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FLOW CYTOMETRIC AND MOLECULAR ANALYSIS OF POSSIBLE PROTOZOAL CONTAMINATION OF DRINKING WATER IN TANTA, EGYPT

By
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Abstract

Protozoal water contamination is an alarming cause of countless waterborne outbreaks. The most eminent causal protozoa are *Cryptosporidium* and *Giardia* species (spp.) as they can endure aquatic environment even with chlorine disinfectants. The currently used traditional techniques cannot permit an easy detection of the waterborne protozoa concerning their count, viability, and pathogenicity. The present work detected protozoal contamination of the drinking water in Egypt with the determination of their load, viability and potential pathogenicity. Four techniques were compared including conventional staining techniques, immunofluorescence (IF) staining, flow cytometry (FC) and molecular study. Also, viability was assessed by conventional trypan blue stain and nucleic acid stain. Along a year, 64 water samples were collected and concentrated from water tanks and tap water of different districts, significant differences ($P < 0.001$) was between the different techniques in each season regarding the detection of *Giardia* cysts and *Cryptosporidium* oocysts. Number of total positive samples was significantly higher in tank water than tap water ($P < 0.001$) especially at summer. Flow cytometry and nested polymerase chain reaction (nPCR) proved to be much more sensitive than IF assay, and conventional staining techniques. Regarding viability, nucleic acid stain was more sensitive than trypan blue stain ($P < 0.001$). Also, *C. parvum* predominate other *Cryptosporidium* genotypes.

Keywords: *Cryptosporidium*, *Giardia,* Immunofluorescence staining, Flow cytometry, water, nested PCR.

Introduction

Unfortunately, safe water, free from pathogens and other contaminants, is not actually obtainable to many of the world population. Being closely associated with human health makes this problem a major public health concern even for the developed countries (McKee and Cruz, 2021). The most recorded protozoal waterborne disease outbreaks (WBDO) were mainly attributed to *Cryptosporidium* spp. and *Giardia* spp. They cause diarrheal diseases in healthy individuals and intractable life-threatening illness in immunocompromised patients, annually accounted for more than 22 million deaths (Rosado *et al*, 2017). In fact, water has a significant risk factor for their transmission as they can survive for several months in aquatic environment and they are resistant to most disinfectants specially chlorination.

Also, they have a low infectious dose, harbbored by many animals (Zahedi *et al*, 2021). The detection of *Cryptosporidium* spp., and *Giardia* spp. in water using the conventional methods and light microscope was a tedious effort and time-consuming, which required a certain level of experience, due to high contamination intensity. So, more sensitive techniques were needed to avoid sudden protozoal WBDO (Hassan *et al*, 2021). Flow cytometry is a highly sensitive and specific technique that can detect low levels of waterborne protozoa and identify their count depending on their size and internal complexity (Silva and Sabogal-Paz, 2020). Nested PCR for genotypes detection of protozoa emerged as powerful source tracking method for public health risk assessment, and pathogenicity determination (Fan *et al*, 2021).

Most genotyping studies of *Cryptosporidium* spp., and *Giardia* spp. on drinking water were conducted in the developed countries whereas, in the developing ones, there were only limited studies (Feng *et al*, 2011;

Yin *et al*, 2021).

This study aimed to assess the currently available advanced techniques in detecting the possible protozoal contamination of the drinking water at Tanta city the capital of Gharbia Governorate (Nile Delta) and thus, identification of their load, viability and potential pathogenicity

Materials and Methods

This descriptive, analytical study was conducted on drinking water samples from different districts of Tanta City and the nearby rural areas over one year.

Sample collection: 64 water samples (5-10 liters) were collected in sterile containers, 32 samples (8/season) were collected from water tanks, and other 32 samples (8/season) were collected from tap water. Data regarding site, date, and source of collection were recorded on each sample container. Boiled distilled water was used as a negative control for FC analysis. Source water and filtered tap one were excluded.

Sample processing: a drop of water was directly examined under light microscope using 10x, 40x & 100xobjectives. Then, all samples were filtered through Whatman cellulose nitrate membrane filters < 2 microns using pumped stainless-steel filtration unit and membrane filters were eluted with PBS (Silva and Sabogal-Paz, 2020). The samples were centrifuged at 4000rpm for 15min., the sediment pellets (1-5ml) were collected in aliquots for each sample and preserved in potassium dichromate (2.5%). The centrifugation force resulted in the recovery of *Giardia* cysts and *Cryptosporidium* oocysts (Fontaine and Guillot, 2003**)**. Each sediment pellet was examined by conventional staining, IF staining, FC and molecular study. Viability was assessed using standard trypan blue stain and 4'6-diamidino-2-phenylindole (DAPI) nucleic acid stain.

Conventional stains as the Lugol's iodine, Modified Zeihl-Neelsen (MZN) and Trypan blue (viability stain) were used (Silva and Sabogal-Paz, 2020). Immunofluorescence staining using a specific kit (A100DFK. Aqua-Glo™ G/C. Direct, Dual Fluorochrome (FL/Cy3), Comprehensive kit Fluorescein & Cy3-labeled Monoclonal Antibody Reagent, Waterborne Inc., New Orleans, LA) was done. Aqua-Glo™ G/C was approved by Environmental Protection Agency (EPA) for use in methods 1622 & 1623 for simultaneous direct immunofluorescence detection of *Giardia* & *Cryptosporidium* (oo) cysts and their viability via DAPI nucleic acid staining by fluorescent microscope (Yoder *et al*, 2008). Protozoal contamination intensity was determined by conventional & IF staining techniques. Live and dead parasites were calculated/100µl using high-power lens. Contamination intensity score was: mild; < 10 (oo) cysts/100µl, moderate; 10-20 (oo)cysts/100µl, and severe; > 20 (oo)cysts/ 100µl.

Detection of *Giardia* and *Cryptosporidium* (oo) cysts in water samples was done by flow cytometric analysis using the previously kit (Vesey *et al*, 1994). 100µl water samples were analyzed on a Flow cytometry Activated Cell Sorter (FACS) Calibur flow cytometer Becton Dickinson equipped with an argonion laser operating at 488nm. FACS was calibrated using calibrated beads (Becton Dickinson, Mississauga, Ontario, Canada) and samples were analyzed using biparametric histograms. A region was set around the particles found in each sample called the gated region from which different signals were used to sort (oo) cysts from the debris. The signals used for sorting were forward angle light scattering (FSC) and side-angle light scattering (SSC). Forward light scatter was responsible for size determination, and right-angle light scatter to detect internal complexity. Signals were based on fluorescence detected by special detectors for fluorescent stains calibrated to detect each stain by using emission wavelength. Flow cytometer expressed results as a percent of the total particles in water sample introduced to device (events) on the histograms' screen. Number of events and percent of gated events in the quadrant of interest determined the contamination intensity according to the follow-

ing score system: mild; 1-30%/100µl, moderate; 31-70%/100µl, and severe; 71-100% / 100µl.

Molecular analysis (Fan *et al,* 2021): Water samples were kept at -20°C to be assessed by nPCR targeting Triose phosphate isomerase (TPI) gene of *Giardia* spp., & *Cryptosporidium* outer wall protein (COWP) gene of *Cryptosporidium* spp. Positive samples were subjected to restriction enzyme length polymorphism (RFLP) to identify its' genotype. The nPCR detected *Giardia* TPI gene via two successive reactions in collaboration with two primers: AL3543: 5'- AAAT IATGCCTGCTCGTCG-'3 & reverse primer AL3546: 5'- CAAACCTTITCCGCAAACC -' 3 for primary reaction to amplify 605bp DNA and a fragment of 530bp for secondary one using AL3544: 5'- CCCTTCATCGGIG GTAACTT-' 3 and reverse primer AL3545: 5'- GTGGCCACCACICCCGTGCC-'3. Mixed reaction consisted of 1µl of each primer (200nM), 5µl of template DNA, and 12.5µl of Dream Taq Green PCR Master Mix (Product No. K1081: Thermo-Scientific, USA) and molecular grade water to achieve 25µl & 2µl of secondary reaction. Cycling conditions were done and the annealing temperature was 45^{o}C for both primary and secondary PCR-assays. Amplified products were visualized with 1.5% agarose gel electrophoresis after ethidium bromide staining. Extraction of COWP gene DNA from water samples was done using Favor-Prep™ stool DNA isolation Mini Kit (Cat. No. FASTI 001, Favorgen Biotech corporation ping-Tung 908, Taiwan). PCR amplified the COWP gene using primers BCOWPF & BCOWPR for E-PCR & Cry-15 and Cry-9 for nPCR.

Reaction master mixture (Rx) for each PCR set was prepared as one reaction, in a volume of 25µl and multiplied by the samples number. Tube was inserted in the thermal cycler, and thermal profile was adjusted as follow; Initial denaturation at 95ºC for 5min., and then 35 cycles of amplification consisted of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 30 seconds. Final elongation was done for 10min at 72ºC. Secondary PCR reaction was done using the primary reaction amplified product as a template. The same reaction conditions were applied. Amplified samples were run in parallel on 2% agarose gel using gel electrophoresis and visualized on a UV transilluminator to confirm the amplified PCR (Ursini *et al*, 2020). After the manufacturer's instructions, following protocol was done to digest nPCR products positive samples for *Cryptosporidium* after amplification (Aghamolaie *et al*, 2016). Components for each reaction included 10µl nPCR product (Target DNA),17µl Nuclease-free water, 2µl Green buffer, 1µl RsaI Enzyme and 30µl to achieve the required total volume. Gentle mixing was done, spinning down for few seconds & incubation at 37ºC for 5min. followed by *Cryptosporidium* genotypes detection using Agarose Gel Electrophoresis and UV Light Transillumination. The PCR marker and volumes of 6µl of amplified product, after digestion, were slowly loaded into the sample wells. Electrophoresis was performed as given before but the power supply was programmed to give 75 volts for 70min. Band detection in Agarose Gel was done using UV Transillumination. *Cryptosporidium* genotypes (Gel interpretation) were done by using restriction enzyme Rsa I, digestion of nPCR product targeting COWP gene. PCR-RFLP showed the 2 genotypes. Sample was C. *parvum* if Rsa I digestion gave 4 bands, namely 34bp, 106bp, 125bp & 285 base pair (bp), and *C. hominis* if Rsa I digestion gave 3 bands namely 34bp, 106bp & 410bp.

Statistical analysis: Data were analyzed by SPSS V.16 using chi-square test for comparison between groups. $P < 0.05$ was significant.

Results

The preliminary examination by direct smear was negative for all samples. Prevalence of *Giardia* cysts using iodine stain was 15.62%, IF staining was 29.69%, FC was 73.43%, and nPCR was 57.8% with signifi-

cant difference (P <0.001). Positive samples detected in the four seasons were significant (P <0.05), with the highest one during summer. Overall prevalence of *Cryptosporidium* oocysts by using MZN stain was 20.31%, IF staining was 39.06%, FC was 81.25%, and nPCR was 62.5% with significant difference (P <0.001). The highest prevalence was during summer with significant difference (P <0.05).

The nPCR was considered the gold standard method to detect the prevalence and seasonal variations of *Giardia* cysts and *Cryptosporidium* oocysts. The relative sensitivity of iodine stain, IF staining and FC in detecting *Giardia* cysts was 77.7%, 79.9%, & 95.5%, respectively, and specificity was 80%, 82.8 % & 92.9%, respectively.

Total positive samples of *Giardia* cysts was significantly higher in the tank than in tap water (P <0.05), but positive samples didn't show a significant difference between both sources seasonally (P >0.05). Seasonal variation showed the highest number of positive tank and tap water samples with significant difference (P <0.05).

Comparing different methods in detecting *Giardia* cysts contamination intensity in tank water in different season, the FC detected the highest positive samples (81.25%) followed by nPCR (68.75%), with significancy (P <0.05). Also, the largest number of positive samples were detected during summer (87.5%) but without significant results (P >0.05). Regarding detection of *Giardia* cysts intensity in tap water using different techniques during different season, FC detected the highest positive samples (65.62%) with significant difference (P <0.001). The highest positive samples for *Giardia* cysts in tap water were detected during summer but without significant difference (P >0.05).

Regarding the viability of *Giardia* cysts in both tank and tap water, results of trypan blue and DAPI stains were significant (P <0.05) with highest number of viable *Giardia* cysts during summer. Also, regarding of *Giardia* cysts intensity contamination, FC was powerful in detection of severe contamination in tank and tap water (P <0.05).

Considering nPCR a gold standard method, the specificity and sensitivity of *Cryptosporidium* oocysts detection in all tank and tap water samples using MZN, IF, FC were compared to its' results. The above techniques' sensitivity was 80%, 81%, & 96.2% respectively, while specificity was 78.9%, 83.3%, & 93.5% respectively. Total number of positive *Cryptosporidium* oocysts samples was significantly higher (P <0.05) in tank water (37.5%) than tap water (25%). The highest number of total positive samples of tank and tap water was during summer with significant results (P<0.05). Flow cytometry showed the highest *Cryptosporidium* oocysts positive samples all tank and tap water, followed by nPCR, with significant differences (P <0.05). The largest *Cryptosporidium* oocysts positive samples was by nPCR during summer but without significant differences (P >0.05).

Regarding the viability of the *Cryptosporidium* oocysts in both tank and tap water, trypan blue and DAPI stains results were significant (P <0.05) with the highest *Cryptosporidium* viable oocysts was during summer. Concerning intensity of *Cryptosporidium* oocysts contamination, IF stain detected more contamination than iodine stain in tank & tap water (P <0.05). FC was powerful in detection of severe contamination in tank and tap water (P <0.05). The nPCR detected 40/64 positive *Cryptosporidium* samples. RFLP products showed *C. parvum* positive samples in 55% followed by *C. hominis* in 12.5%, while both genotypes were detected in 25% of positive samples. Unclassified genotypes were distinguished in 7.5% of positive samples with significant difference (P <0.05).

Details were illustrated in figures (1, 2, 3, 4, 5, 6, 7, & 8).

Discussion

Water pollution is a major global issue which requires regular and continuous evaluation and management. Unsafe water ranks the third among the twenty leading risk fac-

tors for health burden as it provides a favorable environment for many organisms to thrive. This problem has been a major concern of many countries especially in Egypt (Gad *et al*, 2020). Most of the Egyptian governorates depend on surface water as the main drinking source. The most common source of protozoal contamination of water sources in Egypt is the fecal source. This results from the improper disposal of sewage which is usually discarded in seas, rivers, lakes, and canals (Abd El-Latif *et al*, 2020).

The present study reported a significant difference in the number of *Giardia* cysts and *Cryptosporidium* oocysts positive samples detected using different diagnostic techniques in each season as well as in the total number of positive samples detected throughout the year. These results could be attributed to the global warming which stimulates parasite growth or to the use of highly sensitive techniques for testing. It also could be related to where the source of samples, as Delta is highly infiltrated with farms with extensive animal breeding activities. Lastly, it could be caused by the high resistance of these parasites to the usually used water disinfectants as chlorine attributed to their strong (oo)cyst walls (Zahedi *et al*, 2021). Results of this study coincide with some previous similar studies involving drinking water sources of Egypt, as the study conducted in Nile Delta villages over 35 years ago by Khairy *et al*. (1982) which reported a high prevalence of *Giardia* cysts in on both Zir water and tap water (36%) each. Furthermore, a project that was conducted in Nile Delta governorates reported similar results regarding the prevalence of such protozoa (El-Kowrany *et al*, 2016). This indicates that nothing was done either to monitor the prevalence of such protozoa in water nor to treat the water supplies. However, a study conducted in Dakhahlia governorate reported less contamination of the drinking water with *Cryptosporidium* and *Giardia* (oo) cysts 3.1% and 2.1% respectively (El Shazly *et al*, 2007). This could be attributed to the use of a less sensitive technique for detection.

More studies all over the world assessed parasitic contamination of the drinking water, for example, Briancesco and Bonadonna, (2005) performed a study in Italy and reported that *Cryptosporidium* and *Giardia* are present in untreated sewage and surface source water, and they disappear after water treatment. Moreover, in the UK, *Cryptosporidium* was detected in drinking water in 100% of the samples using nPCR and RFLP (Nichols *et al*, 2003). Also, Feng *et al*. (2011) confirmed the same results. On the contrary, in the USA low parasites prevalence was reported by LeChevallier *et al*. (1991) as *Giardia* was only found in 17% and *Cryptosporidium* in only 27% of filtered water samples.

In the current study, revealed that the total protozoal positive samples were significantly higher in tank water than tap water. This agreed with Baldursson and Karanis (2011) and (Rosado-García *et al*. (2017). In the present study, the highest prevalence of *Cryptosporidium* and *Giardia* (oo) cysts was during summer. This agreed with Antonios *et al*. (2010); El-Kowrany *et al*. (2016); Brankston *et al*. (2018) and Xiao *et al*. (2018), who reported that *Cryptosporidium* contamination positive degree correlated with warm weather. But, Ibrahim *et al*. (2020) recorded *Giardia* in river water in rainy seasons.

In the current study, Lugol's iodine identified *Giardia* cysts. Also, MZN stain was proven to be fast, simple, sensitive and the most effective stain for detection of *Cryptosporidium* oocysts. This was confirmed by many studies performed all over the world which compared it to many other stains (Adeyemo *et al*, 2018; Hassan *et al*, 2021). Immunofluorescence assay was proved to be an efficient technique especially with low levels of (oo)cysts as it represents sensitive, specific, and time saving than the traditional stains (Quintero *et al*, 2002). However, its use was limited because it required a fluorescent microscope, expensive reagents, and

didn't distinguish viable and dead organisms (Alles *et al*, 1995). Also, Aghamolaie *et al*. (2016) found that MZN staining gave good accuracy for *Cryptosporidium* diagnosis due to its low costs and inaccessibility of other techniques in all laboratories.

The FC facilitated detection and determination of the intensity of contamination by both protozoa and proved to be more sensitive than the conventional and IF staining. This agreed with Vesey *et al*. (1993) who applied FC as a routine assessment for detection of *Cryptosporidium* and *Giardia* (oo) cysts in water samples. They had proved that FC detected more positive samples than microscopy. Although FC was proved to be a simple rapid and sensitive technique, its use is limited because it is much more expensive than the conventional methods. Moreover, it does not detect any simultaneously present protozoa present in the collected samples as it is confined to the used specific monoclonal antibody (Vesey *et al*, 1994). Moreover, the FC overestimated the intensity of contamination as it could not distinguish the algae that have the same size of protozoa and show the same fluoresce too. However, the percentage of these particles was often negligible, their fluorescence intensity usually very low, and was not incriminate to have a significant effect on results (Valdez *et al*, 1997; Silva and Sabogal-Paz, 2020).

In the current study, nPCR was considered the highly sensitive gold standard method, and detected the DNA of *Giardia* and *Cryptosporidium* spp. This agreed with Nichols *et al*. (2003) who reported its rapid and less liable for post-amplification contamination, with more positive samples than conventional and IF stains ones. Also, Fontaine and Guillot (2003) compared between IF stain and PCR, and found that PCR was more valuable. Moreover, nPCR revealed fewer positive samples than FC regards *Cryptosporidium* and *Giardia* (oo) cysts. This agreed with Valdez *et al*. (1997), where only 25% of FC the positive samples were confirmed by the PCR. No doubt, FC not only detected viable (oo) cysts but also detectd empty ones (ghosts) which led to a higher false result.

In the present study, *C. parvum* and *C. hominis* were the commonest genotypes affecting humans. RFLP showed that *C. parvum* was the dominant species due to high prevalence of bovine cryptosporidiosis in the Nile Delta. This agreed with McLauchlin *et al*. (2000) who reported that *C. parvum* was the major cause of cryptosporidiosis affected bovines and immunocompromised human patients in the UK. But, only 12.5% of *Cryptosporidium* positive samples were due to *C. hominis*. Also, others reported a similar prevalence of the *C. hominis* genotype in water samples and human stool samples (Chalmers *et al*, 2010), as a delicate and less resistant species with relatively lower incidence (Chalmers *et al*, 2011).

In the present study, also the 7.5% unclassified *Cryptosporidium* genotypes in the positive samples, may be *C. baileyi* and *C. meleagridis,* mainly birds' parasites contaminated uncovered water tanks (Gibson *et al*. 2011). *C. baileyi* genotype was found in one sample by either of Nichols *et al*. (2006) and Cardozo *et al*. (2008).

In the present study, the high percentage of viable *Cryptosporidium* and *Giardia* (oo) cysts in drinking chlorinated water. Nakada *et al.* (2019) reported *Giardia* cyst resisted chlorine. The DAPI stain proved to be significantly superior to the trypan blue stain in detecting the viability of both *Cryptosporid ium* and *Giardia* (oo) cysts because only viable nuclei uptake the DAPI. This agreed with Rousseau *et al*. (2018) and Burgt *et al*. (2018) who used fluorochrome DAPI to predict viability of *Cryptosporidium* oocysts.

Since DAPI staining technique only required one step for preparation, it was considered more efficient than the tedious trypan blue stain which consumes much time to detect the percentage of live parasites in the sample under a conventional microscope. To enhance the results, both DAPI stain and FC could be used together to detect the percent-

age of the viable (oo) cysts (Medema *et al*, 1998 and Sammarro *et al*, 2020. Unfortunately, this was not accessible for this study as DAPI had a different wavelength than the used fluorochromes (FITC and Cy3) which was not provided in the FC device.

Conclusion

Regarding detection of *Cryptosporidium* and *Giardia* (oo) cysts in water, the conventional microscope is still the cheapest available technique but with lower sensitivity compared to the recent techniques as FC. Also, nPCR proved to be more sensitive than conventional staining techniques as it detects the parasite DNA and distinguished different genotypes. However, being expensive made it not feasible to be used as a routine assessment. It was recommended to design national standards for detection of all parasitic contaminations of water.

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Explanation of figures

Fig.1: A) Total positive *Giardia* cysts and *Cryptosporidium* oocysts in all 64 tanks and tap water samples in different seasons by using different diagnostic techniques. B) Sensitivity, specificity, PPV, NPV, and accuracy of different diagnostic techniques used. C) Total positive samples of *Giardia lamblia* cysts by nPCR in different seasons in tank and tap water. D) Positive *Giardia* cyst samples in tank water by different diagnostic techniques in different seasons.

Fig.2: A) Positive *Giardia* cyst samples in tap water by different diagnostic techniques in different seasons. B) Viability of *Giardia* cysts by using trypan blue stain and DAPI stain in different seasons in all positive tank and tap water samples. C) Intensity of *Giardia* cysts/100µ by both iodine stain & IF assay. D) Intensity of *Giardia* cysts by FC/100µ.

Fig. 3: A) Sensitivity, specificity, PPV, NPV & accuracy of different diagnostic techniques to detect *Cryptosporidium* oocysts in all water samples. B) Total positive samples of *Cryptosporidium* oocysts by nPCR in different seasons in tank and tap water. C) Positive tank water samples in different seasons by different diagnostic techniques. D) Positive *Cryptosporidium* oocysts in tap water samples in different seasons by different diagnostic techniques.

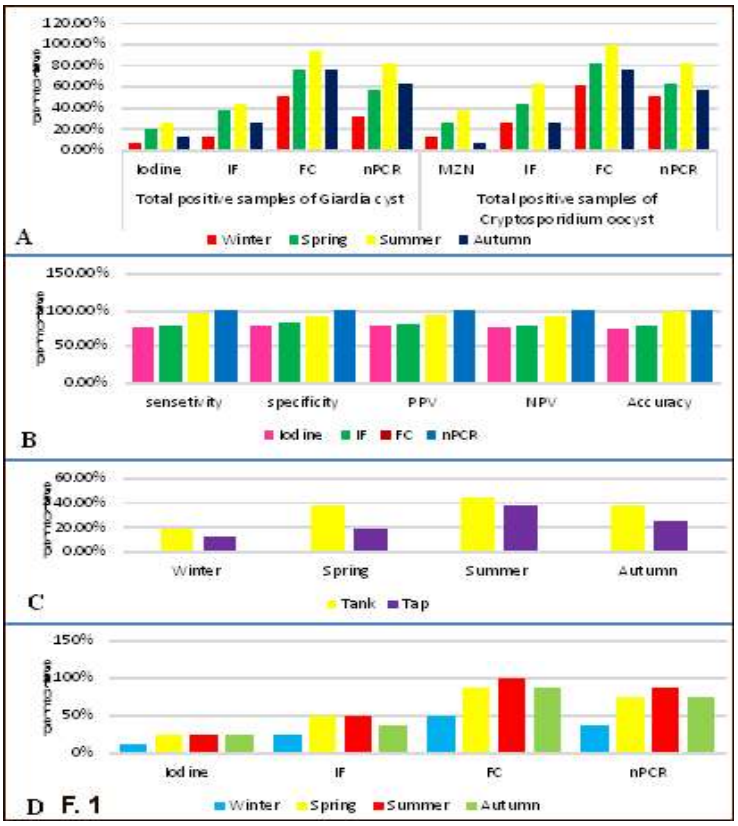
Fig. 4: A) Viability of *Cryptosporidium* oocysts by using conventional trypan blue stain and DAPI stain in different seasons in all positive tank and tap water samples. B) Intensity of *Cryptosporidium* oocysts/100µ by both MZN stain and IF assay. C) Intensity of *Cryptosporidium* oocysts by FC/100µ. D) Total positive *Cryptosporidium* oocysts genotypes in 40 positive tank and tap water samples by nPCR and RFLP.

Fig. 5: A) MZN stained *Cryptosporidium* oocysts round 4-6µ pink in color (acid-fast) against a faint blue-green background (1000x). B) Light trypan blue stained *Cryptosporidium* oocysts (viable) (1000x). C) Viable *Cryptosporidium* oocysts (400x). D) Dark stained (dead) *Cryptosporidium* oocysts by trypan blue (1000x). E) Immunofluorescent bright green *Cryptosporidium* oocysts (1000x). F) DAPI stained *Cryptosporidium* oocysts as bright green with shiny apparent nuclei with a faint wall (1000x). G) *Entamoeba histolytica* cyst distinguished by iodine stain in one sample (1.56%). H) *Cyclospora cayetanensis* oocysts pink (acid-fast) against a faint bluish background in 3 samples (4.7%) by MZN stain (1000x).

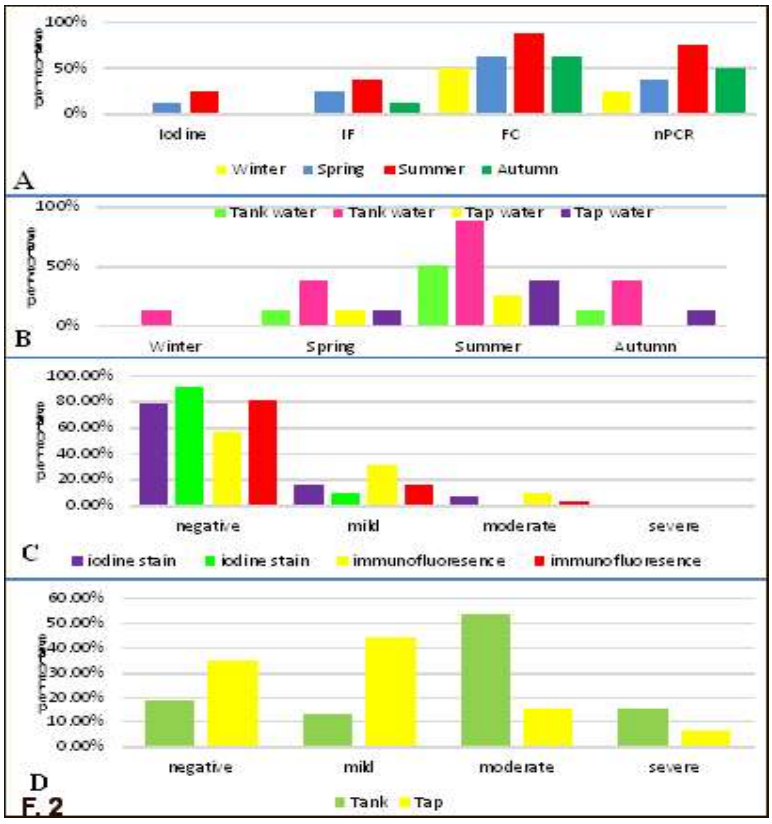
Fig. 6: A) Iodine stained *Giardia* cysts (1000x). B) MZN stained *Giardia* cysts (1000x). C) Trypan blue stained (live) *Giardia* cysts (1000x). D) Dead cysts dark blue by trypan blue stain (400x). D) Immunofluorescence stained *Giardia* cysts (1000x). E) DAPI stained nuclei of *Giardia* cysts (1000x).

Fig. 7: A) Negative sample for both *Cryptosporidium* oocysts and *Giardia* cysts by flow cytometry. B) Positive sample of *Giardia* cysts by FC. C) Positive sample of *Cryptosporidium* oocysts by FC. FC showed all particles of *Giardia* cysts on a graph as dots called events alongside a histogram. X-axis of histogram represented FITC stained *Cryptosporidium* oocysts, and Y-axis represents Cy3 stained *Giardia* cysts, divided into four quadrants. Automatically, device showed each event in histogram by fluorescence intensity. Maximum intensity of FITC stain on X- axis in lower right quadrant *Cryptosporidium* oocysts while maximum fluorescence for Cy3 stain on Y-axis in upper left quadrant *Giardia* cysts.

Fig. 8: A) Agarose gel stained with ethidium bromide corresponding to PCR: Lane L Molecular weight marker (100 bp), Lane 1 Positive control, Lanes 2-6 DNA for products of nPCR of samples targeting TPI gene (530 bp) of *Giardia*. B) Agarose gel electrophoresis for DNA products; Lane L: 100 bp DNA molecular weight marker. Lanes 1-3: RFLP products after digestion with RsaI endonuclease with *C. parvum* genotype 2 digestion products at 34, 106 &410 bp (34 band very small, faint, but difficult to see). Lanes 4-5: RFLP products after digestion with RsaI endonuclease with *C. hominis* genotype 1 digestion products at 34, 106, 125 & 285bp (34 band very small, faint but difficult to see). Lane 6: Undigested product of COW product at 553 bp. Lane 7: Products of nPCR targeting COWP gene of *Cryptosporidium* at 553bp


120.00%
100.00%
80.00%
60.00%
40.00%
0.00%
Iodine
IF
FC
nPCR
MZN
IF
FC
nPCR
Total positive samples of Giardia cyst
Total positive samples of Cryptosporidium oocyst
A
Winter
Spring
Summer
Autumn
150.00%
100.00%
50.00%
0.00%
sensetivity
specificity
PPV
NPV
Accuracy
B
Iodine
IF
FC
nPCR
60.00%
40.00%
20.00%
0.00%
Winter
Spring
Summer
Autumn
C
Tank
Tap
150%
100%
50%
0%
Iodine
IF
FC
nPCR
D
Winter
Spring
Summer
Autumn

F. 1


100%
50%
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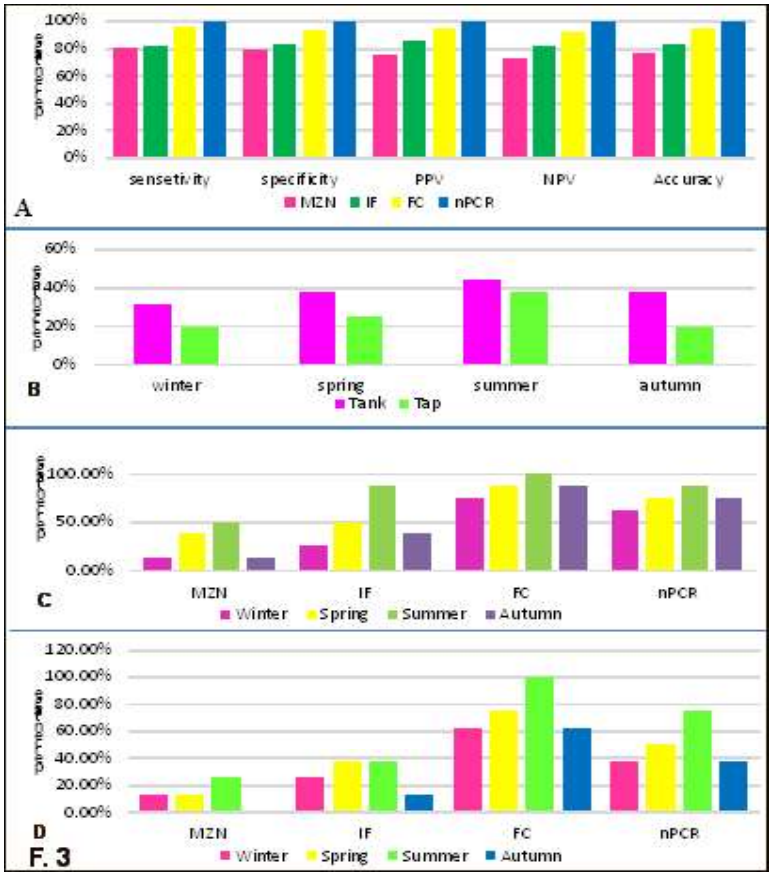
Iodine
IF
FC
nPCR
A
Winter
Spring
Summer
Autumn
100%
50%
0%
Tank Water
Tank Water
Tap Water
Tap Water
Winter
Spring
Summer
Autumn
B
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
negative
mild
moderate
severe
C
Iodine stain
Iodine stain
immunofluoresence
immunofluoresence
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
negative
mild
moderate
severe
D
Tank
Tap

F. 2


100%
80%
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sensetivity
specificity
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Accuracy
MZN
IF
FC
nPCR
A
60%
40%
20%
0%
winter
spring
summer
autumn
Tank
Tap
B
100.00%
50.00%
0.00%
MZN
IF
FC
nPCR
Winter
Spring
Summer
Autumn
C
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
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MZN
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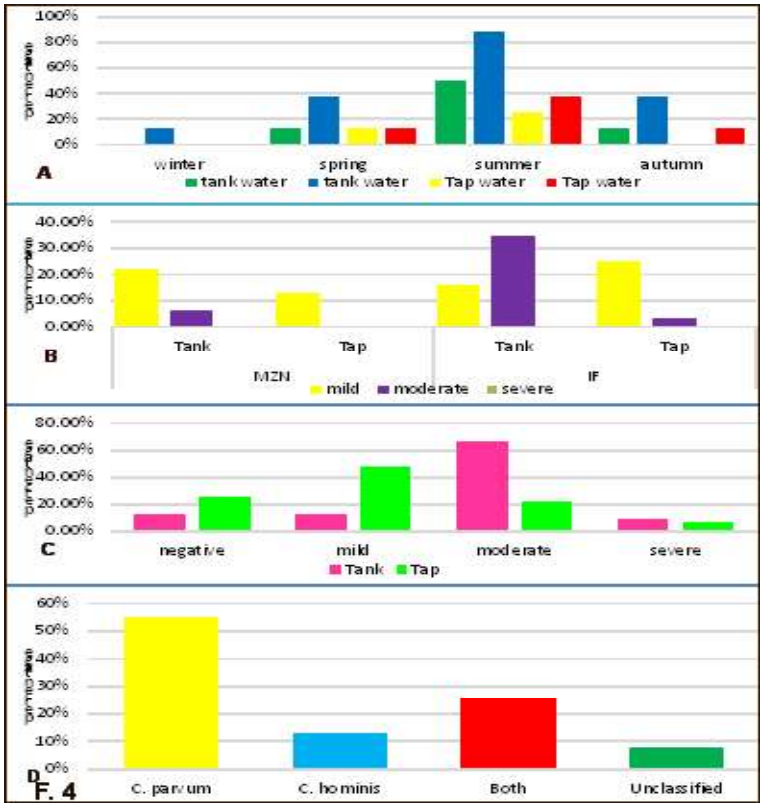
FC
nPCR
Winter
Spring
Summer
Autumn
D

F. 3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80%
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winter
spring
summer
autumn
tank water
tank water
Tap water
Tap water
A
40.00%
30.00%
20.00%
10.00%
0.00%
Tank
Tap
Tank
Tap
MZN
IF
mild
moderate
severe
B
80.00%
60.00%
40.00%
20.00%
0.00%
negative
mild
moderate
severe
Tank
Tap
C
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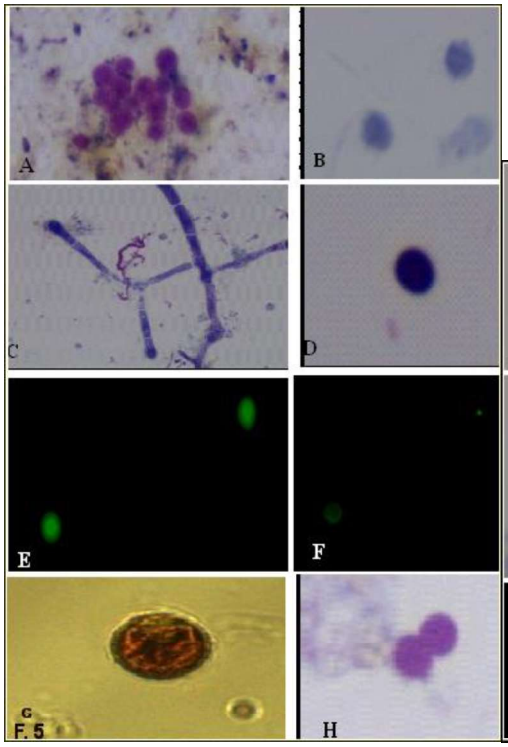
40%
30%
20%
10%
0%
C. parvum
C. hominis
Both
Unclassified
D

F. 4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B
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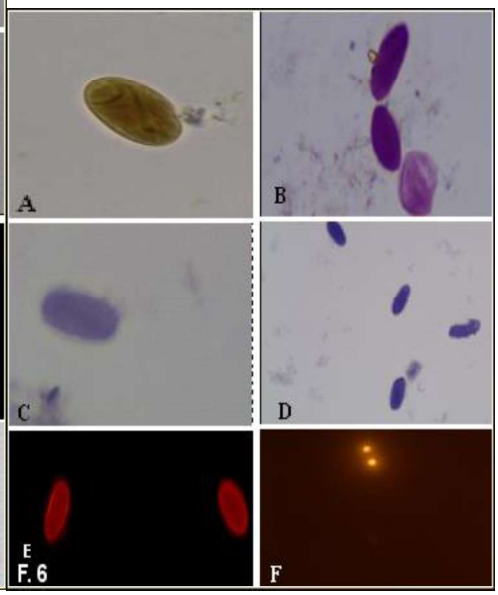
F. 5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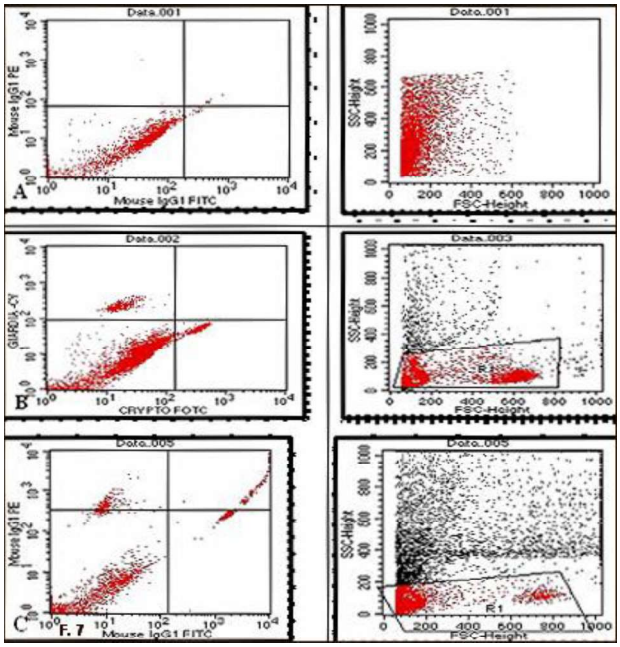
F. 6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Mouse IgG1 FITC
SSC-Height
FSC-Height
A
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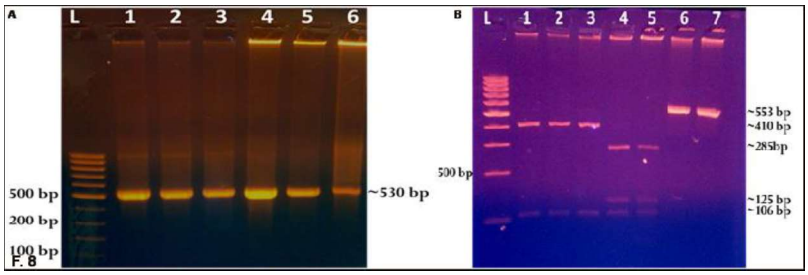
CRYPTO FOTC
B
Data..003
R1
Data..005
C

F. 7


A
L 1 2 3 4 5 6
500 bp
200 bp
100 bp
~530 bp
B
L 1 2 3 4 5 6 7
500 bp
~553 bp
~410 bp
~285bp
~125 bp
~106 bp

F. 8