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Evaluation of antimicrobial and sporicidal activity of hypochlorous acid against clinical isolates from diabetic foot gangrene patients

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Diabetic foot ulcers and gangrene, often infected with multidrug-resistant pathogens and resilient bacterial spores, require effective topical antisepsis to prevent systemic infection and promote healing. This study compared the concentration- and time-dependent bactericidal and sporicidal activity of four common disinfectants against clinical isolates from diabetic foot infections. Forty-five clinical swab samples were collected from DFU patients at Baghdad Teaching Hospital. Pathogens were isolated and identified using the Vitek 2 Compact system. Quantitative time-kill assays and dilution-neutralization methods were employed to determine the $\log_{10}$ reduction of vegetative bacteria against different types of disinfectant and exposure times (1 to 30 minutes). *Staphylococcus aureus* was the most frequent isolate (40.8%), next to *Pseudomonas aeruginosa* (24.5%) and *Acinetobacter baumannii* (20.4%). The time-kill study revealed that HOCl (200 ppm) was the most effective and acted the fastest to kill *S. aureus*, achieving a 6.94 ± 0.71 $\log_{10}$ reduction of bacteria within 1 minute Moreover, HOCl also proved highly effective in killing bacteria spores, achieving a 7.87 ± 0.76 $\log_{10}$ reduction in *Clostridium perfringens* spores in 20 minutes. NaOCl (0.05%) showed high bactericidal activity with a 7.60 ± 1.19 $\log_{10}$ reduction against *S. aureus* but with a long exposure time. HOCl 200 ppm showed rapid bactericidal and sporicidal activity against diabetic foot pathogens, which makes it an effective yet safe clinical-grade wound disinfectant.

Keywords: disinfectant; diabetic foot ulcer; sporicidal activity; *Clostridium perfringens*.

Introduction

Diabetic foot ulcers and gangrene remain serious sequelae of diabetes mellitus, usually due to chronic infections from polymicrobial guilds (Sadeghpour Heravi et al., 2019). Typical pathogens are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and spore-forming anaerobes as *Clostridium perfringens*. Rapid wound disinfection is essential to minimize the microbial load and inhibit systemic infection, as well as induce healing.

HOCl, a natural oxidant generated by neutrophils, is commonly used as a topical disinfectant on account of its broad-spectrum antimicrobial activity, minimal cytotoxicity and rapid killing action (Edwards-Jones, 2025). It is most effective at mild acidity where non-ionized HOCl species dominates and readily penetrates the cell wall of microorganisms (Aherne et al., 2022). Its high oxidative capacity results in a swift membrane translocation and cell inactivation of microorganisms, rendering it very appealing for chronic wound irrigation (Boecker et al., 2023). At low to moderate concentrations (typically 20–200 ppm), HOCl is considered non-toxic, non-irritating, and safe for skin and mucosal contact. It does not produce harmful residues and rapidly degrades into chloride ions and water (Vargová et al., 2020).

Sodium hypochlorite (NaOCl) is a popular chlorine disinfectant. It has an antimicrobial effect by denaturing proteins and destabilizing cell membranes. It is considered effective for vegetative bacteria and some spores (Fukuzaki, 2006), however, high concentration or prolonged exposure can lead to tissue irritation and cytotoxicity (Severing et al., 2019; Chung et al., 2022).

A historical wound disinfectant is Eusol which is a combination of boric acid and chlorinated lime (calcium hypochlorite and with boric acid). It has been applied for discharge washout, slough clearance, and infection control. Its action is based on the liberation of oxidative chlorine and the mild antiseptic action that boric acid possesses (Mung'ong'o & Mugoyela, 2007; Shah et al., 2017).

Hydrogen peroxide (H_2O_2) is an oxidative disinfectant that undergoes ROS formation, which leads to membrane damage and oxidative nucleobase damage (Linley et al., 2012). It is a popular topical wound-cleansing agent, but the rapid breakdown and associated cytotoxicity of the form in which it is most widely available renders it inappropriate for use in chronic wound applications (Zhu et al., 2017; Rai et al., 2023).

In spite of their extensive application, studies that comparatively assess the concentration- and time-dependent bactericidal and sporicidal activity of these disinfectants against potential pathogens isolated from diabetic foot gangrene are limited. Hence, this study investigated time-kill kinetics with $\log_{10}$ reduction and kill rates of selected clinically-relevant bacteria and spores to compare the efficacy of HOCl, NaOCl, H_2O_2 and Eusol.

Material and methods

The study was ethically approved by the Human Ethics Committee of the Al-Habbobi Teaching Hospital based on the Declaration of Helsinki under approval number 558, which was dated January 1, 2025. All the subjects received detailed instructions on the procedures of the study and signed informed consent. All patient data was kept confidential in the course of the research.

Isolation of bacteria was performed in the timeframe spanning from October 2025 to December 2025, in which a total of 45 swabs were collected from Baghdad Teaching Hospital. The sample collection adhered to the protocol outlined (MacFaddin, 2000). All samples were transported to the laboratory without delay. The samples were inoculated on Mannitol salt agar, MacConkey agar, blood agar under aerobic conditions and cooked meat agar under anaerobic condition for 24–48 hrs at 37 °C.

The identification of bacterial isolates involved a comprehensive approach involving microscopic, macroscopic examination and biochemical tests. Then the type of isolate was confirmed by the Vitek 2 compact system (Brooks et al., 2010).

Clostridium perfringens isolated from foot gangrene patients were used as the spore forming bacteria in this study. Spore suspensions were prepared by inoculating 0.1 mL of *C. perfringens* culture into 20 mL of cooked meat medium (Oxoid, England). The inoculated

medium was subjected to heat shock at 90 degrees C for 10 minutes to activate spore germination, followed by overnight incubation at 35 °C under anaerobic conditions using an anaerobic incubator (Memmert, Germany) (Byun et al., 2011).

The cooked meat medium culture was then transferred into 200 mL of sporulation medium (Himedia, India) to promote sporulation and incubated under anaerobic conditions at 30 °C for 8 hours. Subsequently, the culture was inoculated into 1.8 liters of sporulation medium and further incubated at 30 degrees Celsius for 4 days under anaerobic conditions until approximately 90% sporulation was achieved. The transition from vegetative cells to spores was monitored using a light microscope (Zeiss, Germany).

When sporulation reached approximately 90%, the suspension was stirred, and spores were harvested by centrifugation using a refrigerated centrifuge (ESCO, Singapore). The initial centrifugation was performed at 1700 ppm for 10 minutes, followed by three washing steps with sterile distilled water at 4 degrees Celsius using centrifugation at 1200 ppm for 10 minutes. After washing, the spore pellet was resuspended in 5 mL of sterile distilled water.

Aliquots of 100 µL of the *C. perfringens* spore suspension were transferred into sterile microcentrifuge tubes and stored in a deep freeze at –80 °C to maintain spore integrity until use. The concentration of the spore suspension was determined by dilution method on modified tryptone egg yolk extract yeast extract medium (Himedia, India) supplemented with 5% egg yolk and 0.5% yeast extract.

The disinfectants evaluated in this study included hypochlorous acid (HOCl), sodium hypochlorite (NaOCl), hydrogen peroxide (H_2O_2), and Eusol. Test concentrations were prepared according to the experimental design as summarized in Table 1.

Table 1
Disinfectant type and concentration

Disinfectant	Concentrations tested
Hypochlorous acid (HOCl)	20, 50, 100, 200 ppm
Sodium hypochlorite (NaOCl)	0.01%, 0.025%, 0.05%
Hydrogen peroxide (H_2O_2)	1%, 3%, 6%
Eusol	25%, 50%, 100% (v/v)

The sporicidal activity of disinfecting agents was evaluated using a dilution-neutralization method according to the preliminary European Standard prEN 1276:2019. Disinfection testing was performed by thoroughly mixing 1 mL of the *Clostridium sporogenes* spore or bacterial suspension at concentraction $1x10^8$ with 8 mL of the disinfectant agent at different concentrations (Table 1) for bactericidal activity and the highest concentration for sporicidal activity mixed previously with 1 mL of turbidity agent (peptone).

Samples of 1 mL were taken from the reaction mixture at 1, 5, 10 and 20 minutes for bactericidal activity and 1, 5, 10, 20 and 30 minutes for sporicidal activity and immediately added to 9 milliliters of neutralizing solution. A neutralizing solution was prepared by dissolving 3 g of lecithin, 30 g of polysorbate 80 (Tween 80), 5 g of sodium thiosulfate, 1 g of L-histidine, and 1 g of glycine in 800 mL of buffered peptone water (20 g/L). The mixture was stirred until complete dissolution of all components was achieved, after which the volume was adjusted to 1 L with Buffered Peptone Water. The solution was aseptically dispensed into autoclavable tubes in 9 mL aliquots and sterilized by autoclaving at 121 °C for 15 minutes. The tubes were allowed to cool to room temperature prior to use.

1 mL of the final neutralized mixture and ten-fold serial dilutions were then spread on trypticase soy agar and incubated under anaerobic conditions for sporicidal activity of *Clostridium perfringens* spores and under aerobic conditions for other pathogenic bacteria at 35 °C for 48 hours. Colonies arising from germinated spores were counted and expressed as colony-forming units per milliliter. Then calculate log reduction as equation below.

Log_{10} reduction was calculated as:

$$\text{Log reduction} = \log_{10}(N_0) - \log_{10}(N_t)$$

where $N_0 = 1 \times 10^8$ and N_t is the surviving count after treatment.

All quantitative data are expressed as mean ± standard deviation (SD) from three independent replicates. To evaluate the effect of disinfectant type and exposure time on the log reduction of microbes, a Two-Way Analysis of Variance (ANOVA) was performed. Following the ANOVA, Fisher's Least Significant Difference (LSD) post-hoc test was used to determine significant differences between specific pairs of means (interaction effects). A probability value of $P < 0.05$ was considered statistically significant. In the results, means that share a common superscript letter are not significantly different from each other.

Results

Forty-five bacterial isolates were isolated from the foot diabetic ulcers. *Staphylococcus aureus* was the predominant species, accounting for 40.8% (n = 20) of the total isolates. The next most frequent species were Gram-negative non-fermenting bacteria, *P. aeruginosa* (24.5%, n = 12) and *A. baumannii* (20.4%, n = 10). These species comprised the majority of the isolates and accounted for 85.7% of the total bacterial burden. All other bacterial species were isolated at lower rates. *Clostridium perfringens* as an anaerobic spore-forming bacterium represented 8.2% (n = 4) of the isolates. Members of the Enterobacteriaceae family had the lowest frequency of detection, represented by *E. coli* (4.1% (n = 2) and *Serratia* spp., which related to only 2.0% (n = 1) of the total isolates. Statistical analysis confirmed that there was a highly significant difference in the frequency of all bacterial species ($\chi^2 = 32.34$, $P < 0.001$) and the observed distributions of *S. aureus*, *P. aeruginosa*, and *A. baumannii* was not attributable to random variation.

The bactericidal efficacy of four disinfectants against the predominant pathogens at different concentrations (Table 2). Hypochlorous acid showed reduction at various concentrations. Reductions for *S. aureus* at 20 and 200 ppm were 2.02 ± 0.13 and 5.83 ± 0.38 log_{10}, respectively. Reductions against *P. aeruginosa* and *A. baumannii* were 1.77 ± 0.20 to 5.65 ± 0.66 log_{10} and 1.41 ± 0.08 to 5.43 ± 0.20 log_{10}, respectively. These findings suggest that hypochlorous acid possesses a potent antimicrobial activity at relatively low concentrations. The bactericidal action of NaOCl showed low reductions at 0.01% (1.98 ± 0.12 log_{10} for *S. aureus*) and high reductions with increased concentration at 0.05% (4.35 ± 0.39 log_{10} for *S. aureus*). H_2O_2 was moderately active, with the maximum log_{10} reduction of 4.57 ± 0.17 against *P. aeruginosa* at 3.0%. Eusol had the least antibacterial action even at high concentration (100%).

Table 2
Frequency of bacterial isolates
from diabetic foot ulcer and gangrene samples

Bacterial Species	Number of isolates	Percentage
Staphylococcus aureus	20	40.8
Pseudomonas aeruginosa	12	24.5
Acinetobacter baumannii	10	20.4
Escherichia coli	2	4.1
Serratia sp.	1	2.0
Clostridium perfringens	4	8.2
Total	49	100.0

Note: $\chi^2 = 32.34$, $P < 0.001$.

The disinfectant efficacy test was assessed by time-dependent tests for bactericidal effects at the highest concentrations as shown in Table 4. HOCl (200 ppm) had an almost broad spectrum effect on bacterial loads, showing 6.94 ± 0.71 log_{10} reduction of *S. aureus* at 1 min and over 8.26 ± 0.49 log_{10} reduction at 20 min. Complete microbial eradication for both *P. aeruginosa* and *A. baumannii* was achieved within 20 min. Sodium hypochlorite (0.05%) also showed bacteria reductions at a slightly slower rate. Initial log_{10} reductions ranged from 3.82 ± 0.28 to 4.67 ± 0.30, reaching maximal reductions of 7.60 ± 1.19 only at 20 min. In contrast, H_2O_2 (3.0%) displayed slower bactericidal activity, with reductions reach to 6.46 ± 0.18 with *S. aureus* at 20 min. Eusol (100%) exhibited the slowest kinetics, achieving maximum reductions of 5.92 ± 0.19 log_{10} for *P. aeruginosa* in 20 minutes, indicating that limited rapid bactericidal efficacy requires prolonged contact time.

The disinfectants were tested at maximum concentrations (Table 5) for the ability to inactivate spores. HOCl (200 ppm) showed an im-

mediate sporicidal effect with a 3.96 ± 0.12 log_{10} and a 7.87 ± 0.76 log_{10} reduction at 1 to 20 min respectively. The efficiency of NaOCl (0.05%) for spore inactivation was slightly lower (7.13 ± 1.35 log_{10} reduction) but required a longer contact time (30 min). In contrast, H_2O_2 (3.0%) and Eusol (100%) showed a gradual and less pronounced effect with maximum log_{10} reductions of 5.3 ± 0.68 and 3.42 ± 0.07 at 30 min, respectively, confirming the potency of HOCl against resistant spores.

Table 3
Bactericidal activity (Log_{10} reduction) at different concentrations (mean ± SD)

Disinfectant	Concentration	*S. aureus*	*P. aeruginosa*	*A. baumannii*
HOCl	20 ppm	2.02 ± 0.13	1.77 ± 0.20	1.41 ± 0.08
	50 ppm	3.69 ± 0.12	3.32 ± 0.23	2.79 ± 0.08
	100 ppm	5.09 ± 0.60	4.81 ± 0.58	4.68 ± 0.04
	200 ppm	5.83 ± 0.38	5.65 ± 0.66	5.43 ± 0.20
NaOCl	0.01%	1.98 ± 0.12	1.97 ± 0.22	1.59 ± 0.13
	0.025%	3.55 ± 0.42	3.43 ± 0.20	2.71 ± 0.19
	0.05%	4.35 ± 0.39	4.82 ± 0.33	3.55 ± 0.35
H_2O_2	0.5%	1.11 ± 0.03	1.93 ± 0.03	1.60 ± 0.03
	1.0%	2.82 ± 0.08	2.58 ± 0.13	2.38 ± 0.16
	3.0%	3.40 ± 0.19	3.17 ± 0.05	4.57 ± 0.17
Eusol	25%	1.01 ± 0.09	0.91 ± 0.12	0.85 ± 0.12
	50%	1.50 ± 0.12	1.38 ± 0.03	1.13 ± 0.08
	100%	4.06 ± 0.19	4.86 ± 0.14	4.58 ± 0.27

Discussion

The bacteriological profile observed in this study confirms that diabetic foot ulcer and gangrene infections are predominantly polymicrobial, with a statistically significant variation in the distribution of bacterial isolates ($\chi^2 = 32.34$, $P < 0.001$). The predominance of *S. aureus* (40.8%) is consistent with numerous studies that identify this organism as the most common pathogen in diabetic foot infections followed by *P. aeruginosa* and *A. baumannii* with 24.5% and 20.4%, respectively. The study results are in agreement with other studies which indicate the *S. aureus* most predominant pathogen in diabetic foot ulcers (British Standards Institution, 2019; Stańkowska et al., 2022). A study conducted by Son et al. (2017) reported that, among 745 isolates, MRSA was the most frequently identified organism (13.7%), followed by *Enterococcus faecalis* (12.6%) and methicillin-sensitive *Staphylococcus aureus* (MSSA) (12.5%).

Table 4
Time-kill assay data table (at highest concentrations) (mean ± SD)

Disinfectant	Concentration	Time, min	*S. aureus*	*P. aeruginosa*	*A. baumannii*
HOCl	200 ppm	1	6.94 ± 0.71	6.03 ± 0.40	5.49 ± 0.66
		5	7.03 ± 0.40	6.49 ± 0.33	6.44 ± 0.49
		10	7.76 ± 0.99	7.12 ± 0.67	7.09 ± 0.65
		20	8.26 ± 0.49	7.65 ± 0.38	7.59 ± 0.42
NaOCl	0.05%	1	4.67 ± 0.30	4.13 ± 0.21	3.82 ± 0.28
		5	5.57 ± 0.42	5.12 ± 0.37	4.93 ± 0.42
		10	6.40 ± 0.72	6.06 ± 0.41	5.56 ± 0.32
		20	7.60 ± 1.19	6.55 ± 0.68	6.15 ± 0.41
H_2O_2	3.0%	1	2.72 ± 0.22	2.08 ± 0.20	1.94 ± 0.18
		5	3.58 ± 0.47	3.12 ± 0.31	2.87 ± 0.29
		10	5.47 ± 0.27	4.63 ± 0.26	5.32 ± 0.21
		20	6.46 ± 0.18	6.07 ± 0.23	5.85 ± 0.22
Eusol	100%	1	2.16 ± 0.06	1.95 ± 0.08	1.59 ± 0.11
		5	2.49 ± 0.14	3.36 ± 0.12	2.08 ± 0.14
		10	3.69 ± 0.18	4.61 ± 0.09	4.35 ± 0.11
		20	5.70 ± 0.26	5.08 ± 0.15	5.92 ± 0.19

Table 5
Sporocidal activity of disinfectants against *Clostridium perfringens* spores

Disinfectant	1 min	5 min	10 min	20 min	30 min
HOCl (200 ppm)	3.96 ± 0.12^{cdfg}	5.01 ± 0.23^{cd}	6.01 ± 0.66^{b}	7.87 ± 0.76^{a}	7.55 ± 0.96^{a}
NaOCl (0.05%)	0.87 ± 0.05^{jk}	2.71 ± 0.36^{hi}	4.02 ± 0.43^{ef}	5.85 ± 0.85^{bc}	7.13 ± 1.35^{a}
H_2O_2 (3.0%)	0.30 ± 0.01^{jk}	1.99 ± 0.41^{i}	3.44 ± 0.17^{fgh}	4.43 ± 0.09^{de}	5.39 ± 0.68^{bc}
Eusol (100%)	0.20 ± 0.01^{k}	0.64 ± 0.06^{jk}	1.09 ± 0.20^{j}	3.13 ± 0.23^{gh}	3.42 ± 0.07^{fgh}

Another study in Iraq indicates that the three most common pathogens isolated from patients with diabetic foot were *S. aureus* (30.1%) *Acinetobacter* spp. (19.1%) and *Pseudomonas aeruginosa* (15%) (Habeeb et al., 2021). Another study found that the the most frequently isolated bacteria were 27 (38.0%) for *P. aeruginosa* (Matar & Saleh, 2024).

At the General University Hospital José María Morales Meseguer, Spain, 55% of the isolated microorganisms were Gram-positive. Among these, *Staphylococcus aureus* was the most common (33%), followed by *Pseudomonas aeruginosa* (12%) (Mercado et al., 2007).

The current study provides a detailed comparison of the bactericidal and sporicidal efficacy of HOCl, NaOCl, H_2O_2, and Eusol. The study results demonstrate that HOCl exhibits the most potent antimicrobial and sporicidal activity, achieving rapid and high reductions in bacterial counts and spores. This finding is consistent with previous studies reporting that HOCl effectively disrupts microbial membranes and penetrates biofilms, leading to rapid bacterial death (Romanowski et al., 2018; da Cruz Nizer et al., 2020).

In another study significant antimicrobial activity was demonstrated, with key pathogens including MRSA, MSSA, *Pseudomonas aeruginosa*, and *Candida albicans* exhibiting >6 log_{10} reductions for bacteria and >3 log_{10} reductions for yeast within less than one minute of exposure (Anagnostopoulos et al., 2018). Standard hypochlorous acid typically achieve a more than 5.0 log_{10} reduction of *Clostridium* spores only after 15 to 30 minutes of contact time (Goda et al., 2017).

Similarly, NaOCl showed strong bactericidal activity, although its killing kinetics were slightly slower than HOCl, which aligns with reports that NaOCl is effective against both Gram-positive and Gram-negative bacteria but requires slightly longer exposure times (Fabrizio et al., 2024).

H_2O_2 exhibited moderate activity in our study, with slower reductions and lower maximum log_{10} reductions. This is in agreement with previous findings that H_2O_2's antimicrobial effect can be limited by the presence of catalase-producing bacteria, which degrade H_2O_2 and reduce its effectiveness (Bezsenyi et al., 2021; Anwar et al., 2024). However, some studies have reported the hydrogen peroxide achieved reductions ≥ 4 log10 CFU/mL) against *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus hirae*, and *Pseudomonas aeruginosa* (Ríos-Castillo et al., 2017).

Eusol showed the weakest activity, consistent with historical data indicating its limited rapid antimicrobial action. The slow kinetics and low maximum reductions suggest that Eusol may be insufficient for situations requiring fast disinfection, although it may still have a role in low-risk applications or for extended exposure (Newsom & Ridgway, 2014).

When comparing the time-kill results of the current study with other studies, the rapid bactericidal effect of HOCl supports its practical application in clinical and laboratory disinfection, particularly in environments with high contamination risks as well as usage of hypochlorous acid for wound management due to safety characteristic (Ulfig & Leichert, 2021).

HOCl is an endogenous molecule, naturally synthesized by the human immune system; during phagocytosis, neutrophils produce HOCl via the myeloperoxidase enzyme to destroy invading pathogens. Because it mirrors this natural physiological process, topically applied HOCl-even at the highly effective concentration of 200 ppm

utilized in our study – is non-cytotoxic to human cells (Lewandowski et al., 2024).

The localized safety profile suggests that HOCl is an ideal candidate for the extensive and repeated irrigation needed for chronic diabetic wound care, providing a therapeutic superiority to traditional antiseptic agents (Irawan et al., 2024). On the other hand, sodium hypochlorite (NaOCl) showed significant antibacterial activity in this study, but the clinical use of NaOCl is limited because of the small margin of safety. The pH and strong oxidative action when NaOCl is used at concentrations needed for adequate reduction of microbes, such as the 0.05% concentration we tested, can rapidly injure host tissues (Slaughter et al., 2019). In addition to this, NaOCl is a potent cytotoxic agent that has the potential to inhibit cellular proliferation in a concentration-dependent manner that could potentially disrupt the normal wound healing process (Hidalgo & Dominguez, 2000).

Contemporary clinical guidelines also discourage use of hydrogen peroxide (H_2O_2) for chronic wound care. H_2O_2 releases non-specific reactive oxygen species that in various studies have been demonstrated to be cytotoxic to fibroblasts and endothelial cells involved in tissue healing (Yung et al., 2006; Ricci et al., 2025).

Eusol is safer and also more suitable by modern standards. The chlorinated lime component causes less tissue damage than sodium hypochlorite (Coaguila-Llerena et al., 2026), whereas boric acid may have a part in wound healing via an increase in extracellular matrix formation and angiogenesis (Kaymaz et al., 2016). Despite these theoretical advantages, the current findings provide clear evidence of the dramatic inferiority of Eusol in comparison to modern HOCl formulations in the effective and rapid killing of polymicrobial flora and hardy spores, the key pathogens associated with diabetic foot gangrene.

Conclusion

Diabetic foot gangrene presents a unique clinical conundrum requiring simultaneous control of a heavy burden of polymicrobial, spore-forming pathogens whilst preserving the cell matrix which is critical to wound healing. We present quantitative evidence for the accelerated kinetics of hypochlorous acid (HOCl at high concentration of 200 ppm), which makes it significantly superior as a disinfectant over conventional disinfectants like sodium hypochlorite (NaOCl), hydrogen peroxide (H_2O_2) or Eusol, through the various time-kill studies undertaken in this research.

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