



Resistance of bacterial pathogens to calcium hypochlorite disinfectant and evaluation of the usability of treated filter paper impregnated with nanosilver composite for drinking water purification



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ABSTRACT

Objectives: Innovative techniques are urgently required to remove pathogenic bacterial contamination of drinking water. This study aimed to evaluate the biocidal activity of calcium hypochlorite [Ca(OCl)₂], silver nanoparticles (AgNPs) and Ca(OCl)₂/AgNPs composite against bacteria isolated from drinking water supplies (tap and hand pump water). A field trial was subsequently performed to evaluate the efficacy of a biocidal filter paper containing Ca(OCl)₂/AgNPs composite against existing pathogenic bacteria and indicator coliform bacteria.

Methods: A total of 100 water samples were collected from the main source and water troughs used for cattle drinking and were examined for the presence of pathogenic bacteria. The susceptibility of 60 isolated strains to Ca(OCl)₂, AgNPs and Ca(OCl)₂/AgNPs was evaluated by the broth macrodilution method. The field trial examined different water samples collected from water supplies (pre- and post-treatment) using filter paper impregnated with Ca(OCl)₂/AgNPs.

Results: Ca(OCl)₂ loaded on AgNPs at a concentration of 1.5 mg/L showed a lethal effect (100%) on *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* following 180 min of exposure. Furthermore, filter paper impregnated with Ca(OCl)₂/AgNPs exhibited a biocidal effect (100%) against existing pathogenic bacteria, bacterial total viable count, total coliform count and faecal coliform count.

Conclusions: Use of the disinfectant Ca(OCl)₂ against isolated bacteria revealed the existence of bacterial resistance. Enhancement of the biocidal effect of Ca(OCl)₂ using AgNPs was reliable, proving that Ca(OCl)₂/AgNPs composite had a biocidal effect against isolated bacteria at 1.5 mg/L as well as inactivation of coliforms and pathogenic bacteria during percolation through bactericidal filter paper.

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1. Introduction

Despite advances in the field of water management practices, waterborne pathogens remain one of the main challenges and threats both to human and animal health [1]. *Escherichia coli* is the main recognised indicator micro-organism of faecal contamination in water sources [2,3]. The disinfection process is considered the main step to ensure water safety and to protect public and animal health, and many scientists have been exploring various methods to improve its performance through disinfection strategies for water treatment [4].

Different methods of disinfection (chemical, physical and photochemical) are essential for the treatment of contaminated water. Chemical disinfectants such as calcium hypochlorite [Ca(OCl)₂] act by oxidation of the chemical substance itself, which

determines the extent of damage of bacterial cell walls [5]. Chlorine remains a popular disinfectant owing to its ease of application, low cost [6], reaction with the cell membrane, and induction of cell lysis and microbial death [7].

In terms of the resistance profile of bacteria isolated from water supplies to common disinfectants used for water purification, it has been found that free chlorine at 0.5 mg/L was ineffective in eliminating bacterial isolates and that a high level of *E. coli* were tolerant to chlorine [8]. Further treatment at higher chlorine concentrations was more effective in inactivating *E. coli* isolates, with an optimum dose of 1.5 mg/L. From all of the available hypochlorites, calcium hypochlorite and sodium hypochlorite are most commonly used within the industry for domestic, industrial and commercial water applications [9]. The mode of action of Ca(OCl)₂ in water is attributed to the dissociation of hypochlorous acid, which is formed when chlorine dissolves in water, into hydrogen ions and hypochlorite (OCl⁻). Since two hypochlorous acid molecules are produced from one Ca(OCl)₂ molecule, this

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disinfectant is considered a strong oxidant [10]. *Salmonella* spp. and *E. coli* are more resistant to chlorine disinfection than total coliforms and enterococcal bacteria; use of chlorine at a concentration of 0.2–3.0 mg/L for 30 min led to coliform reductions of 3.5 log, where dosages and exposure times differ due to the varying water qualities [11].

To control waterborne pathogenic bacteria resistant to disinfectants, the use of nanosilver as an antimicrobial agent is attractive as it has no unpleasant effects on the odour, taste and colour of drinking water and its activity against different microorganisms has been shown [12]. Furthermore, the sensitivity of bacterial strains to nanosilver varies within the same species, and nanosilver has almost no adverse impacts on either human or animal health if digested in low amounts. There are numerous applications of silver and silver compounds as antimicrobial agents, portable water filters and medical devices. Sondi and Salopek-Sondi first studied nanosilver as an antimicrobial agent against *E. coli* bacteria [13]. In that study, nanosilver was synthesised through reduction of silver nitrate using ascorbic acid [14]. The antimicrobial activity of nanosilver at a dose of 10 mg/L against the Gram-negative bacterium *E. coli* resulted in 70% inhibition of bacterial growth, whereas a lethal effect (100% inhibition) occurred at 50 mg/L. Gong et al. successfully synthesised magnetic Fe₃O₄@Ag nanoparticles ranging in size from 40–80 nm and showed their ability to inhibit the growth of *E. coli* (Gram-negative) and *Staphylococcus epidermidis* (Gram-positive) [15]. Dankovich et al. found that paper filters impregnated with silver or copper nanoparticles exhibited complete reduction and inactivation of indicator coliform bacteria [16].

This study was therefore designated to evaluate the biocidal efficacy of Ca(OCl)₂ disinfectant for drinking water treatment and to explore its efficacy against waterborne pathogenic bacteria. Subsequently, a field trial was performed using a biocidal filter paper impregnated with Ca(OCl)₂ loaded on silver nanoparticles (AgNPs) to evaluate its lethal effect on pathogenic bacteria and indicator bacterial counts for water purification.

2. Methods

2.1. Study area and period

This study was conducted on small cattle breeder farms located in Beni-Suef (coordinates 29°04'N31°05'E) and El-Fayoum (coordinates 29°30'374"N–30°844'105'E) provinces, Egypt, during the period June 2017 to January 2018. Representative water samples were collected both from the main source and water troughs of two supplies (tap and hand pump water) on examined farms ($n = 10$) in the study areas. Despite these farms continuously using Ca(OCl)₂ disinfectant for water treatment, they were suffering from waterborne diseases both of animals and farm workers.

2.2. Study design

The study protocol included two steps to investigate and improve the hygienic quality of drinking water at small cattle breeder farms. The first protocol aimed to evaluate the biocidal activity of Ca(OCl)₂ disinfectant for drinking water and to determine the extent of its efficacy against waterborne bacteria. Then, a nanosilver composite containing Ca(OCl)₂ was used with the aim of enhancing the disinfectant performance. Stratified water samples were collected from investigated farms for isolation and identification of pathogenic bacteria using biochemical tests. The susceptibility of 60 bacterial strains was evaluated with the tested disinfectant [Ca(OCl)₂], AgNPs and the Ca(OCl)₂/AgNPs composite using the broth macrodilution method. The second protocol was a field trial to evaluate the efficacy of bactericidal filter paper against indicator coliforms and existing pathogenic bacteria. Water samples were examined

bacteriologically prior to and post application of treated filter paper for bacterial total viable counts (TVCs), total coliform counts (TCCs) and faecal coliform counts (FCCs). All collected data were recorded and analysed statistically.

2.3. Water sampling

Under aseptic conditions, a total of 100 water samples were collected from the main source and water troughs used for cattle drinking. The collected samples were obtained from two water supplies (tap and hand pump water) in a 250-mL sterilised tightly capped bottle and were properly labelled. The outlet of both water supplies was thoroughly disinfected using 70% ethyl alcohol and then water samples were taken after allowing water flow. Samples were transferred immediately to the laboratory in an ice box for further microbiological examination according to standard guidelines [17].

2.4. Isolation and identification of bacterial pathogens

Samples were cultured on plate count agar (CM 0325; Oxoid Ltd., Basingstoke, UK) for detection and enumeration of bacterial TVCs using a pour plate method as described by the American Public Health Association (APHA) [17]. TCCs were enumerated on m-Endo LES agar (Difco, Sparks, MD), whereas FCCs were enumerated on M-FC agar (EM Science, Gibbstown, NJ) using a membrane filtration technique according to the APHA [17]. *Klebsiella* spp. and *E. coli* were isolated on MacConkey agar (CM 0115; Oxoid Ltd.) and eosin methylene blue agar (CM 69; Oxoid Ltd.) plates. *Staphylococcus aureus* was isolated on Baird–Parker agar base (CM 0275; Oxoid Ltd.) with egg yolk supplement. Furthermore, for purification, all isolated bacterial colonies were subcultured on nutrient agar medium. Enteric bacteria were identified on the basis of their colony morphology and using API 20E (bioMérieux, Craaponne France). *S. aureus* strains were identified phenotypically by the tube coagulase test and Staph-D32API systems (bioMérieux, Paris, France).

2.5. Evaluating the biocidal activity of calcium hypochlorite

The selected disinfectant [Ca(OCl)₂] is a chemical substance (white powder containing 65% available chlorine and considered a strong oxidant) that is used for disinfection of drinking water as it is effective, easy to use and stable. Furthermore, it has been proven to be effective under field conditions [10]. The efficacy of Ca(OCl)₂ disinfectant was tested against 60 bacterial strains isolated from two different water supplies using the broth macrodilution method as described by Li et al. [18] with minor modifications related to disinfectant concentrations and exposure times. The disinfectant was tested at different concentrations (1.0, 1.5 and 2.0 mg/L) and exposure times (30, 60, 120 and 180 min).

2.6. Synthesis and characterisation of silver nanoparticles

AgNPs were synthesised using a chemical reduction method according to Šileikaitė et al. [19]. AgNPs were morphologically characterised by Fourier-transform infrared spectroscopy (FTIR) and by transmission electron microscopy (TEM) using a JEM-100CX II transmission electron microscope (JEOL Ltd.) in the National Research Center (Cairo, Egypt).

2.7. Evaluation of silver nanoparticles (AgNPs) and Ca(OCl)₂/AgNPs composite

Sixty bacterial isolates were tested against AgNPs at concentrations of 1.5, 3.0 and 5.0 mg/L at different exposure times (30, 60, 120 and 180 min) using the broth macrodilution method. Next, a Ca

(OCl)₂/AgNPs composite was prepared according to Ahmed et al. [20] in order to enhance the disinfectant performance against the tested bacterial strains. A total of 5 mg/L of AgNPs was added to 1.5 mg/L of Ca(OCl)₂ disinfectant. The Ca(OCl)₂/AgNPs composite was shaken well using a magnetic stirrer for 4 h continuously to avoid agglomeration of nanoparticles over the incubation period. The nanocomposite was then centrifuged at 3000 rpm for 15 min and was washed three times with distilled water. Finally, 100 µL (1 × 10⁵ CFU/mL) of freshly prepared bacterial suspension in normal saline was added to 1 mL of Muller–Hinton broth (MHB) and then 1 mL of Ca(OCl)₂/AgNPs composite at different concentrations (0.5, 1.0 and 1.5 mg/L) and exposure times (30, 60, 120 and 180 min) was added and was assayed using the broth macro-dilution method as described previously [18]. Moreover, two sterilised test tubes were used as controls, one containing bacterial inoculum and MHB (negative control) and the second containing Ca(OCl)₂/AgNPs composite and MHB without bacterial inoculum (positive control). Then, 100 µL (1 × 10⁵ CFU/mL) of the tested mixture was spread on the selective agar media, was incubated at 37 °C for 24 h and was investigated for the presence of bacterial growth to detect susceptible and resistant strains of the tested isolates to the nanocomposite. Susceptible strains showed an absence of growth, whilst resistant strains showed bacterial growth on the agar plates.

2.8. Preparation of biocidal filter paper using Ca(OCl)₂/AgNPs composite

The filter paper used was porous and highly absorbent pure cellulose paper of 0.45 mm diameter (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The porosity of the filter paper allows micro-organisms to come into contact with the Ca(OCl)₂/AgNPs composite during water purification. The filter paper was prepared according to the method described by Dankovich and Gray [21] with some modifications. Filter paper was soaked overnight in 15 mL of Ca(OCl)₂ loaded on AgNPs at concentrations of 1.0 mg/L and 1.5 mg/L and was then removed from the solution and rinsed with 70% ethanol, followed by soaking in water for 5 min to remove excess unabsorbed nanocomposite. Finally, the paper was dried in an oven at 60 °C for 1 h. The shape of nanoparticles and their distribution on the filter paper were examined by TEM. Moreover, FTIR was used to determine the structure of Ca(OCl)₂ disinfectant, AgNPs and the Ca(OCl)₂/AgNPs composite.

2.9. Field trial to evaluate the biocidal filter paper

The biocidal activity of the filter paper was tested against total viable bacteria and indicator coliform bacteria (total and faecal coliform) in water samples. Twenty representative water samples were collected from water troughs of both water supplies (tap water and hand pump). Samples were examined bacteriologically prior to and post treatment during passing 100 mL of water

samples on either non-treated filter paper or Ca(OCl)₂/AgNPs-impregnated filter paper for 10 min. All non-treated and treated filter papers were incubated on specific agar media at 37 °C for 24 h. Thereafter, the incubated plates were examined for the absence or growth of pathogenic and indicator bacteria (total and faecal coliform) on filter papers on pre- and post-treated plates using a magnifying lens. Furthermore, all water samples were cultured for TVCs on plate count agar. The targeted bacteria were enumerated on specific media as mentioned above to evaluate the efficacy and usability of the biocidal filter paper. Furthermore, effluent water (water drain after examination of water samples passing through treated filter paper) was examined bacteriologically and the shape of coliform bacteria in the effluent water was observed by scanning electron microscopy (SEM).

2.10. Data analysis

All data were recorded using Microsoft Excel (Microsoft Corp., Redmond, WA) and were then prepared for analysis. The percentages of pathogenic bacteria isolated from different water supplies in the investigated areas as well as the biocidal activity of Ca(OCl)₂, AgNPs and Ca(OCl)₂/AgNPs composite against water-borne pathogenic bacteria were calculated using non-parametric tests (χ^2 test, linear-by-linear association) and one-way analysis of variance (ANOVA) using IBM SPSS Statistics v.22.0 (IBM Corp., Armonk, NY).

3. Results

The percentage of *E. coli* isolates was significantly higher both in tap and hand pump (ground) water trough samples [21/37 (56.8%) and 11/26 (42.3%), respectively; $\chi^2 = 12.32$, $P < 0.05$]. Meanwhile, both *Klebsiella pneumoniae* and *S. aureus* were isolated at the highest percentage in hand pump water [9/26 (34.6%) and 6/26 (23.1%), respectively] compared with tap water [9/37 (24.3%) and 7/37 (18.9%), respectively]. On the other hand, the main sources were free from bacterial contaminants, especially the hand pump supply (Table 1).

The biocidal efficacy of Ca(OCl)₂ disinfectant against *E. coli* and *S. aureus* isolates did not exceed 50% at a concentration of 2.0 mg/L after 120 min of exposure, whilst its efficacy against *K. pneumoniae* was 70% at the same concentration and exposure time. In contrast, exposure of pathogenic bacteria to Ca(OCl)₂ disinfectant at a concentration of 1.0 mg/L showed that the susceptibility both of *E. coli* and *S. aureus* strains was 30% compared with 50% for *K. pneumoniae* after 120 min of exposure ($P \leq 0.001$) (Table 2).

The susceptibility of pathogenic bacteria isolated from different water supplies to AgNPs (Table 3) showed the susceptibility of *K. pneumoniae*, *E. coli* and *S. aureus* to nanosilver was 100%, 90% and 90%, respectively, at a concentration of 5.0 mg/L after 180 min of exposure compared with 90%, 80% and 70%, respectively, after 120 min of exposure ($P \leq 0.001$). Meanwhile, all of the isolated

Table 1
Percentage of pathogenic bacteria isolated from different examined water sources.

Water sample	Total no. of samples examined	No. (%) of samples positive	No. (%) of bacterial isolates		
			<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i>
Tap water					
Main source	10	2 (20.0)	0	0	2 (100.0)
Water trough	40	37 (92.5)	21 (56.8)	7 (18.9)	9 (24.3)
Hand pump					
Main source	10	0	0	0	0
Water trough	40	26 (65.0)	11 (42.3)	6 (23.1)	9 (34.6)
Total	100	65 (65.0)	32 (49.2)	13 (20.0)	20 (30.8)

The association between different bacterial isolates is statistically significant: $\chi^2 = 12.32$, $P < 0.05$.

Table 2
Biocidal activity of calcium hypochlorite [Ca(OCl)₂] disinfectant against isolated bacterial pathogens.

Bacterial species (n)	Ca(OCl) ₂ concentration (mg/L)	Biocidal activity of Ca(OCl) ₂ (%) at:								P-value
		30 min		60 min		120 min		180 min		
		S	R	S	R	S	R	S	R	
<i>Escherichia coli</i> (20)	1.0	10	90	30	70	30	70	60	40	0.001
	1.5	10	90	30	70	40	60	60	40	
	2.0	30	70	40	60	50	50	70	30	
<i>Staphylococcus aureus</i> (20)	1.0	10	90	10	90	30	70	50	50	0.001
	1.5	20	80	20	80	30	70	50	50	
	2.0	20	80	40	60	40	60	60	40	
<i>Klebsiella pneumoniae</i> (20)	1.0	40	60	50	50	50	50	60	40	0.05
	1.5	50	50	60	40	60	40	70	30	
	2.0	60	40	60	40	70	30	70	30	

S, susceptible (absence of bacterial growth); R, resistant (presence of bacterial growth).

The χ^2 association between *E. coli* strains and the biocidal activity of Ca(OCl)₂ at concentrations of 1.0 mg/L and 2.0 mg/L and exposure times of 30 min and 120 min was statistically significant ($P \leq 0.001$). For *S. aureus*, the χ^2 association was statistically significant ($P \leq 0.001$) at concentrations of 1.0 mg/L and 2.0 mg/L and exposure times of 60 min and 180 min. Whilst the susceptibility of *K. pneumoniae* strains to Ca(OCl)₂ was statistically significant at concentrations of 1.0 mg/L and 2.0 mg/L and exposure times of 30 min and 120 min ($P \leq 0.05$).

Table 3
Biocidal activity of silver nanoparticles (AgNPs) against isolated bacterial pathogens.

Bacterial species (n)	AgNPs concentration (mg/L)	Biocidal activity of AgNPs (%) at:								P-value
		30 min		60 min		120 min		180 min		
		S	R	S	R	S	R	S	R	
<i>Escherichia coli</i> (20)	1.5	40	60	50	50	60	40	80	20	0.001
	3.0	40	60	40	60	50	50	70	30	
	5.0	50	50	60	40	80	20	90	10	
<i>Staphylococcus aureus</i> (20)	1.5	50	50	60	40	60	40	70	30	0.05
	3.0	50	50	60	40	70	30	70	30	
	5.0	60	40	70	30	70	30	90	10	
<i>Klebsiella pneumoniae</i> (20)	1.5	40	60	60	40	80	20	80	20	0.001
	3.0	50	50	60	40	80	20	80	20	
	5.0	60	40	80	20	90	10	100	0	

S, susceptible (absence of bacterial growth); R, resistant (presence of bacterial growth).

The χ^2 association between *E. coli* strains and the biocidal activity of AgNPs was statistically significant at concentrations of 1.5 mg/L and 5.0 mg/L and exposure times of 60, 120 and 180 min ($P \leq 0.001$). For *S. aureus*, the χ^2 association was statistically significant at concentrations of 1.5 mg/L and 5.0 mg/L and an exposure time of 180 min ($P \leq 0.05$). Whilst the susceptibility of *K. pneumoniae* strains to AgNPs was statistically significant at concentrations of 1.5 mg/L and 5.0 mg/L and exposure times of 30, 60 and 180 min ($P \leq 0.001$).

bacteria showed resistance to AgNPs that exceeded 30% at concentrations of 1.5 mg/L and 3.0 mg/L at exposure times of 30 min and 60 min. Characterisation of AgNPs by TEM revealed the morphological shape and size of nanosilver. Furthermore, the nanoparticle size ranged between 3.45–28.85 nm and its shape was spherical and elongated (Fig. 1A,B).

The biocidal efficacy of Ca(OCl)₂/AgNPs composite against bacterial isolates showed that the bacterial isolates from different water sources were highly susceptible (100%) to Ca(OCl)₂/AgNPs composite at a concentration of 1.5 mg/L after 180 min of exposure. Meanwhile, the efficiency after 120 min of exposure was 90% for *S. aureus* and 80% each for *K. pneumoniae* and *E. coli*, respectively ($P < 0.001$) compared with those exposed to lower dose (concentration of 0.5 mg/L) at different exposure times (Table 4).

The efficacy of Ca(OCl)₂/AgNPs-impregnated filter paper against indicator bacteria in different water sources is shown in Table 5. It was found that prior to treatment with biocidal filter paper the TVCs, TCCs and FCCs in the tap water source ($4.52 \times 10^5 \pm 2.1 \times 10^3$ CFU/mL, 46.0 ± 3.1 CFU/100 mL and 14.0 ± 0.24 CFU/100 mL, respectively) were high exceeding the World Health Organization (WHO) limits of 1.0×10^2 CFU/mL for TVCs and 0.0 CFU/mL for TCCs, compared with hand pump water ($2.23 \times 10^3 \pm 1.3 \times 10^2$ CFU/mL, 18 ± 1.2 CFU/100 mL and 4.0 ± 0.36 CFU/100 mL, respectively), in addition to the presence of bacterial growth (*E. coli*, *S. aureus* and *K. pneumoniae*) in water samples. Meanwhile, the use of treated filter paper impregnated with Ca(OCl)₂/AgNPs showed a high reduction both in existing pathogenic

bacteria and indicator bacterial counts close to the maximum permissible limit. Moreover, the entire water samples required 1.5 mg/L of Ca(OCl)₂/AgNPs for complete destruction of pathogenic bacteria (*E. coli*, *S. aureus* and *K. pneumoniae*) in examined water samples in addition to filtrated effluent water (drain water) compared with those exposed to a lower dose (a concentration of 1.0 mg/L induced $2.1 \times 10^2 \pm 1.0 \times 10$ CFU/mL and $1.1 \times 10^2 \pm 0.81 \times 10$ CFU/mL). Furthermore, the shape of the nanoparticles and their distribution on the Ca(OCl)₂/AgNPs-impregnated filter paper was determined by TEM, showing that the morphological shape of Ca(OCl)₂/AgNPs on the biocidal filter paper was spherical and elongated. Furthermore, the diameter of the nanoparticles ranged between 7.68–14.34 nm (Fig. 1C,D). Meanwhile, SEM of coliform bacteria before exposure to Ca(OCl)₂/AgNPs-impregnated filter paper and indicator coliform bacteria in the effluent (water drain) after percolation on treated filter paper showed changes in the bacterial cell wall structure, which became scaly, as well as cytoplasm leakage (Fig. 1E,F). Furthermore, characterisation of Ca(OCl)₂/AgNPs by FTIR indicated comparable peaks and new peaks corresponding to the Ca(OCl)₂ (2480 cm^{-1}) appeared in FTIR spectrum, confirming the successful loading of Ca(OCl)₂ on the AgNPs (Fig. 2).

4. Discussion

In this study, the relationship between the presence of waterborne pathogenic bacteria in water troughs and their

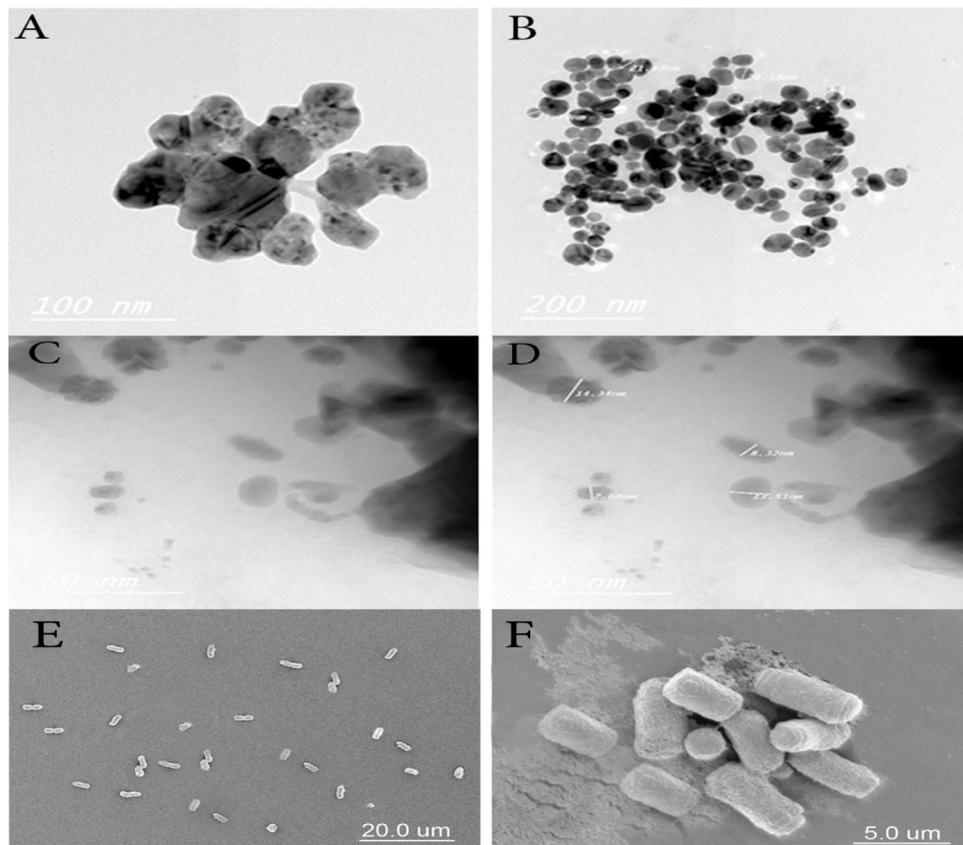


Fig. 1. (A,B) Transmission electron microscopy (TEM) images of silver nanoparticles showing the morphological shape (A) and size (B). (C,D) TEM images of the calcium hypochlorite/silver nanoparticles [$\text{Ca}(\text{OCl})_2/\text{AgNPs}$] composite showing the morphological shape (C) and diameter (D) on the biocidal filter paper. (E,F) Scanning electron microscopy images of indicator coliform bacteria prior to exposure to $\text{Ca}(\text{OCl})_2/\text{AgNPs}$ -impregnated filter paper (E) and after percolation on biocidal filter paper (F).

Table 4
Biocidal activity of calcium hypochlorite loaded on silver nanoparticles [$\text{Ca}(\text{OCl})_2/\text{AgNPs}$] against isolated bacterial pathogens.

Bacterial species (n)	$\text{Ca}(\text{OCl})_2/\text{AgNPs}$ concentration (mg/L)	Biocidal activity of $\text{Ca}(\text{OCl})_2/\text{AgNPs}$ (%) at:								P-value
		30 min		60 min		120 min		180 min		
		S	R	S	R	S	R	S	R	
<i>Escherichia coli</i> (20)	0.5	40	60	60	40	70	30	90	10	0.001
	1.0	40	60	70	30	70	30	90	10	
	1.5	50	50	70	30	80	20	100	0	
<i>Staphylococcus aureus</i> (20)	0.5	50	50	50	50	70	30	80	20	0.001
	1.0	50	50	60	40	70	30	90	10	
	1.5	60	40	70	30	90	10	100	0	
<i>Klebsiella pneumoniae</i> (20)	0.5	50	50	70	30	80	20	100	0	0.05
	1.0	60	40	70	30	70	30	100	0	
	1.5	70	30	70	30	80	20	100	0	

S, susceptible (absence of bacterial growth); R, resistant (presence of bacterial growth).

The χ^2 association between *E. coli* strains and biocidal activity of $\text{Ca}(\text{OCl})_2/\text{AgNPs}$ composite was statistically significant at concentrations of 0.5 mg/L and 1.5 mg/L and an exposure time of 180 min ($P \leq 0.001$). For *S. aureus*, the χ^2 association was statistically significant at concentrations of 0.5 mg/L and 1.5 mg/L and exposure times of 60, 120 and 180 min ($P < 0.001$). Whilst the susceptibility of *K. pneumoniae* strains to $\text{Ca}(\text{OCl})_2/\text{AgNPs}$ composite was statistically significant at concentrations of 0.5 mg/L and 1.5 mg/L and an exposure time of 30 min ($P \leq 0.05$).

susceptibility to $\text{Ca}(\text{OCl})_2$ disinfectant, AgNPs and $\text{Ca}(\text{OCl})_2$ loaded on AgNPs was studied. It was found that drinking water troughs were highly contaminated with *E. coli* bacteria at small cattle breeder farms that used tap water as the main source. Meanwhile, hand pump (ground) water troughs had the highest percentage of isolated bacteria such as *K. pneumoniae* and *S. aureus*, which was attributed to the absence of hygienic measures and the absence of sanitisers used for cleaning of water troughs that should be applied for protecting drinking water supplies from bacterial contaminants. These findings are in accordance with Mohammed [22] who

found that the highest percentage of isolated bacteria from tap water of small cattle breeders was *E. coli* (48.0%) and *Streptococcus faecalis* (44.0%), followed by *K. pneumoniae* (28.0%) and *S. aureus* (20.0%), whereas the percentages of *E. coli* and *S. aureus* in ground water were 28.0% and 24.0%, respectively. On the other hand, Hassouna et al. showed that tap water was free from *E. coli* bacteria, whereas ground water contained *E. coli* (16.6%) [23]. Aragan revealed the importance of drinking water for dairy cattle in transmission of pathogenic bacteria that caused the occurrence of some diseases such as enteritis, mastitis and calf diarrhoea [24].

Table 5
Biocidal activity of filter paper impregnated with calcium hypochlorite loaded on silver nanoparticles [Ca(OCl)₂/AgNPs] against indicator bacteria.

Water supply	Ca(OCl) ₂ /AgNPs concentration (mg/L)	TVC (CFU/mL)		TCC (CFU/100 mL)		FCC (CFU/100 mL)	
		Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Tap water	1.0	$4.52 \times 10^5 \pm 2.1 \times 10^{3a}$	$2.1 \times 10^2 \pm 1.0 \times 10^a$	46 ± 3.1^a	0.0	14.0 ± 0.24^a	0.0
	1.5		0.0		0.0		0.0
Hand pump	1.0	$2.23 \times 10^3 \pm 1.3 \times 10^{2b}$	$1.1 \times 10^2 \pm 0.81 \times 10^b$	18 ± 1.2^b	0.0	4.0 ± 0.36^b	0.0
	1.5		0.0		0.0		0.0

TVC, total viable count; TCC, total coliform count; FCC, faecal coliform count.

Within the same column, proportions with different superscript letters differ significantly at $P \leq 0.01$.

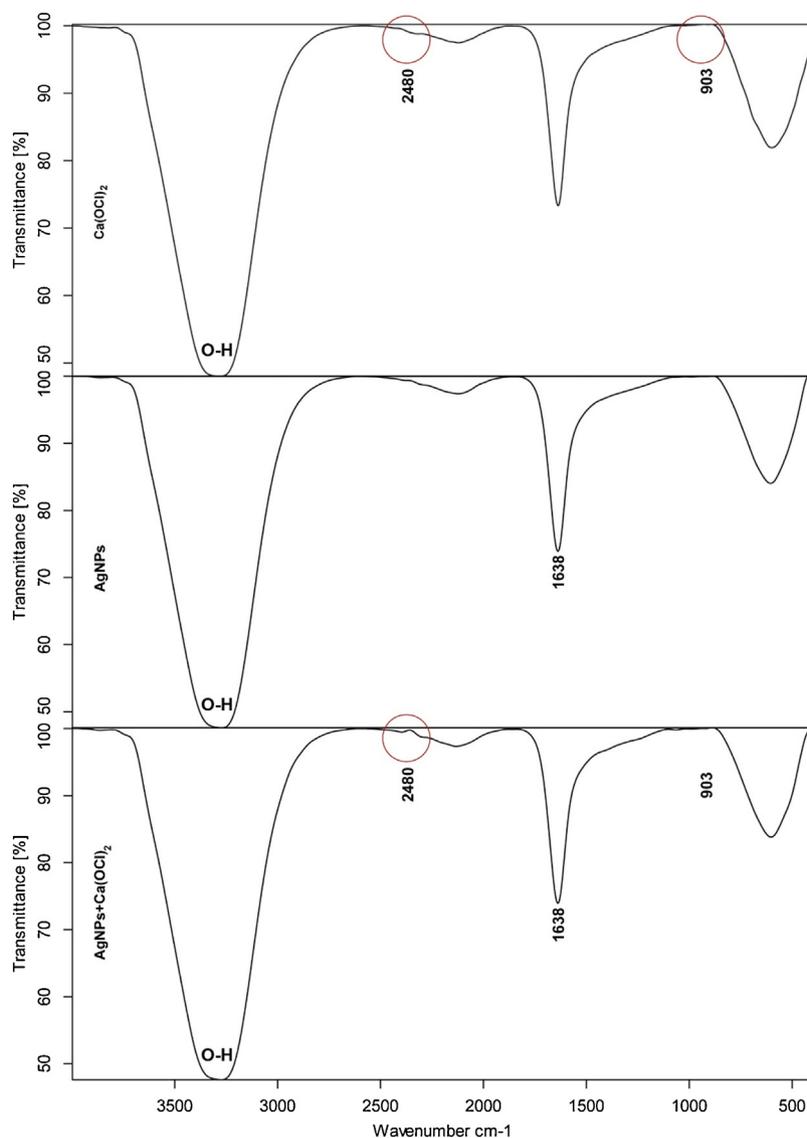


Fig. 2. Fourier-transform infrared spectroscopy (FTIR) spectrum of calcium hypochlorite loaded on silver nanoparticles [Ca(OCl)₂/AgNPs].

E. coli is the most reliable indicator bacteria of water faecal contamination and provides an indication of the hygienic status of freshwater sources [25].

In the present study, the biocidal activity of Ca(OCl)₂ against bacterial strains isolated from different water supplies showed that the susceptibility of *K. pneumoniae* and *E. coli* did not exceed 70% at the highest concentration after 180 min of exposure. Furthermore, its efficacy against *S. aureus* strains was 60%. These results showed that the susceptibility of bacterial strains was

variable within the same species in addition to between other isolated strains from different water sources. Bacterial susceptibility was influenced by Ca(OCl)₂ concentration and exposure time; moreover, continuous usage of Ca(OCl)₂ for water purification could result in the development of resistant bacterial strains. These results are in accordance with Wojcicka et al. who showed that the variability in bacterial susceptibility to disinfection events and their ability to develop a resistant profile might be due to continuous exposure of micro-organisms to disinfectant [26].

Other literature reported that the main components of the bacterial outer membrane, including proteins, fatty acids and phospholipids, could reduce the disinfection performance [27,28].

The biocidal efficacy of AgNPs exceeded 80% both against *E. coli* and *S. aureus*, whilst it reached a lethal effect (100%) against *K. pneumoniae* at the highest concentration (5.0 mg/L) after 180 min of exposure compared with other concentrations and exposure times. In contrast, Moustafa found that the use of AgNPs at a concentration of 676.5 mg/L led to complete removal of total coliform bacteria in water, reaching zero after 15 min of exposure [29]. Furthermore, Tran et al. and Nawaz et al. showed that there was a positive correlation between the AgNP concentration and inhibition of *E. coli* bacteria [30,31]. Moreover, Yang et al. found that the concentration of AgNPs used for inactivation of bacteria in water ranged from 5.4–108 ppm [32]. The minimum inhibitory concentrations (MICs) were dependent upon the diameter of the nanoparticles, the concentration of bacteria and exposure. The present study aimed to improve the performance of $\text{Ca}(\text{OCl})_2$ used for drinking water treatment by loading it on AgNPs to allow the use of lower doses with decreased contact times to achieve the best results. TEM and FTIR were performed to confirm the successful loading of $\text{Ca}(\text{OCl})_2$ disinfectant onto the AgNPs. In the FTIR spectrum, the chemical bonds in the $\text{Ca}(\text{OCl})_2$ were identified by the band that is centred at 903 cm^{-1} , which is attributed to $\text{M}-\text{O}-\text{M}$ vibration; this band, like the $\text{M}-\text{O}-\text{H}$ bending, involves the oxygen metal ion translational motion in the $\text{Ca}(\text{OCl})_2$ solution [33]. The strong broad band at 3470 cm^{-1} is related to the stretching vibrations of the H-bond of the OH group (ν O-H) in the nanostructures. The bending vibration (δ H_2O) of the H_2O molecules in the interlayers appeared at 1638 cm^{-1} [34]. The peak located at 1638 cm^{-1} in the FTIR spectrum of AgNPs showed its characteristic peak [35]. Furthermore, it has been revealed that the lethal effect of nanocomposite $\text{Ca}(\text{OCl})_2/\text{AgNPs}$ was significantly high (100%) against tested bacterial isolates from different water sources at a concentration of 1.5 mg/L after 180 min of exposure. This effect could be attributed to the ability of silver ions to bind to and penetrate the cell membrane of bacteria and increase its permeability. These findings are in accordance with Morones et al. [36] and Sondi and Salopek-Sondi [13] who revealed that using AgNPs in the treatment of water led to increasing cell membrane permeability and leakage of the cytoplasm of *E. coli* bacteria. Furthermore, the antibacterial effect of AgNPs has been attributed to release of Ag ions from the AgNP surface and binding on thiol groups in membrane proteins and subsequently causing DNA aggregation [37] and inhibiting enzymatic systems of bacteria [38]. To the best of our knowledge, the current study is the first report of the use of $\text{Ca}(\text{OCl})_2$ loaded on AgNPs and testing its efficacy against bacterial isolates. The lethal effect was very obvious against pathogenic bacteria with a short contact time. Moreover, implementation of a field trial using biocidal filter paper impregnated with $\text{Ca}(\text{OCl})_2/\text{AgNPs}$ composite against pathogenic bacteria in water samples, indicator bacteria and bacterial TVCs exhibited a lethal effect (100%) on *E. coli*, *S. aureus* and *K. pneumoniae* in addition to a high reduction in indicator bacterial counts that was close to the maximum permissible limit.

5. Conclusions

Control of waterborne pathogenic bacteria in drinking water sources using common types of disinfectants without evaluating the susceptibility of pathogenic bacteria to these disinfectants may be useless in their control. Enhancement of the disinfectant performance of $\text{Ca}(\text{OCl})_2$ using a $\text{Ca}(\text{OCl})_2/\text{AgNPs}$ composite was performed and the composite had a biocidal effect (100%) against pathogenic bacteria at a concentration of 1.5 mg/L after 180 min of exposure and led to inactivation of coliform bacteria in water

during percolation through a biocidal filter paper impregnated with the nanocomposite. Further studies are needed to improve the performance of common disinfectants used in water treatment through the use of nanoparticles with antimicrobial effect.

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Competing interests

None declared.

Ethical approval

Not required.

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