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Antiprotozoal activity of silver nanoparticles against *Cryptosporidium parvum* oocysts: New insights on their feasibility as a water disinfectant

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ABSTRACT

Cryptosporidium is a protozoan of extremely medical and veterinary impact; whose oocysts donate a considerable resistant to the water treatment processes. Therefore, this study aimed to explore the impacts of silver nanoparticles (AgNPs) on count and viability of the *Cryptosporidium parvum* (CP) isolated from different tap water samples. The oocysts were exposed to AgNPs at different dosages of 0.05, 0.1 and 1 ppm for several contact times (30 min to 4 h). The results showed a significant decrease in oocyst count and viability in a dose-dependent manner. Additionally, AgNPs at a conc. of 1 ppm for 30 min and 0.1 ppm for 1 h reduced the oocysts by 97.2 and 94.4%, respectively. Comparatively, there was a noticeable increase in the oocyst's viability at 2 and 4 h, which emphasized that the time of contact between AgNPs and CP was not a major influencing factor for successful application of AgNPs in the nano-water treatment.

1. Introduction

Waterborne parasitic diseases are one of the most significant environmental contributors to the human and livestock disease worldwide; accounting in 1.6 million deaths and 4 billion cases of diarrhea annually (Zahedi et al., 2016). *Cryptosporidium* spp. are considered as a major contributor to this problem. The prevalence of Cryptosporidiosis in human and different domestic animals varies considerably among studies, ranging from 0 to 100% (Shrivastava et al., 2017), with a severity ranging from severe or mild to asymptomatic, depending on the immune status, age and nutrition of the infected host (Samie et al., 2015). In Egypt, zoonotic cryptosporidiosis is continually reported in nearly all the Egyptian Governorates (El-Sherbini and Mohammad, 2006; Elshazly et al., 2007; Khalifa et al., 2014; Massoud et al., 2008; Rayan et al., 2009). The protozoan is transmitted by the fecal-oral way, either directly by inter individual or animal-to-person transmission or indirectly via assimilation of *Cryptosporidium* contaminated water or food (Ryan and Hijjawi, 2015). The water-borne pathway is the most common and critical route of cryptosporidiosis, where *Cryptosporidium* has been involved in 60.3% of the parasite's waterborne outbreaks, worldwide between 2004 and 2010 (Baldursson and Karanis, 2011). The removal and inactivation of *C. parvum* from water supplies prevails as an extremely difficult problem in both the developed and developing countries due to several factors. First, water-borne *Cryptosporidium* oocysts is of great resistance to the most commonly used water disinfectants (Alum et al., 2016; Castro-Hermida et al., 2015; Tam et al., 2012) such as UV irradiation, hypochlorous acid, and chloramine (Korich et al., 1990; Montemayor et al., 2005), in addition to their potential to remain active in water environments for over a year. Second, its low infective doses pose a major challenge in delivering and monitoring safe drinking water (King and Monis, 2007). Ultimately, the disinfection methods currently used in drinking water treatment through chlorination to control microbial pathogens yield harmful disinfection byproducts (Li and Mitch, 2018; Wang et al., 2015). Therefore, recent research efforts tried to find a new alternative to both the removal and inactivation of *Cryptosporidium* oocysts.

Silver nanoparticles (AgNPs) can be one of the most promising technology to combat the pathogens. AgNPs have been used in numerous pharmaceutical and biological applications owing to their well-recognized antibacterial and antifungal activity (Dosoky et al., 2015; Kailasa et al., 2019; Prabhu and Poulose, 2012). The antimicrobial mechanism of nano-Ag is attributed to the release of silver ions (Ag^+) which trigger oxidative stress via the release of reactive oxygen species (ROS) (Fauss et al., 2014). Additionally, nanoparticles (NPs) have an

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extensive surface, which allows them for greater contact to the bacterial binding site and slow release of Ag^+ and ROS (Rai et al., 2009). There is a little data on the mechanism of AgNPs for protozoal inactivation. For instance, Cameron et al. (2016) reported a duplicate mode of interaction between AgNPs and CP oocyst, where released Ag ions can enter the oocyst and demolish the sporozoites while nanosized particles of silver can react with the cell wall, resulting in leakage. Interestingly, the use of NPs in water treatment exemplifies one of its most important applications, being relatively cheap, and safe for the environment (Brame et al., 2011; Qu et al., 2012). However, the adoption of AgNPs as antiprotozoal agent for water treatments still needs further investigation. Therefore, this study aimed to explore the impacts of different doses and contact times of AgNPs on waterborne isolates of *Cryptosporidium parvum* count and viability, in a promising trail to overcome the existing difficulties of removing the parasite from drinking water.

2. Material and methods

This study involved the effectiveness of AgNPs as one of the most recent promising water disinfectants in reducing both total number and viability of *Cryptosporidium parvum* that has been isolated from drinking water in Assiut city.

2.1. Samples collection

Drinking water samples were assembled as pooled samples from 6 different regions in Assiut City and surrounding villages including: Area 1: Manqabad, a village located 3 miles north to Assiut City. Area 2: Arab-Almadabegh, a village located north-west to Assiut City. Area 3: Assiut University, including Assiut University Hospital, Veterinary Teaching Hospital and Faculty of Agriculture. Area 4: Al Moalemeen area. Area 5: Al-Sadat and Al-Shader areas, located within the south-west part of Assiut City.

Water samples collection was performed according to Method 1623 implemented by the Environmental Protection Agency for the Examination of *Cryptosporidium* from water samples (EPA, 2005). Twenty-five liters sterile plastic containers were used for collection of water samples. In each area, at least 3 samples were gathered from 3 different points for better coverage. The source of all water samples was tap water intended for human and animal drinking.

2.2. Samples preparation

2.2.1. Filtration

Each 20-lL water sample was completely filtered using filter apparatus designed and executed by Sayed et al. (2016a) as illustrated in Fig. 1. Concentration and elution were the second step after the filtration process, where the membrane filter was processed, following the instructions of the manufacturer, according to Pezzana et al. (2000), by replacing the Laureth 12 elution buffer with phosphate-buffered saline (PBS).

2.3. Detection of Cryptosporidium parvum in water sample

2.3.1. Modified acid-fast stain

Positive samples for CP were detected by modified carbolfuchsin (Kinyoun's) with methylene blue (KMb) stain, according to Cole (1997). Evaluation of each slide was done with an optical microscope at ×400 and ×1000 magnification. *C. parvum* oocysts stained as bright to pale pink spheres against blue background.

2.3.2. Molecular identification of C. parvum

Using nested polymerase chain reaction (nPCR) technique, oocysts were processed for Genomic DNA extraction by FavorPrep Stool DNA Isolation Mini Kit (Favorgen Biotech Co., Taiwan) according to the manufacturer's guidelines. DNA extracted by nPCR was amplified, using two sets of primers as shown in Table 1. *Cryptosporidium* oocyst wall protein (COWP) fragments digestion was determined by electrophoresis in 3.2% agarose gels comprising ethidium bromide, thereafter, fragments visualization was done under UV light to explore the *Cryptosporidium* genotype.

2.3.3. Oocyst count

C. parvum positive oocyst were counted before and after AgNPs exposure according to Suresh and Rehg (1996) using hemocytometer slide under bright-field microscopy, according to the following equation:

$$C = (T.\ D\ )/W$$

where, C is the oocyst count, T is the total number of counted oocysts, D represents the dilution factor, and W is the volume of tested water sample in (mL).

2.3.4. Acridine orange staining

Acridine orange staining was performed to each positive sample to compare parasite viability before and after AgNPs exposure following the method initiated by Khalifa et al. (2011). Acridine orange binds to DNA of the organisms and ignite various colors that help distinguish cellular organelles as shown in Fig. 2. *Cryptosporidium* oocysts viability percentage was determined using hemocytometer as explained by Cadena-Herrera et al. (2015) and Othman et al. (2003).

2.4. Nano-silver synthesis and exposure technique

After processing, positive samples for *C. parvum* oocysts confirmed by nPCR were transferred in ice box to the Animal Hygiene Lab, Faculty of Veterinary Medicine, Assiut University for further treatment by AgNPs.

2.4.1. Synthesis of nano-Ag solution

One-step method implemented by Vigneshwaran et al. (2006) was followed to synthesize AgNPs. Briefly, one gram of soluble starch was dissolved in 100 mL of deionized water. Thereafter, 1 mL of a 100 μM of silver nitrate aq solution (AgNO3, 99.0% purity) was added and mixed well. This mixture was stored in dark bottles and autoclaved for 5 min at 121 °C.

2.4.2. Characterization of AgNPs

Transmission Electron Microscopy (JEOL-100CX) was used to determine the particle size of synthesized AgNPs, as shown in Fig. 3. Additionally, the entire AgNPs concentration was estimated by 210VGP Model Atomic Absorption Spectrophotometer.

2.4.3. AgNPs-CP exposure experiments

For in-vitro exposures, PBS containing stock of *C. parvum* oocysts was thoroughly mixed and equally distributed in 4 sterile tubes marked as C, T1, T2, and T3. AgNPs was added to T1, T2, and T3 tubes to obtain a final concentration of 0.05, 0.1 and 1 ppm, respectively. The fourth tube (C) served as a control (without AgNPs treatment). All tubes were kept at room temperature, where AgNPs were allowed to interact with *C. parvum* oocyst for several contact times; 30 min, 1, 2 and 4 h. At the end of each contact times, 0.5 mL from each tube was transferred to eppendorf tubes in triplicates and quenched with 5 g/L sodium thiosulfates to stop the toxic action of AgNPs (Dosoky et al., 2015). Finally, a phase-contrast light microscopy and fluorescence microscopy were used to asses both *C. parvum* oocyst count and viability, respectively.

2.5. Infectivity assay using the animal challenge

In this experiment, isolated *C.parvum* oocyst from water samples and those subjected to different concentrations of AgNPs was

Fig. 1. Parts of water filter apparatus adopted by (Sayed et al., 2016a). [1]Vacuum Air Pump; [2]stainless steel tank; [3&4]2 stages filter cartridge; [5]filtration-drainage box; and [6]membrane filter housing.

Table 1
Primers sets used for nPCR targeting COWP gene.

	Sequences	bp	Annealing temp.	References
1st PCR (E-COWP = Extended COWP)				
BCOWPF	5'-ACCGCTTCTCAACAACCATCTTGTCCTC-3′	769	65	Pedraza-Díaz et al. (2001)
BCOWPR	5'-CGCACCTGTTCCCACTCAATGTAAACCC-3′			
2nd PCR (N-COWP = Nested COWP)				
cry-15	5′-GTA GAT AAT GGA AGA GAT TGT G-3'	553	54	Spano et al. (1997)
cry-9	5′-GGA CTG AAA TAC AGG CAT TAT CTT G-3′			

inoculated to albino male mice to examine the viability of *C. parvum* in-vivo. Briefly, laboratory bred Swiss albino male mice ($n = 6$ animals/group) of 8 weeks old and each of an average weight of approximately 30 g was allocated randomly into four groups, G1–G4. All mice were examined to confirm their free from any parasitic infection through fecal samples and by direct wet smear and modified Ziehl–Neelsen technique for three consecutive days (Abdou et al., 2013). The first 3 groups (G1–G3) were infected with *C.* oocyst previously exposed to 0.05, 0.1 and 1 ppm of AgNPs in-vitro. The fourth group was the control (infected with oocysts not exposed to AgNPs). G1–G3 were subsequently subdivided into four subgroups depending on AgNPs contact times. According to Sayed et al. (2016b); all mice were infected per os with 600 *Cryptosporidium parvum* oocysts suspended in 75 µL distilled water, either AgNPs exposed or AgNPs non-exposed. Thereafter, fresh fecal pellets from each mouse in the study groups including the control were collected separately every day throughout the experimental period (from the 2nd to 14 days post-inoculation). Ultimately, infection rate at all 13 groups and subgroups was determined by detection of oocysts by both acid-fast stain (Cole, 1997) and Direct Fluorescent Antibody Staining (DFA) complemented by 4,6-diamidino-2-phenylindole (DAPI) staining using the Aqua-Glo™ kit (A100FLK, Waterborne, USA) (Jex et al., 2008) as shown in Fig. 5. Excreted *C. parvum* oocysts

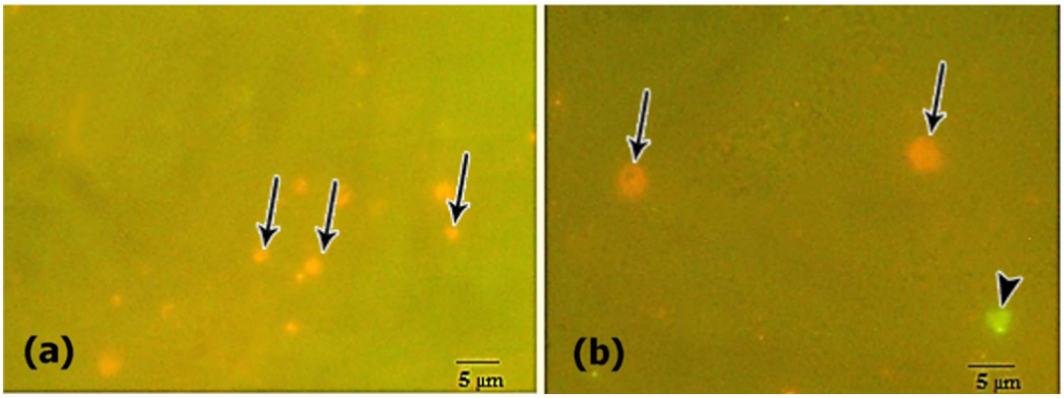

Fig. 2. *C. parvum* oocysts labeled with acridine orange vital dye (a) viable oocysts (arrows) at × 400 (b) viable oocysts (arrows) and inviable oocyst (arrowhead) at × 1000.

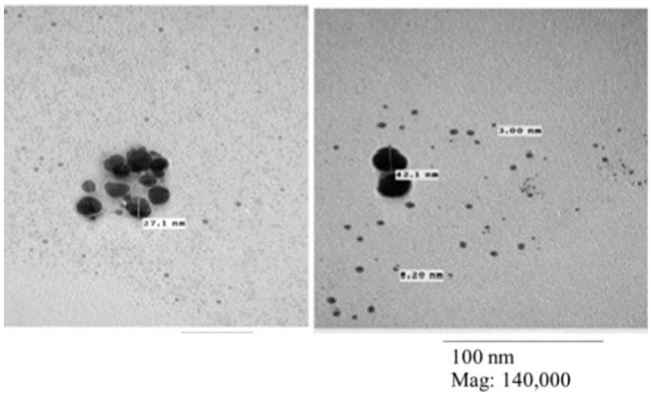

Fig. 3. TEM images of synthesized AgNPs, diameters in nm.

were counted for each mice every day throughout the experiment using hemocytometer slide under bright-field microscopy (Suresh and Rehg, 1996).

2.6. Statistical analysis of data

Data was collected, tabulated and statistically analyzed using SAS program version 9.3 (SAS, 2011). One-way ANOVA was performed through Duncan's multiple range test to estimate the significant differences between treated groups and the control. *P*-value was statistically significant at $p^{<}0.05$.

3. Results and discussions

Cryptosporidium oocysts are currently among the most important contaminants of drinking water being second only to Rotavirus (Jaskiewicz et al., 2018). In recent years, research has focused not only on the resistance of *Cryptosporidium* oocysts to commonly used disinfectants but also tried to find new effective disinfection methods. Therefore, study was carried out to determine the usefulness of AgNPs as a water disinfectant in reducing the total number and viability of *Cryptosporidium parvum* and to finding an alternative solution to overcome its resistance to chlorine. These objectives were raised from the facts that the local drinking water in Assiut City shows high levels of *C. parvum* contamination along with other bacteria and parasites (Dyab et al., 2015; Sayed et al., 2016b).

The total *Cryptosporidium* count in nano-exposed samples and the control groups are presented in Fig. 4. The results denoted a non-significant difference in the control samples at different time periods. Nevertheless, the application of AgNPs, at all used concentration and during all time periods, significantly reduced the CP oocysts ($p^{<}0.05$) compared to the control group. Additionally, the supplementation of AgNPs at 0.1 ppm for 30 min yielded the highest inhibition of CP oocyst count of 87.0%. The results illustrate that after 30 min contact time, the inhibition of total *Cryptosporidium* count was reduced at all used concentrations, which supposed slight reactivity of oocyst again after 30 min exposure, as shown in Fig. 4.

Table 2
Viability of *Cryptosporidium* after treatment with AgNPs at different concentrations.

AgNPs concentration	Contact time	Viable *Cryptosporidium* oocyst		Death rate (%)
		Treated sample	Control	
0.05 ppm	30 min	6.0 ± 1.4^{c}	28.6 ± 3.0^{ab}	79.0
	1 h	2.9 ± 0.5^{d}	28.6 ± 3.0^{a}	89.9
	2 h	4.0 ± 0.8^{cd}	31.6 ± 2.8^{b}	78.3
	4 h	2.8 ± 0.6^{d}	28.3 ± 2.9^{a}	90.1
0.1 ppm	30 min	1.9 ± 0.8^{cd}	28.6 ± 3.0^{ab}	93.3
	1 h	1.6 ± 0.1^{c}	28.6 ± 3.0^{a}	94.4
	2 h	2.3 ± 0.3^{d}	31.6 ± 2.8^{b}	92.7
	4 h	1.9 ± 0.2^{cd}	28.3 ± 2.9^{a}	93.3
1.0 ppm	30 min	0.8 ± 0.4^{c}	28.6 ± 3.0^{ab}	97.2
	1 h	2.6 ± 0.7^{d}	28.6 ± 3.0^{a}	90.9
	2 h	5.6 ± 0.5^{de}	31.6 ± 2.8^{b}	82.3
	4 h	5.1 ± 0.5^{e}	28.3 ± 2.9^{a}	82.0

Values within the column of each conc. Differ significantly ($P < .05$) when they are not sharing any common superscript letter.

Table 2 illustrates the viability of the *Cryptosporidium* oocyst of treated samples and the control group along the different contact times. Overall, there was a significant reduction of the viability between difference between all nano-silver treated samples at all used doses over all contact times and the control ($P < .05$). Additionally, dosing of AgNPs at a rate of 0.05 ppm significantly reduced the viable oocyst oocyst count particularly at 1 and 4 h than those of 30 min. Besides, we noticed an increase in the viability of the oocysts after treatment by AgNPs at 2 and 4 h than those of 30 min contact time. The inhibition percent of the oocyst viability ranged from 78.3 to 97.3 for 0.05 concentration at 2 h and 1 ppm at 30 min, respectively, which represents a great reduction in the viability of CP oocyst considering the lower dosages of AgNPs as shown in Table 2.

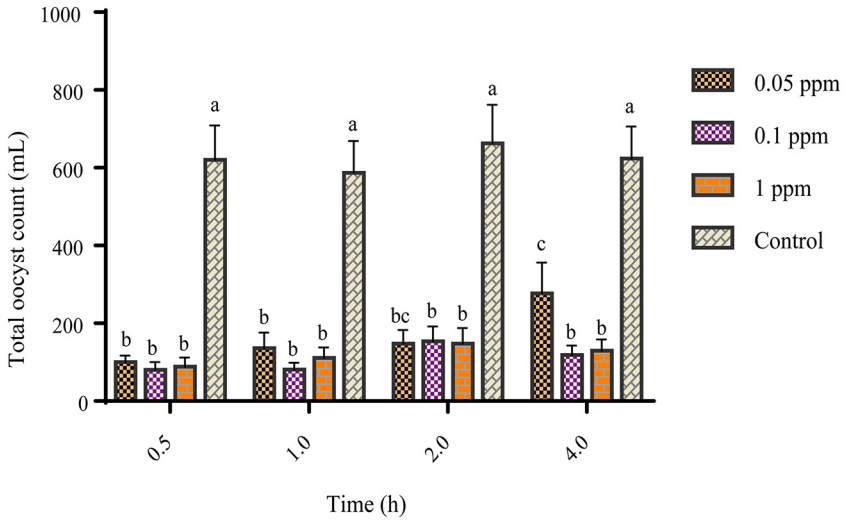

Fig. 4. Comparison of the total *Cryptosporidium* count between AgNPs treated and control groups. ^{a,b,c}superscript differ significantly ($P < .05$), when they are not sharing any common letter.

Table 3
C. parvum infection rate and count in laboratory mice inoculated with oocysts exposed to AgNPs in relation to dose and contact times.

AgNPs concentration	Contact times	Infection rate	Mean oocysts counts/ infected mouse
Control (G4)	–	6/6	1450 ± 13.83
0.05 ppm (G1)	30 min	2/6	266 ± 2.65
	1 h	1/6	357 ± 1.5
	2 h	2/6	390 ± 3. 3
	4 h	1/6	728 ± 6.45
0.1 ppm (G2)	30 min	1/6	232 ± 0.96
	1 h	0/6	0
	2 h	1/6	413 ± 1.12
	4 h	1/6	319 ± 2.22
1.0 ppm (G3)	30 min	0/6	0
	1 h	1/6	290 ± 1.80
	2 h	2/6	267 ± 3.76
	4 h	2/6	335 ± 4.54

Interestingly, the in-vivo study through experimental infection of *C.* oocyst to mice showed a slight dissimilar trend than the in vitro study. In fact, all groups from G1 to G3 and over all contact times showed a significant reduction of *C.* oocyst count compared to the control that allowed the establishment of infection in all the mice inoculated (6/6) as shown in Table 3. However, the groups infected with *C. parvum* exposed to AgNPs at 1 ppm for 30 min and 0.1 ppm for 1 h failed to achieve infection in laboratory animals (0/6 infection rate), with non-detected *C.* oocyst. This make a robustness to our in-vitro study and might confirm that the dye permeability assays slightly underestimated inactivation in comparison to mouse infectivity assay (Table 3 and Fig. 5).

In this study, nano-Ag showed variable but effective reduction in both *C. parvum* count and viability in different water samples. These effects are probably exerted through its cytotoxic and cell inhibitory action, which are supported by the well-established antibacterial effects of AgNPs (Zheng et al., 2008). The anti-protozoal property of AgNPs has been previously discussed on various parasites, including the *Giardia*, *Leishmania*, *Entamoeba*, *Plasmodium*, *Toxoplasma* and insect larvae (Adeyemi et al., 2017; Said et al., 2012; Ullah et al., 2018). AgNPs also showed significant anti-leishmanial effects by inhibition of promastigotes propagation and metabolic activity (Allahverdiyev et al., 2011). In this context, two hypotheses have been adapted to explain the destructive effect of NPs over *C. parvum* oocysts; First, it acts on the surface of the parasites as theorized by Choi and Hu (2008) who suggested that NPs, via ROS generations, could impair parasites surface lipophosphoglycan and glycoprotein molecules, responsible for infection, thereby resulting in suppression of parasitic infection. Chang et al. (2012) concluded that NPs can directly diffuse into the cell through the pores present in cell membrane due to their smaller size particles, which are supposed to induce toxic effects on eukaryotic cells including protozoal cells. Second; Intracellularly, the nanoparticles might bind with DNA molecules and disturb the helical structure by cross-linking within and between nucleic acid strands (Stohs and Bagchi, 1995). Additionally, NPs inside the eukaryotic cells can disrupt biochemical processes (Kim et al., 2000).

In the current study, both *C. parvum* oocysts count and viability were reduced by different AgNPs doses in an ascending manner. In both in-vitro and in-vivo experiments, the overall best reduction was obtained at AgNPs concentration of 1 ppm and the least reduction was encountered at 0.05 ppm. The obtained results agreed with those of Cameron et al. (2016) who reported that AgNPs at dosages of 0.005 and 500 μg/mL significantly reduced the viability of *C. parvum* oocyst in a dose-dependent way by excystation assay and shell/sporozoite ratio. Similarly, Saad et al. (2015) found that *C. parvum* oocysts isolated from humans and animals' feces became inactive after treatment by NPs. The oocyst inactivation was attributed to the changes imposed by NPs in the structure of its wall.

The dose-response pattern obtained in the study may refer typically to a hormetic effect. Hormesis is a dose-response phenomenon that describes growth stimulation at low doses and growth inhibition at high doses (Choi et al., 2018). Hormesis is considered to be an adaptive response of biological systems to moderate environmental challenges (Jiao et al., 2014). A hormetic response has been previously encountered with low concentrations of Ag^+ ions and AgNPs, which enhanced the growth of *E. coli* (Xiu et al., 2012; Fauss et al., 2014). The hormetic effects of AgNPs were also observed in several studies on different human cell lines (Arora et al., 2008; Jiao et al., 2014; and Sthijns et al., 2017). Moreover, Iavicoli et al. (2018) found that the stimulatory action of low concentrations of AgNPs was greater for nanoparticles with a smaller diameter than those with a greater size. In the current study, the average diameter of the used AgNPs was from 8.20 to 42.1 nm.

Regarding contact time effects, the present study showed variable effects within each AgNPs concentration at different contact times. In vitro study, the highest inactivation occurred at a concentration of 1 ppm at 30 min contact time (97.2%), followed by the concentration of 0.1 ppm at 1-h contact time (94.4%). However, at 1 ppm concentration, the viability of the oocysts showed a significant increase at 2 and 4 h contact times in comparison to the viability at 30 min (Table 2). However, in the vivo study, the highest inactivation of 100% was obtained at a concentration of 1 ppm at 30 min and 0.1 ppm at 1 h contact time. Overall, there were rebound increase in the count and the viability of CP at 2 and 4 h contact times and this was also confirmed through the experimentally infected amice. The exact explanations are still uncertain and may be attributed to the source of the collected water samples, which came from ordinary chlorinated drinking water sources in Assiut City and this water was not de-chlorinated before usage. It is

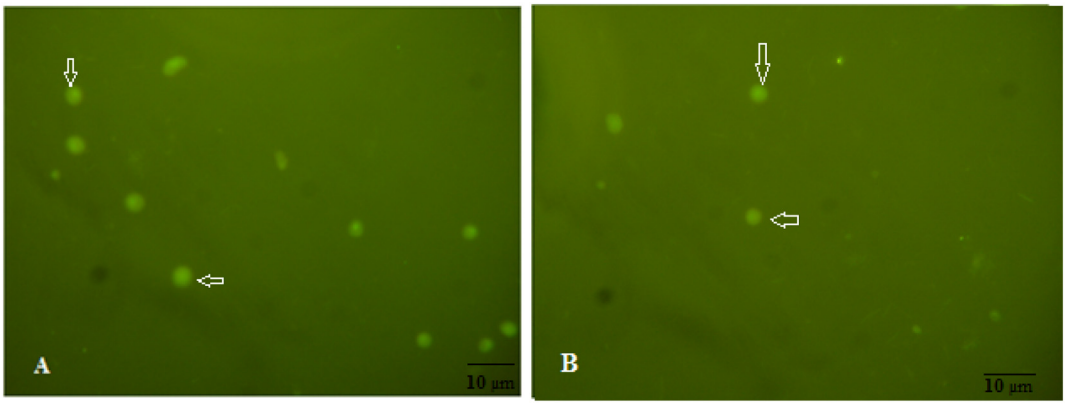

Fig. 5. *C. parvum* oocysts labeled with DFA, detected in feces of mice infected with *C. parvum* not exposed to AgNPs (A) and *C. parvum* exposed to AgNPs (B) ×400.

well-known that the stability of nanoparticles in natural or wastewater is related to the interactions between the nanoparticles and micro-environmental factors. The presence of natural organic matter or inorganic ligands in water may modify the bioavailability and toxicological effects of nanoparticles (Cameron et al., 2016; Zheng et al., 2017). This fact should also be considered when analyzing the results of this study. According to Fabrega et al. (2011) and Farghali et al. (2019) higher amounts of chlorides or organic matter, could produce largely insoluble aggregation with NPs in long contact, which reduces the physical contact between pathogen and the AgNPs' surfaces, thereby reduce its disinfectant effects on *C. parvum* oocysts. Xiu et al. (2012) also reported that AgNPs may lead to different effects in different biological systems. Additionally, after AgNPs treatment, Su et al. (2014) identified two subpopulations of *C. parvum* oocysts; a persistent and a sensitive subpopulation, which was phenotypically distinct. While, the persistent subpopulations of oocysts were found to contain intact sporozoites, the sensitive oocysts contained sporozoites with altered structure, but with intact oocyst wall. In our opinion, possible phenotypic heterogeneity of the *C. parvum* oocysts in our samples may explain the initial oocysts inactivation at short contact time followed by an increase in oocysts viability at long contact times. Walker et al. (2001) also stated that subpopulations within *C. parvum* sample can lead to substantial variations in the oocyst viability.

Ultimately, there are two main reasons that make the present study distinctive and novel. First, this study was conducted on *C. parvum* isolated from the local water samples and not from laboratory preserved species. Second, this study investigated the disinfectant effects of recent promising element at nanoparticles scale (AgNPs) on one of the most resistant pathogens (*C. parvum*). This in our opinion gives a true evidence of practicality and realism of using AgNPs to treat and disinfect drinking water on the real world.

4. Conclusion

The extreme biological hazard of *Cryptosporidium* oocysts enabled these experiments to be conducted in real drinking water to investigate the impact of nanoparticles at concentrations close to their allowable limit in the water. Our results indicated that shorter contact times are recommended for better *C.* oocyst inactivation (either 30 min at 1 ppm, or 1 h at 0.1 ppm concentrations). Additionally, gaining of safe, effective, and cheap water disinfectant against *C. parvum* contamination and perhaps against many other parasites and microbial ones is possible using nanotechnologies. In this sense, further larger-scale research is recommended to determine more effective and safe concentrations of AgNPs with proper contact times for the control of *C. parvum* oocysts.

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Declaration of competing interests

None.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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