

Bactericidal effect of performic acid on salivary bacteria

Hiroki Morioka¹, Takayuki Nambu² and Kazuya Takahashi³

¹Department of Geriatric Dentistry, Graduate School of Dentistry, ²Department of Bacteriology, and ³Department of Geriatric Dentistry, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan

Although heat-sensitive semi-critical medical devices (e.g., transnasal endoscopes) should undergo high-level disinfection between patients, these devices are often harmful to humans and the environment due to their residual toxicity caused by disinfectants. We analyzed the bactericidal activity against salivary bacteria of performic acid (PFA) which has strong antibacterial activity and has shown a consistent absence of genotoxic and mutagenic effects on human cells. Collected saliva samples were mixed with various concentrations of PFA. After incubation, bactericidal activity was assessed with colony-forming assays and quantification of ATP produced by metabolically active cells. Even for a 1-minute exposure, PFA showed a strong bactericidal effect against salivary bacteria, in which more than 99.9% and 67.1% of bacterial colonies were suppressed at 8×10^{-4} % (w/v) and 8×10^{-6} % (w/v) PFA, respectively. This effect was enhanced by prolonged incubation. Bacterial viability assay based on ATP measurement also showed concentration-dependent bactericidal effects of PFA. PFA showed comparatively strong antibacterial activity against salivary bacteria, even with short duration exposure. (J Osaka Dent Univ 2018; 52: 11-15)

Key words : Performic acid ; Bactericidal activity ; Salivary bacteria

INTRODUCTION

With the progression of population aging in developing countries, there is an increasing need to prevent opportunistic infections in the elderly.^{1,2} This is important in the field of dentistry, where there is a focus on reducing infection when providing oral care for elderly patients and in disinfecting dentures and semi-critical medical devices.³⁻⁶ Highly effective disinfectants should be selected appropriately for use with dental devices. In addition, high-level disinfection such as chemical sterilization is required for semi-critical medical devices including endoscopes that are used to diagnose patients with difficulties ingesting and swallowing.

Disinfectants used in high-level disinfection include glutaraldehyde, phthalaldehyde, and peracetic acid. Such disinfectants are effective in eliminating all microbes except bacterial spores. However, their use is limited as they need to be applied for a

long period of time to be effective in eliminating spores at room temperature, do not have a neutral pH, and can cause mucosal irritation.⁷⁻⁹ While these high-level disinfectants are currently used to disinfect medical devices such as endoscopes, their use has been linked to concerns including a limited effectiveness against spores and acid-fast bacteria, allergic reactions, and potential risks such as inflammation caused by residual disinfectants that can remain on the devices after treatment. Therefore, there is a need to develop alternative disinfectants.

Performic acid (PFA) has superior bactericidal effects compared with conventional high-level disinfectants. Furthermore, it does not produce toxic secondary products as it is decomposed into carbon dioxide, oxygen, and water. These characteristics have made PFA an attractive option for use in high-level disinfection in aquatic environments.¹⁰⁻¹⁴ However, there are limitations associated with its

use. Formic acid used to synthesize PFA remains following the synthesis reaction, the stock solution of PFA is highly acidic ($\text{pH} < 3$), and it has shown poor stability. Therefore, PFA has yet to be used clinically. However, a recent study developed a novel method to synthesize PFA by a plasma reaction that may overcome these limitations.^{15, 16} We assessed the applicability of PFA in clinical practice by investigating the bactericidal effect of chemically-synthesized PFA against oral bacteria.

MATERIALS AND METHODS

Preparation of performic acid

PFA was prepared according to the method described by Chhetri *et al.*¹⁰ Briefly, 1.1 mL of formic acid (85% w/w, Wako, Osaka, Japan) was mixed with 0.1 mL of sulphuric acid (95%, Sigma-Aldrich, Tokyo, Japan) to produce a strongly acidic solution. Next, 0.63 mL of this mixture and 0.27 mL of deionized water were added to 1.1 mL of hydrogen peroxide (35% w/w, Wako) in a test tube and incubated in a 20°C bead bath for 10 min. The resulting solution was immediately quantified and used for experiments.

Quantitative determination of PFA

The concentration of PFA was quantified with ABTS (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]) according to the method described by Chhetri *et al.*¹⁰ ABTS is oxidized by PFA to an intensely green radical cation, which is measured by absorption at 405 nm. Three hundred fifty μL of ABTS solution (1 mg/mL), which was prepared with Active-Cl Test (Wako), was mixed with 350 μL of 1 M acetic acid (adjusted to pH 3.5 with NaOH). This mixture was added to 350 μL of the PFA sample, and then the color was allowed to develop for 20 min. The absorbance was measured at 405 nm on a microplate reader (M 50, Molecular Devices, Sunnyvale, CA, USA), and recalculated to 6.74 mg L^{-1} Abs⁻¹.

Bacterial susceptibility assays

Saliva collected from one healthy subject, without stimulation, was used to verify the bactericidal activity of PFA. This research protocol was approved

by the Ethics Committee of Osaka Dental University (Approval no.110864), and the subject signed informed consent forms. The subject refrained from eating and drinking for at least 2 h before saliva collection. Two milliliters of saliva were collected in sterile plastic tubes and then homogenized by repetitive pipetting through a narrow-bore tip. After serial dilution of saliva, 20 μL aliquots of the sample were mixed with 80 μL of various concentrations of PFA (8×10^{-4} to $8 \times 10^{-6}\%$ [w/v]) or hydrogen peroxide (31.6 to $9 \times 10^{-2}\%$ [w/v]), and then incubated at room temperature for 1 or 5 min. The bactericidal reaction was terminated by 1 : 100 dilution in phosphate buffered saline followed by immediate spreading of this suspension on modified Gifu anaerobic medium (GAM) agar (Nissui Pharmaceutical, Tokyo, Japan). The plates were stored aerobically for 48 h at 37°C, and the resultant colonies were counted and expressed as colony-forming units (CFU)/mL. Bacterial cell viability was also measured with the BacTiter-Glo™ Microbial Cell Viability Assay, a luciferase-based assay that quantifies ATP produced by metabolically active cells, from Promega (Madison, WI, USA), according to the manufacturer's instructions.

RESULTS

Stability evaluation of on-site synthesized PFA

Since PFA is an unstable chemical that can decompose when exposed to heat and impurities even in mild conditions. It must be prepared by mixing hydrogen peroxide and formic acid shortly before each experiment. To confirm the efficacy of PFA for disinfection under more stable and controlled conditions for PFA, we monitored the concentration of on-site synthesized PFA for 60 min. In the case of undiluted PFA stock solution, the concentration of PFA was gradually increased 1.26-fold from 5.3 to 6.68 g/L for 60 min (Fig. 1). In contrast, the concentration of 800-fold diluted PFA was gradually decreased from 5.3 to 3.77 g/L. Because 1.1-fold diluted PFA did not show any significant change of concentration over 60 min, we chose this concentration as the stock solution for the following experiments.

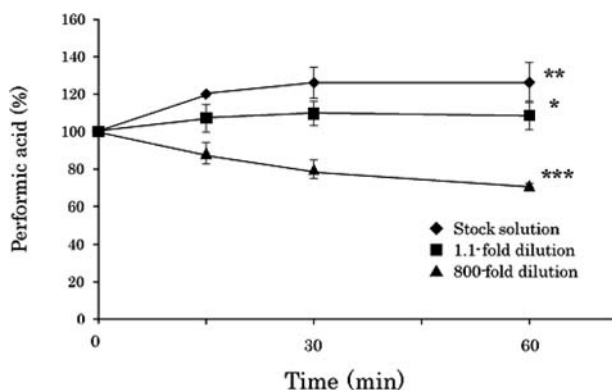


Fig. 1 Stability of chemically synthesized PFA. This shows the concentration changes of the undiluted PFA (◆, 5.3 ± 0.15 g/L), 1.1-fold diluted PFA (■), and 800-fold diluted PFA for 60 min. Each data point represents the average value of three independent experiments and the standard deviation.

*no significant difference ($p > 0.05$) between 0 and 60 min after PFA synthesis, **significant increase ($p < 0.05$), ***significant decrease ($p < 0.01$).

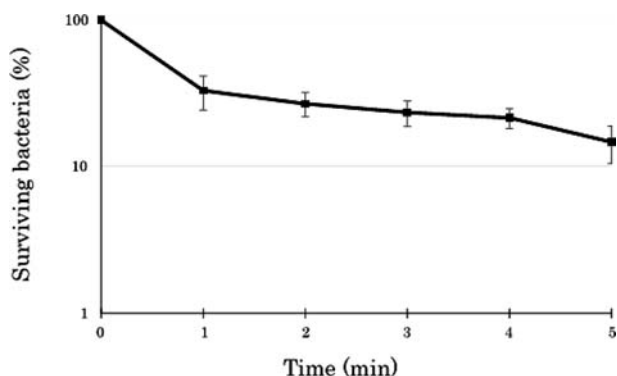


Fig. 2 Time-dependent bactericidal profile of PFA. The bactericidal effect of PFA was determined by exposing saliva to 8×10^{-6} (w/v) PFA for 0 to 5 min and then inoculating on an agar plate. Colony-forming units (2.1×10^6 /mL) are designated as 100% in cases where no disinfectant was used. Each data point represents the average value of three independent experiments and the standard deviation.

Rapid bactericidal effect of PFA on salivary bacteria

Bactericidal activity of PFA against salivary bacteria was initially assessed with colony-forming assays. Collected saliva samples were mixed with various concentration of PFA, incubated at room temperature for 1 or 5 min, and plated on GAM agar for enumeration. Even after only a one-minute exposure, PFA showed a strong bactericidal effect against salivary bacteria, in which more than 99.9%

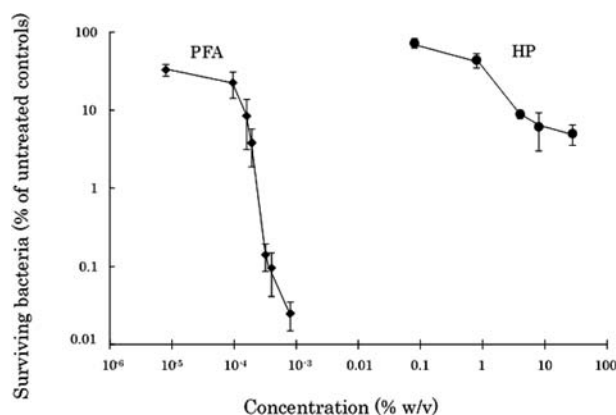


Fig. 3 Bactericidal effect of PFA and hydrogen peroxide on salivary bacteria quantified by colony counting. Survival rates of salivary bacteria were measured by exposing saliva to different concentration of PFA and hydrogen peroxide for 1 min and then inoculating on an agar plate. Colony-forming units of (6×10^6)/mL are designated as 100% in cases where no disinfectant was used. Each data point represents the average value of three independent experiments and the standard deviation. HP : Hydrogen peroxide

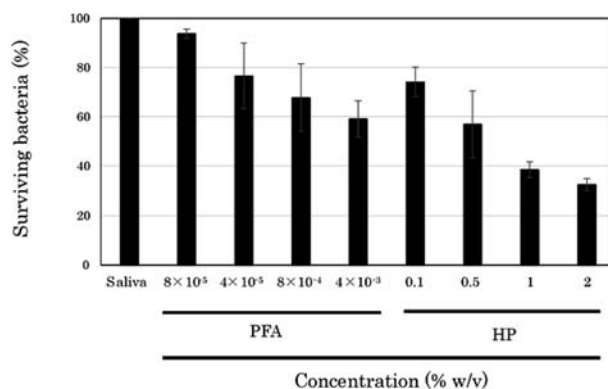


Fig. 4 Bactericidal effect of PFA and hydrogen peroxide on salivary bacteria quantified by ATP measurement. Survival rates of salivary bacteria were measured by exposing saliva to different concentrations of PFA and hydrogen peroxide for 1 min and then measuring the amount of ATP using BacTiter-Glo™. ATP counts of 3167 are designated as 100% in cases where no disinfectant was used. Each data point represents the average value of three independent experiments and the standard deviation.

and 67.1% of bacterial colonies were suppressed at 8×10^{-4} (w/v) and 8×10^{-6} (w/v) PFA, respectively. In the case of 8×10^{-6} (w/v) PFA, this effect was enhanced to 85% by prolonging incubation to 5 min (Fig. 2). In contrast, a similar rate of bactericidal activity required a relatively high concentration of hydrogen peroxide, in which 95% and 27.5% of

the bactericidal effect was observed at 31.6% (w/v) PFA and $9 \times 10^{-2}\%$ (w/v) hydrogen peroxide, respectively (Fig. 3).

Bactericidal activity was also assessed by the quantification of ATP produced by metabolically active cells. Although PFA and hydrogen peroxide both exhibited concentration-dependent killing of salivary bacteria, concentrations showing bactericidal activity were lower for PFA (Fig. 4).

DISCUSSION

Chemically-synthesized PFA has been applied for wastewater treatment as it has strong and immediate bactericidal effects, and its decomposition products, which include water, oxygen, and carbon dioxide, cause little harm to the human body.¹⁰⁻¹⁴ Given these characteristics of chemically-synthesized PFA, we examined its bactericidal activity against oral bacteria. A colony-forming assay was performed to assess the bactericidal activity of PFA, demonstrating that it is approximately 100 times more effective than hydrogen peroxide. Previously, Chhetri *et al.* and Tondera *et al.* demonstrated that chemically-synthesized PFA has a high bactericidal effect against *Escherichia coli* at a relatively low concentration (4×10^{-4} – $8 \times 10^{-4}\%$, w/v).^{10, 11} We also demonstrated a 99.9% toxicity against salivary bacteria with a 1-minute exposure to low-concentration PFA ($8 \times 10^{-4}\%$). Thus, in addition to *E. coli*, this finding suggests that PFA has strong and immediate bactericidal effects against oral bacteria. In accordance with Chhetri *et al.*, we further demonstrated that increasing the dilution factor of PFA results in its accelerated decomposition.¹⁰ As the bactericidal activity of PFA decreases with time due to the reduction of the PFA concentration, low-concentration PFA should be used immediately after its synthesis.

The quantification of ATP produced by metabolically active bacteria further demonstrated bactericidal activity of 23% with $4 \times 10^{-4}\%$ PFA. The finding that PFA is approximately 100 times more effective than hydrogen peroxide was in agreement with the results from the colony-forming assay. However, the actual measure of bactericidal activity differed between the colony-forming assay and the

quantification of ATP production. While the exact reason for this discrepancy is unknown, we observed that a mixture of saliva and highly concentrated PFA, which would almost completely eliminate salivary bacteria, achieved the same level of ATP production as a control that did not include PFA (data not shown). In addition, a study by Green *et al.* suggested that the measured amount of ATP production can be higher than the actual amount when disinfectants come in contact with the reagents for ATP quantification.¹⁷ Therefore, one must consider the possibility that the results may not reflect the true bactericidal effects depending on the experimental conditions when using ATP production by metabolically active bacteria as a measure of the bactericidal effect of a disinfectant.

PFA is highly reactive and unstable.¹⁰ Therefore, we sought to determine the dilution factor for PFA facilitating a chemical reaction equilibrium between its synthesis and decomposition. Under our experimental conditions, we used a 1:1 dilution with pure water as it facilitated the equilibrium of the PFA reaction after its synthesis. The unstable nature of the PFA concentration remains its limitation when considering its applicability. However, a recent study by Kawasaki *et al.* described the synthesis of PFA by plasma reaction and demonstrated that it achieved a neutral pH and stable concentration.^{15, 16} Future investigations are required to examine whether PFA synthesized by the plasma reaction is as effective as chemically-synthesized PFA in terms of its bactericidal activity. In addition, the effects of plasma- and chemically-synthesized PFA on medical devices such as endoscopes and dental materials such as dentures must be investigated further.

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Competing interests

The authors declare that they have no competing interests.

REFERENCES

1. Krone CL, van de Groep K, Trzciński K, Sanders EA, Bogaert D. Immunosenescence and pneumococcal disease: an imbalance in host-pathogen interactions. *Lancet Respir Med* 2014; **2**: 141-153.
2. Molony RD, Malawista A, Montgomery RR. Reduced dynamic range of antiviral innate immune responses in aging. *Exp Gerontol* 2017; pii: **S0531-5565**: 30483-7.
3. Brondani MA, Samim F, Feng H. A conventional microwave oven for denture cleaning: a critical review. *Gerodontology* 2012; **29**: e 6-15.
4. Spach DH, Silverstein FE, Stamm WE. Transmission of infection by gastrointestinal endoscopy and bronchoscopy. *Ann Intern Med* 1993; **118**: 117-128.
5. Shin SP, Kim WH. Recent update on microbiological monitoring of gastrointestinal endoscopes after high-level disinfection. *Clin Endosc* 2015; **48**: 369-373.
6. Adachi M, Ishihara K, Abe S, Okuda K. Professional oral health care by dental hygienists reduced respiratory infections in elderly persons requiring nursing care. *Int J Dent Hyg* 2007; **5**: 69-74.
7. Russell AD. Bacterial spores and chemical sporicidal agents. *Clin Microbiol Rev* 1990; **3**: 99-119.
8. Sagripanti JL, Bonifacino A. Comparative sporicidal effects of liquid chemical agents. *Appl Environ Microbiol* 1996; **62**: 545-551.
9. Takigawa T, Endo Y. Effects of glutaraldehyde exposure on human health. *J Occup Health* 2006; **48**: 75-87.
10. Chhetri RK, Thornberg D, Berner J, Gramstad R, Øjstedt U, Sharma AK, Andersen HR. Chemical disinfection of combined sewer overflow waters using performic acid or peracetic acids. *Sci Total Environ* 2014; **490**: 1065-1072.
11. Tondera K, Klaer K, Koch C, Hamza IA, Pinnekamp J. Reducing pathogens in combined sewer overflows using performic acid. *Int J Hyg Environ Health* 2016; **219**: 700-708.
12. Karpova T, Pekonen P, Gramstad R, Øjstedt U, Laborda S, Heinonen-Tanski H, Chávez A, Jiménez B. Performic acid for advanced wastewater disinfection. *Water Sci Technol* 2013; **68**: 2090-2096.
13. Ragazzo P, Feretti D, Monarca S, Dominici L, Ceretti E, Viola G, Piccolo V, Chiucchini N, Villarini M. Evaluation of cytotoxicity, genotoxicity, and apoptosis of wastewater before and after disinfection with performic acid. *Water Res* 2017; **116**: 44-52.
14. Luukkonen T, Heyninck T, Rämö J, Lassi U. Comparison of organic peracids in wastewater treatment: disinfection, oxidation and corrosion. *Water Res* 2015; **85**: 275-285.
15. Kawasaki M, Morita T, Tachibana K. Facile carbon fixation to performic acids by water-sealed dielectric barrier discharge. *Sci Rep* 2015; **5**: 14737.
16. Kawasaki M, Nakamura T, Morita T, Tachibana K. Catalyst-free one-pot plasma chemical conversion of carbon dioxide to performic acid by water-sealed dielectric barrier discharge. *Plasma Process and Polym* 2016; **13**: 1230-1241.
17. Green TA, Russell SM, Fletcher DL. Effect of chemical cleaning agents and commercial sanitizers on ATP bioluminescence measurements. *J Food Prot* 1999; **62**: 86-90.