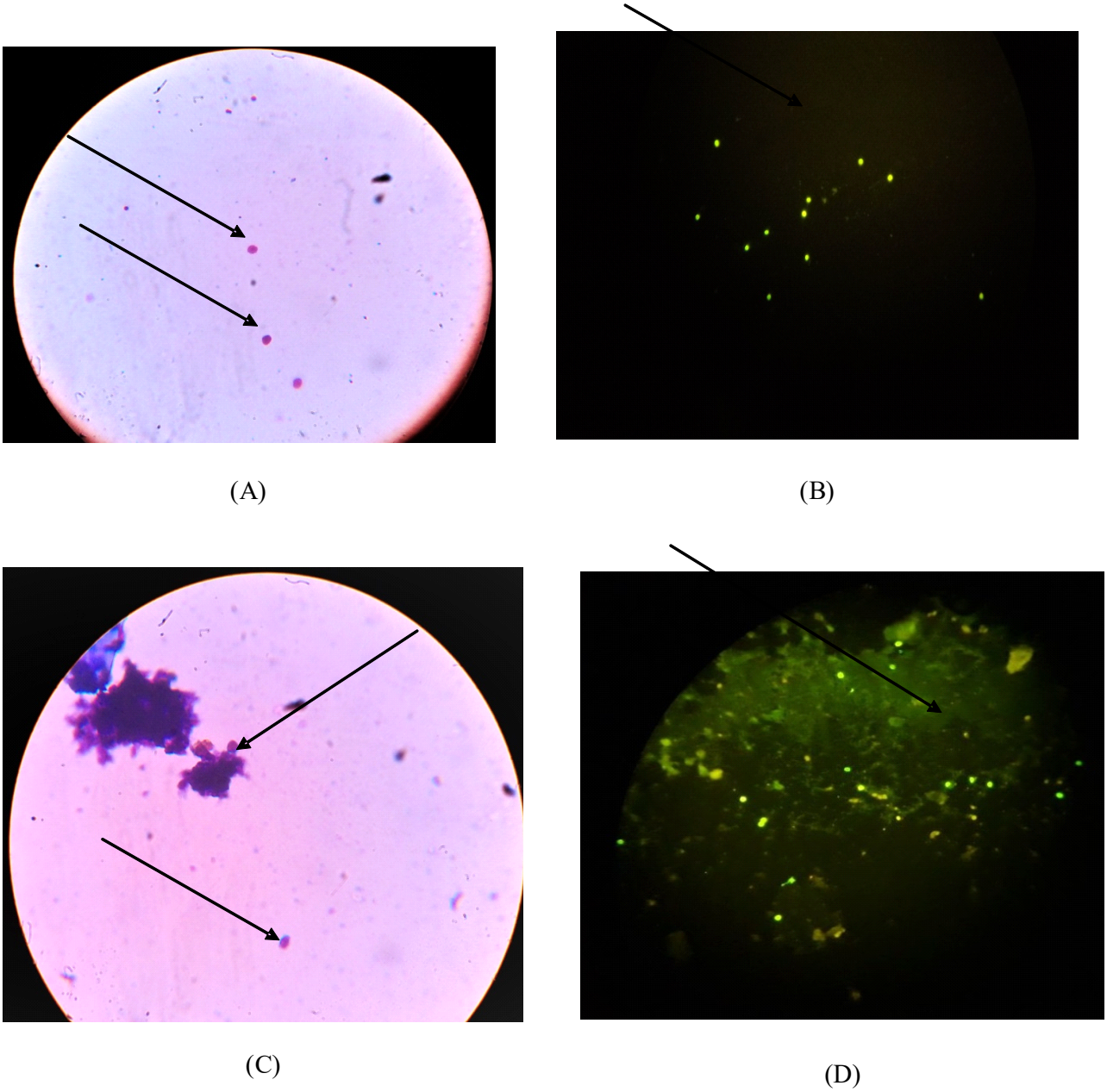

Fig 1: Microscopic Comparison of staining methods. (A): ZNCF stained oocysts (100X) from water. (B): FITC stained oocysts (40X) from the water showing distinct oocysts under clear background. (C): ZNCF stained oocysts (100X) from coriander rinsate. (D): FITC stained oocysts (40X) from coriander rinsate showing distinct oocysts against high background noise. Arrows indicate the position of C.parvum oocysts.

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