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The in vitro effect of hypochlorous acid-metal nanoparticles combination on Salmonella under different temperature conditions

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Abstract: Hypochlorous acid (HClO) is an excellent surface disinfectant and classified as nonhazardous but maintaining a steady HClO solution is extremely difficult. This study aimed to mix HClO with various metal nanoparticles (NPs) to improve stability. The efficiency of the prepared solutions against *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Dublin, and *Salmonella* Infantis was assessed using the culture method in five distinct experimental groups at varying temperatures (4 °C, 10 °C, 25 °C, 40 °C, and 50 °C). The type of metal NPs, HClO, and application temperatures utilized in the combined solutions for bacterial decontamination did not result in a significant difference between the investigated *Salmonella* serogroups ($p > 0.05$). At 50 °C, the highest effective antibacterial activity was detected. There was no statistically significant difference across metal NPs effectiveness ratings ($p > 0.05$). The antibacterial activity was highest in the 200 ppm HClO + 100 nm AgONP application. According to our findings, we propose mixing these metal NPs with HClO. Using these particles in conjunction with HClO may be an innovative and cost-effective strategy for increasing antimicrobial activity and combating antibacterial resistance in *Salmonella*.

Key words: Copper, hypochlorous acid, nanoparticles, *Salmonella* spp., silver, zinc

1. Introduction

Hypochlorous acid (HClO) is an endogenous, potent oxidizing agent generated by neutrophils, eosinophils, mononuclear phagocytes, and B lymphocytes in response to damage and infection via the mitochondrial membrane [1,2]. It denatures and aggregates proteins in aqueous solutions by dissociating into H^+ and $HOCl^-$, notably at pH 3 to 6, where it is most active. HClO is effective against a wide spectrum of bacteria at doses ranging from 0.1 to 2.8 g/mL [2,3]. It is an excellent surface disinfectant since it is nontoxic, nonirritating, noncorrosive, effective in various ways, reasonably easy to manufacture, and relatively affordable. The US Environmental Protection Agency classifies it as nonhazardous. Maintaining a steady HClO solution, on the other hand, is exceptionally challenging. When the active chemicals are in solution, they soon return to the brine and lose their disinfectant power. It has a short shelf life and should not be stored for an extended period. It has also been noted that in the presence of organic debris, its efficiency is reduced [3–6].

Metal nanoparticles (NPs) have been employed as antibacterial agents for many years. Metal NPs resistance is less probable than resistance to narrow-spectrum antibiotics [7]. Nanoparticles' antibacterial activities are based on their nano size and unique electrical, chemical, mechanical, and optical characteristics [8,9]. The fact that they have a vast surface area resistant to intense heat treatments increases their demand [10]. Because of their excellent solubility, retention, absorption, or surface binding, naturally or synthetically produced metal NPs have increased chemical activity [11–13]. They are the most promising antibacterial nanomaterials due to their high surface-to-volume ratios and crystalline structures [8].

Salmonella is a gram-negative, rod-shaped, facultatively anaerobic, nonspore-forming, and motile (excluding *Salmonella* typhi and *Salmonella* paratyphi) bacterium, and it belongs to the *Enterobacteriaceae* family. *Salmonella* serovars from various animals can all be regarded as potential human infections. Foodborne *Salmonella* infections frequently result in gastrointestinal symptoms such as diarrhoea, nausea, stomach cramps, and abdominal discomfort [14,15]. *Salmonella* infections and enteric fever are serious public health issues all over the world [16,17]. Raw materials derived from animals and human contamination represent a considerable risk of *Salmonella* infections. The rapid growth of the convenience food industry, the destruction of the microbiota due to

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improper antibiotic use, and an increase in the number of immunocompromised people in the community are thought to be the causes of this problem [17]. Salmonellosis, which progresses with morbidity and mortality at varying rates depending on countries, causes severe financial losses, including medical care and treatment costs, money spent on epidemiological analysis, loss of production, loss of workforce, costs for legal proceedings, and operational losses, as a result of product recalls [18].

In recent years, increasing concerns about antibiotic resistance in bacterial pathogens have led to research on new antimicrobial agents. The results of such research have revealed that applying alternative and more effective sanitization techniques against resistant pathogen bacteria is vital for public health. This study has aimed to evaluate how effective a mixture of HClO and metal NPs was against *Salmonella* at various temperatures.

2. Materials and methods

2.1. Experimental design of this study

Our study consisted of 5 experimental groups.

Group 1:

0.9% saline water (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
200 ppm HClO (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
100 nm CuO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
100 nm ZnO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
100 nm AgO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C).

Group 2:

0.9% sterile saline water (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
200 ppm HClO + 100 nm CuO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
200 ppm HClO + 100 nm ZnO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
200 ppm HClO +100 nm AgO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C).

Group 3:

0.9% sterile saline water (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
100 nm CuO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
200 ppm HClO + 100 nm CuO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C).

Group 4:

0.9% sterile saline water (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
100 nm ZnO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
200 ppm HClO + 100 nm ZnO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C).

Group 5:

0.9% sterile saline water (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
100 nm AgO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C);
200 ppm HClO +100 nm AgO NPs (4 °C, 10 °C, 25 °C, 40 °C, 50 °C).

2.2. Preparation of metal NPs

Three different metal NPs (CuO NPs, ZnO NPs, AgO NPs) with sizes 100 nm (Sigma-Aldrich 792004), (Sigma-Aldrich 721077), (Sigma-Aldrich 730777) were used. A free chlorine concentration of (v/v) 200 ppm, which is used in the disinfection of equipment and surfaces in the poultry industry, is preferred [19].

All HClO solutions were enriched with 200 ppm metal NPs. The solutions were cooled in a refrigerator till 4 °C and 10 °C; heated in a water bath to 25 °C, 40 °C and 50 °C by checking with a thermometer.

2.3. Preparation of bacterial cultures

The reference strains of *Salmonella* Typhimurium (ATCC 14028), *Salmonella* Enteritidis (ATCC 13076), *Salmonella* Dublin (ATCC 15480), and *Salmonella* Infantis (ATCC 51741) were obtained from ISS Collection, Italy. Each *Salmonella* strain was inoculated into 5 mL Brain Heart Infusion Broth (BHI, CM1032, Oxoid, England) and incubated at 37 ± 2 °C overnight. All fresh *Salmonella* cultures were subcultured twice on Plate Count Agar (PCA, CM0463, Oxoid, England). Subcultures on PCA were collected in a mini centrifuge tube using sterile swabs wetted with sterile saline solution. The collected subcultures were diluted with 1000 µL of sterile saline solution and centrifuged at 3000 rpm for one min. The supernatant was discharged, and this step was repeated twice. The obtained pellet was stored for microbiological analysis.

2.4. Application of CuONPs-HClO, ZnONPs-HClO, AgONPs-HClO combinations, and microbiological analyses

According to the experimental design of our study, 1000 µL of each prepared solution (heated/cooled) was added to the *Salmonella* serovars culture pellets. They were homogenised and incubated for 5 min after the homogenate was serially diluted and 100 µL from each dilution was spread on PCA in two parallels. All Petri dishes were incubated at 37 ± 2 °C for 24 h under aerobic conditions, and all colonies were counted. Metal NPs or HClO free sterile 0.9% saline water was used to control all groups.

2.5. Statistical analysis

The trials were performed in duplicate. The bacterial counts were expressed on $\log_{10}$ CFU. *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Dublin, and *Salmonella* Infantis were studied in relation to HClO concentration, CuONPs, ZnONPs, AgONPs, and application temperatures (4 °C, 10 °C, 25 °C, 40 °C, and 50 °C). Bacterial counts were analyzed using a general linear model univariate test (SPSS, Chicago, IL, USA, 1997).

3. Results

This study aimed to assess the antibacterial impact of metal NPs-HClO combinations with various heat treatments. Three metal NP solutions and hypochlorous acid combinations were examined in five experimental groups on four *Salmonella* serotypes (*Salmonella* Typhimurium,

Salmonella Enteritidis, *Salmonella* Dublin, and *Salmonella* Infantis). According to our present knowledge, the impact of HClO mixed with various metal NPs on *Salmonella* serogroups was explored for the first time. Bacterial colonies were counted after 5 min of treatment with several metal NPs and HClO combinations at different temperatures (4 °C, 10 °C, 25 °C, 40 °C, and 50 °C). Metal NPs combined with HClO decreased bacterial viability in a metal NP type and heat treatment-dependent way. All findings were transformed to logarithmic values due to the uneven distribution of colony numbers.

The kind of metal NPs, HClO, and application temperatures utilized in the combined solutions for bacterial decontamination did not result in a significant difference between the studied *Salmonella* serogroups ($p > 0.05$). All groups had the most effective antibacterial activity at 50 °C (Table 1). In the first group, the bactericidal activity of 0.9% saline water, 200 ppm HClO, and each metal NPs individually was evaluated. There was no statistically significant difference in metal NPs effectiveness ($p > 0.05$) (Table 2).

When the combined effects of HClO and metal NPs in the second experimental group were assessed, it was discovered that the application of 200 ppm HClO + 100 nm AgONPs had the strongest antibacterial activity (Table 2).

In groups 3, 4, and 5, the combined effects of metal NPs and HClO were assessed. When the results were analyzed, the difference between CuONPs and the 200 ppm HClO + 100 nm CuO NPs combination was determined to be statistically significant, but no significant difference was discovered between the outcomes of the other applications (Table 2).

4. Discussion

HClO or metal NPs were utilized by a few studies to inactivate or suppress the microbiological development of harmful bacteria [20,21,22,23]. Nonetheless, to the best of our knowledge, no research has been conducted to investigate the combined impacts of HClO and metal NPs, as well as their efficiency at various application temperatures. Although HClO is regarded as a safe antibacterial agent, numerous drawbacks, such as diminished antimicrobial action in the presence of organic matter and poor storage stability, restrict its application [21]. To mitigate these drawbacks, we tested the efficacy of using HClO-metal NPs in conjunction with four *Salmonella* serotypes (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Dublin, and *Salmonella* Infantis).

For an antimicrobial drug to be regarded as appropriate, a 3 $\log_{10}$ CFU (99.9% bactericidal effect) reduction in bacterial colonies must be obtained in vitro [24,25]. Mild heat is frequently used in commercial fruit and vegetable processing operations [26]. Temperature increases are known to cause a reduction in microbiota [27,28].

The control in our investigation was 0.9% saline water with all-temperature applications. The effects of HClO and metal NPs have been studied alone and in combination. Furthermore, only the heat impact was investigated using free 0.9% saline water. The efficacy of various applications was then compared. The logarithmic reduction in 0.9% saline water was determined to be 0.23, 0.23, 0.26, 1.17, and 1.21 at 4 °C, 10 °C, 25 °C, 40 °C, and 50 °C, respectively. Delaquis et al. [29] also revealed that modest heat treatment had been shown to improve the bactericidal efficacy of electrolyzed water sanitisers.

Table 1. Comparison of the activity means of all application temperatures in all experimental groups (Log_{10} CFU±SD)*.

	Colony Count				
Application Temperature	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
4 °C	$5.030^{a} \pm 0.054$	$4.408^{a} \pm 0.058$	$5.021^{a} \pm 0.062$	$5.023^{a} \pm 0.070$	$4.969^{a} \pm 0.067$
10 °C	$4.501^{b} \pm 0.054$	$4.478^{a} \pm 0.058$	$4.751^{b} \pm 0.062$	$4.667^{b} \pm 0.070$	$4.714^{b} \pm 0.067$
25 °C	$4.413^{b} \pm 0.054$	$4.319^{a} \pm 0.058$	$4.664^{b} \pm 0.062$	$4.619^{b} \pm 0.070$	$4.542^{b} \pm 0.067$
40 °C	$3.731^{c} \pm 0.054$	$3.959^{b} \pm 0.058$	$4.074^{c} \pm 0.062$	$4.100^{c} \pm 0.070$	$3.631^{c} \pm 0.067$
50 °C	$3.265^{d} \pm 0.054$	$3.503^{c} \pm 0.058$	$3.402^{d} \pm 0.062$	$3.687^{d} \pm 0.070$	$3.442^{c} \pm 0.067$

*Each column was evaluated within itself separately, values in the same column with different letters are significantly different ($p < 0.05$).
Group 1: 0.9% Saline Water, 200 ppm HClO, 100 ppm CuO NPs, 100 ppm ZnO NPs, 100 ppm AgO NPs
Group 2: 0.9% Saline Water, 200 ppm HClO + 100 ppm CuO NPs, 200 ppm HClO + 100 ppm ZnO NPs, 200 ppm HClO + 100 ppm AgO NPs
Group 3: 0.9% Saline Water, 100 ppm CuO NPs, 200 ppm HClO + 100 ppm CuO NPs
Group 4: 0.9% Saline Water, 100 ppm ZnO NPs, 200 ppm HClO + 100 ppm ZnO NPs
Group 5: 0.9% Saline Water, 100 ppm AgO NPs, 200 ppm HClO + 100 ppm AgO NPs

Table 2. Comparison of the efficient means of the solutions used in all experimental groups at all application temperatures (Log_{10} CFU ± SD)*.

	Experiment solution	**Colony count**
Group 1	0.9% saline water	6.262^{a} ± 0.054
	200 ppm HClO	4.554^{b} ± 0.054
	100 ppm CuO NPs	3.360^{c} ± 0.054
	100 ppm ZnO NPs	3.456^{c} ± 0.054
	100 ppm AgO NPs	3.309^{c} ± 0.054
Group 2	0.9% saline water	6.262^{a} ± 0.051
	200 ppm HClO+100 ppm CuO NPs	3.525^{b} ± 0.051
	200 ppm HClO+100 ppm ZnO NPs	3.539^{b} ± 0.051
	200 ppm HClO+100 ppm AgO NPs	3.208^{c} ± 0.051
Group 3	0.9% saline water	6.262^{a} ± 0.048
	100 ppm CuO NPs	3.360^{c} ± 0.048
	200 ppm HClO+100 ppm CuO NPs	3.525^{b} ± 0.048
Group 4	0.9% saline water	6.262^{a} ± 0.055
	100 ppm ZnO NPs	3.456^{b} ± 0.055
	200 ppm HClO+100 ppm ZnO NPs	3.540^{b} ± 0.055
Group 5	0.9% saline water	6.262^{a} ± 0.052
	100 ppm AgO NPs	3.309^{b} ± 0.052
	200 ppm HClO+100 ppm AgO NPs	3.208^{b} ± 0.052

*Each group was evaluated within itself separately, values in the same column with different letters are significantly different ($p < 0.05$).

When we examined the effect of HClO on our test organisms, we found that the average of the logarithmic reduction values at 4 °C, 10 °C, 25 °C, 40 °C, and 50 °C was 2.33 ± 0.28. This data indicates that 200 ppm HClO is insufficient for 99.9% bactericidal action. The treatment with HClO resulted in the greatest logarithmic decrease at 4 °C (2.48 log_{10} CFU/mL) (Figure 1). Delaquis et al. [30] discovered that cold chlorinated water (4 °C) was more successful in inhibiting *E. coli* O157:H7 growth. The researchers ascribed this to the fact that a 4 °C temperature boosted the sanitiser's stability by reducing chlorine gas evaporation. Some *Salmonella* isolates, according to Mokgatla et al. [31], may survive in the presence of HClO. HClO has been found to have a selective impact on *Salmonella* isolates proliferating exponentially in a poultry slaughterhouse. This is believed to be the first report on HClO-resistant *Salmonella*. Researchers observed a 2.6 log_{10} CFU/ml decrease in *E. coli* and *Salmonella* Infantis colony counts in mildly acidic hypochlorous acid water (containing 50/100 ppm chlorine, pH 6) within 5 s of exposure in a study done by Hakim et al. [32]. According to Klabunde and Richards [33], reactive metal oxide nanoparticles have good bactericidal properties; therefore, investigating other inorganic nanoparticles as antibacterial materials is intriguing.

The maximum antibacterial effect was seen in testing with all-metal NPs. Furthermore, when the application temperature rises, so does the antibacterial activity. Their efficacy at 4 °C did not deliver the expected 3 log_{10} CFU level inhibition of an antibacterial agent. However, it was discovered that the greatest impact was exhibited at 50 °C in all combinations (Figure 1). Metal NPs are expensive technical products; thus, their usage in the food industry is restricted due to cost concerns. In this context, we wanted to create an experimental model based on HClO-metal NP combinations that would give a feasible antibacterial action with HClO acid at lower concentrations.

Electrolyzed water was applied to *E. coli*-inoculated beef carcasses and *L. monocytogenes*-inoculated sausages in research designed by Veasey and Muriana [6]. When compared to controls, infected goods showed little or no decline. Protein concentration as low as 0.1% has been demonstrated to decrease free accessible chlorine and diminish antibacterial action. Metal NPs did not inhibit bacteria at the desired 3 log_{10} CFU level at 4 °C.

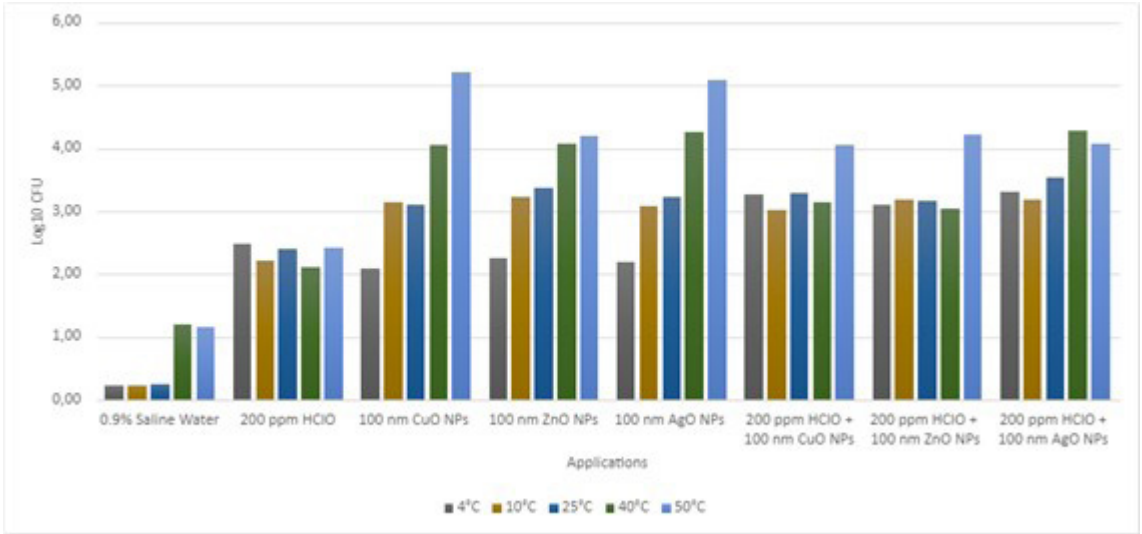

Figure. Logaritmic reduction (Log_{10} CFU) of *Salmonella* serovars in all applications at different temperatures.

The required inhibition was detected in all-metal NPs paired with 200 ppm HClO at all examined application temperatures, including 4 °C. This is due to the fact that HClO is more stable at 4 °C ambient temperature than at higher application temperatures.

5. Conclusion

Since the activities of metal NPs at refrigerator temperatures are limited, it is advised that these particles be combined with HClO. It has been determined that it is an alternate method for eliminating *Salmonella* serotypes, which are a major issue, particularly in the poultry business and the public health issues they would create.

When coupled with metal NPs, HClO acts as a disinfectant, preventing the spread of important pathogens such as *Salmonella*. It offers many of the required properties of an excellent disinfectant: it is simple to use, affordable and has a high safety profile. Further research into the efficiency and stability of HClO and metal NPs in the presence of organic materials is planned.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial and financial relationships that could be construed as a potential conflict of interest.

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