



Original Article

Slightly acidic electrolyzed water disrupts biofilms and effectively disinfects *Pseudomonas aeruginosa*[☆]Takashi Okanda^{a,*,1}, Ryo Takahashi^{b,1}, Tomoko Ehara^a, Kiyofumi Ohkusu^a, Nobuhiko Furuya^b, Tetsuya Matsumoto^{a,c}^a Department of Microbiology, Tokyo Medical University, Tokyo, Japan^b Health Care Science, Graduate School of Bunkyo Gakuin University, Tokyo, Japan^c Department of Infectious Diseases, International University of Health and Welfare, Chiba, Japan

ARTICLE INFO

Article history:

Received 24 December 2018

Received in revised form

21 January 2019

Accepted 28 January 2019

Available online 16 February 2019

Keywords:

Pseudomonas aeruginosa

Slightly acidic electrolyzed water

Biofilm

Removal effect

Bactericidal effect

ABSTRACT

Introduction: Biofilm formation is an important issue in the healthcare industry, but conventional disinfectants are not effective for biofilms formed in the hospital environment and on medical instruments. In this study, aim at determine the effectiveness of slightly acidic electrolyzed water (SAEW) on biofilm removal and the disinfection of biofilm-forming *Pseudomonas aeruginosa*.

Methods: Mucoïd and non-mucoïd strains were used for biofilm formation. Biofilms were incubated with SAEW and the reduction in biofilm volume was determined based on the optical density. Furthermore, to investigate the mechanism underlying the effects of SAEW, a biofilm was produced with alginate and structural changes in response to incubation with SAEW were observed by fluorescence microscopy. The minimum bactericidal chlorine concentration of SAEW for *P. aeruginosa* cells was evaluated.

Results: The amounts of alginate and biofilm decreased by 99.9% and 56.8% immersed by 30 ppm of SAEW at 25 °C for 10 min. The effectiveness of SAEW increased as the temperature increased, and the biofilm volume was reduced by 85.4% at 45 °C. Furthermore, 30 ppm SAEW completely disinfects *P. aeruginosa* in the biofilm, even for immersion at 15 °C for 5 min.

Conclusion: Our results suggest that SAEW, a low-cost and safe chlorine disinfectant, is a useful disinfectant for biofilm-forming bacteria.

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1. Introduction

Pseudomonas aeruginosa, a non-fermenting gram-negative rod, is the most common cause of opportunistic and refractory infections. *P. aeruginosa* is an important bacterium for infection

control because it has the ability to form biofilms around water in the hospital environment and on the surface of medical equipment, and colonization persists for long periods. Biofilms formed from exopolysaccharides of mucous substances produced by *P. aeruginosa* protect bacterial cells not only from disinfectants and antimicrobial agents but also from host immune responses [1–3]. In principle, all disinfectants are effective against *P. aeruginosa* [4]. However, biofilm formation prevents a sufficient disinfecting effect, even for intermediate-level disinfectants [5,6]. Commonly used sodium hypochlorite solution (SHS) is a strong irritant to the skin and mucous membranes; accordingly, it is necessary to dilute the chlorine concentration to 100–300 parts per million (ppm). However, a bactericidal effect against biofilm-forming *P. aeruginosa* has not been reported at this chlorine concentration [7,8]. Biofilms cannot be easily removed once formed, and no effective disinfection method has been established.

Abbreviations: SAEW, Slightly acidic electrolyzed water; SHS, Sodium hypochlorite solution; ppm, Parts per million; HOCl, Hypochlorous acid; CFU, Colony forming units; OD, Optical density; DW, Distilled water; FITC-ConA, Fluorescein isothiocyanate-conjugate concanavalin A; MBC, Minimum bactericidal chlorine concentrations.

* All authors meet the ICMJE authorship criteria.

* Corresponding author. Department of Microbiology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan.

E-mail addresses: okd.taka@gmail.com, okanda@tokyo-med.ac.jp (T. Okanda), ryo.takahashi.tmg@gmail.com (R. Takahashi), e-tomo@tokyo-med.ac.jp (T. Ehara), ohkusu@tokyo-med.ac.jp (K. Ohkusu), nfuruya@bgu.ac.jp (N. Furuya), tetsuya.m@iuhw.ac.jp (T. Matsumoto).

¹ These authors contributed equally to this work.

Slightly acidic electrolyzed water (SAEW) has attracted recent attention as a food cleaning and disinfection agent in the food manufacturing industry. SAEW is a chlorine disinfectant with pH 5.0–6.5 containing non-dissociated hypochlorous acid (HOCl) as the main component obtained by the electrolysis of hydrochloric acid. It has a strong bactericidal effect, even at an effective chlorine concentration of 10–30 ppm. SAEW is effective for cleaning contact lens cases and food products [9,10]. However, it has not been utilized extensively in the healthcare industry. In this study, we examined the biofilm removal ability and mechanism, bactericidal ability, and optimal conditions to determine the effectiveness of SAEW against biofilm-forming *P. aeruginosa*.

2. Materials and methods

2.1. *P. aeruginosa* isolates

Seven strains of mucoid-type *P. aeruginosa* (PaM) isolated at Tokyo Medical University Hospital in Japan were used. As a control, *P. aeruginosa* PAO1 (non-mucoid type) was used.

2.2. Induction of biofilm formation

After incubating *P. aeruginosa* at 35 °C overnight, the number of cells in the culture broth was adjusted to 1×10^5 colony forming units (CFU/mL) with Luria broth. The diluted culture solution was added to a 48-well microtitre plate at 500 μ L per well and then shake-cultured (30 rpm) at 35 °C for 48 h.

2.3. Measurement of the biofilm volume

The total amount of biofilm formed was determined by a modified version of the microtitre plate biofilm assay [11,12]. Cultures in each well were stained with 0.1% crystal violet solution for 10 min at room temperature (20 °C) and then filtered with a Falcon® 40- μ m cell strainer. The sample on the cell strainer was washed with distilled water and air-dried, and then crystal violet was extracted with 95% ethanol. After adding 125 μ L of the

extracted crystal violet solution to a microtitre plate, the optical density (OD) at an absorbance of 580 nm was measured with a microplate reader (Mithras LB940; Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany).

The PaM6 strain had the highest biofilm OD among seven PaM strains and was used for further investigations (Fig. 1).

2.4. Incubation with SAEW under various conditions

SAEW used in this study was obtained by connecting the SAEW generator (Purester μ -Clean; Morinaga Milk Industry Co., Ltd., Tokyo, Japan) to a tap water source. Biofilms of strains PAO1 and PaM6 were collected in a cell strainer and then incubated with SAEW (5, 10, 15, 20, and 30 ppm) and distilled water (DW) at 25 °C for 10 min. In addition, to examine the effects of incubation temperature, the biofilms of PAO1 and PaM6 were incubated with SAEW (15 ppm) at 15 °C, 35 °C, and 45 °C.

2.5. Fluorescent staining for alginate

Biofilms were prepared using alginate and the influence of SAEW was confirmed based on the fluorescence intensity. Biofilm samples were prepared by mixing 1% sodium alginate and 8 mM CaCl₂. The sample (300 μ L) was added to 1.2 mL of SAEW and then centrifuged at 15,000 rpm for 20 min. The pellet was washed with distilled water and stained with 50 μ g/mL fluorescein isothiocyanate-conjugate concanavalin A (FITC-ConA; EY Laboratories, Inc., San Mateo, CA, USA) for 30 min [13–15]. The morphology of the biofilm was observed with a fluorescence microscope (ECLIPSE E600; Nikon, Tokyo, Japan), and fluorescence intensity was measured using a microplate reader.

2.6. Observation of microbial structure by transmission electron microscopy

Biofilm-forming bacteria before and after incubation with SAEW were fixed with 2.5% glutaraldehyde at 4 °C for 2 h and then centrifuged at 3000 rpm for 15 min. The obtained pellet was fixed

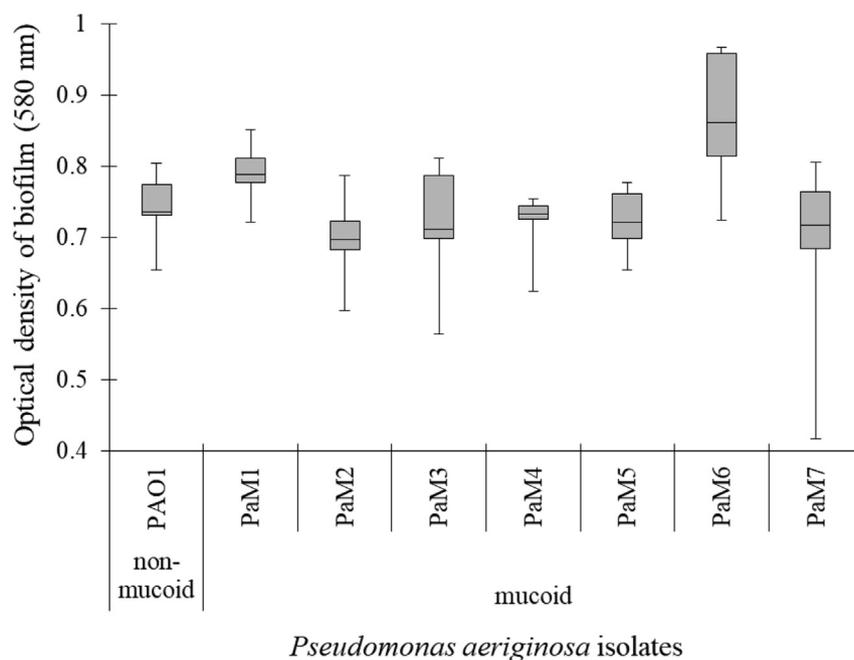


Fig. 1. Quantitative analysis of biofilm formation in a non-mucoid strain (PAO1) and mucoid strains (PaM1–7).

with 2% osmium tetroxide for 2 h. The fixed pellet was washed with a phosphate buffer and embedded in 2% agarose. The embedded sample was dehydrated with a 50–100% graded ethanol series and acetone and then embedded and polymerised in Spurr resin. Resin-embedded samples were subjected to serial sectioning using an Ultra-Microtome (Poter-Blum MT-1; Sorvall, Newtown, CT, USA) and electron stained with 3% uranyl acetate and lead citrate. The prepared sample was observed using a transmission electron microscope (H-7000; HITACHI).

2.7. Evaluation of the bactericidal ability of SAEW on *P. aeruginosa* cells

Strains were analysed after biofilm formation and biofilm removal. After biofilm removal, the strain was prepared by centrifugation (3000 rpm, 15 min) with a 10-fold volume of physiological saline solution. The obtained sediment was dissolved in Luria broth.

First, the conditioned *P. aeruginosa* cells were incubated at 15 °C–45 °C for 5 or 10 min with SAEW. Next, the incubated sample with SAEW was cultured on heart infusion agar medium at 35 °C for 24 h, and bactericidal activity was evaluated based on the presence or absence of growth.

2.8. Statistical analysis

Biofilm amount and alginate content were determined median and standard deviation by analysis of variance.

3. Results

3.1. Influence of SAEW on biofilms

Changes in biofilm OD values after incubation with SAEW under each condition are summarised in Fig. 2. For the PAO1 strain, the reductions in OD after 10 min of incubation with DW at 15 °C, 25 °C, 35 °C, and 45 °C were 3.9%, 6.3%, 27.9%, and 44.4%, respectively. The reductions in OD after incubation with 30 ppm SAEW at each temperature were 31.8%, 44.0%, 77.2%, and 80.4%, respectively (Fig. 2a). For the PaM6 strain, the reductions in OD after 10 min of incubation with DW at 15 °C, 25 °C, 35 °C, and 45 °C were 5.8%, 11.5%, 46.1%, and 52.3%, respectively. The reductions in OD after incubation with 30 ppm SAEW at each temperature were 58.5%,

59.6%, 63.1%, and 85.4%, respectively, and it was confirmed that the higher the chlorine concentration, the greater the effect (Fig. 2b).

3.2. Influence of SAEW on alginate produced by biofilms

After incubation with DW, there was no difference in the fluorescence intensity of FITC-ConA (Fig. 3a), or the structure of the biofilm (Fig. 3b). However, SAEW at 15 ppm and 30 ppm decreased the fluorescence intensity by 90.1% and 99.9%, respectively (Fig. 3a), and a broken biofilm structure was confirmed (Fig. 3c).

3.3. Bactericidal effect of *P. aeruginosa* cells by SAEW

Transmission electron microscopy images of PAO1 before and after SAEW immersion are shown in Fig. 4. Compared with cells not treated with SAEW (Fig. 4a), PAO1 cells treated with 15 ppm SAEW exhibited a partially destroyed cell wall and membrane structure and decreased cytoplasm density (Fig. 4b). Disruption of the cell structure was observed in most PAO1 cells, and similar destruction was observed in PaM6 cells.

The minimum bactericidal chlorine concentrations (MBC) of SAEW against biofilm-forming *P. aeruginosa* are shown in Fig. 5. The MBCs of SAEW were 5 ppm without biofilms. With biofilms, the MBC of SAEW was 30 ppm for 5 min of immersion at any temperature, but immersion time extended to 10 min decreased the MBC of SAEW to 10 ppm at 45 °C.

4. Discussion

Our results provide the first evidence that SAEW efficiently degrades alginate, the main component of biofilms, and disinfects *P. aeruginosa* cells in biofilms. Biofilm removal is the first step in the disinfection of biofilm-forming bacteria. Biofilms increase the durability of bacteria against various stresses; they become dormant by undergoing local nutritional restriction. Bacteria that detach from the biofilm return to a proliferative state and are susceptible to antibiotics and antiseptics [13,16]. The bactericidal effect of chlorinated disinfectants strongly depends on the concentration of HOCl. The HOCl content in the chlorine disinfectant is related to the pH, and the contents for strongly acidic electrolyzed water (pH 2–3), SAEW (pH 5–6.5), and SHS (pH 9–9.5) are about 80%, 100%, and 10%, respectively. Commonly used SHS has a weak biofilm removal effect; only 58.3% of biofilms can be removed at an

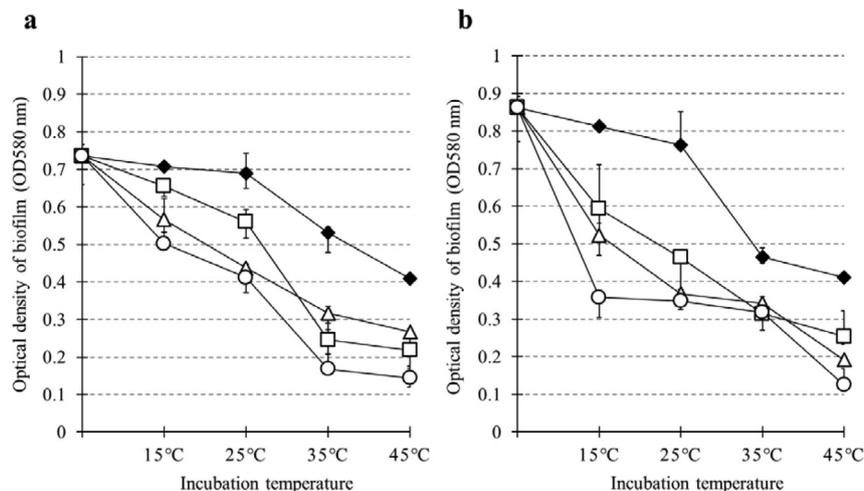


Fig. 2. Change in biofilm amounts for PAO1 (a) and PaM6 (b) in response to distilled water (DW) and slightly acidic electrolyzed water (SAEW) at various temperatures for 10 min. SAEW results are shown according to the chlorine concentration (10, 15, and 30 ppm). ◆: DW, △: 10 ppm, □: 15 ppm, ○: 30 ppm.

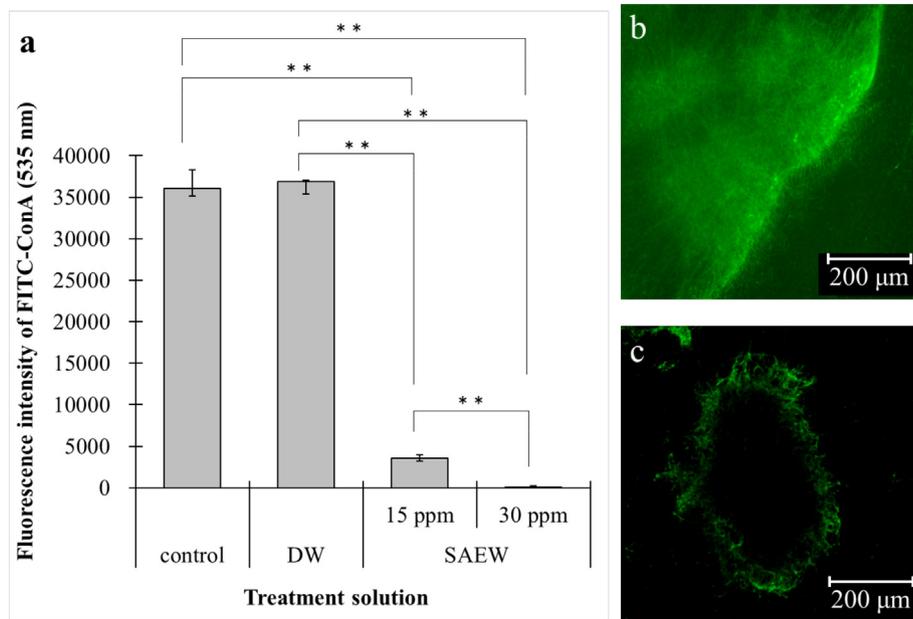


Fig. 3. Image of alginate before and after immersion with slightly acidic electrolyzed water (SAEW), and changes in the alginate amount in response to distilled water (DW) and SAEW. The amount of alginate, as determined by fluorescent staining with FITC-ConA, was significantly reduced by SAEW immersion at 15 and 30 ppm (a). In the control, a film of fibrous alginate was observed in green fluorescent colour (b). After immersion with 15 ppm SAEW, the alginate membrane was broken and the contents were hollow (c). ** $P < 0.01$.

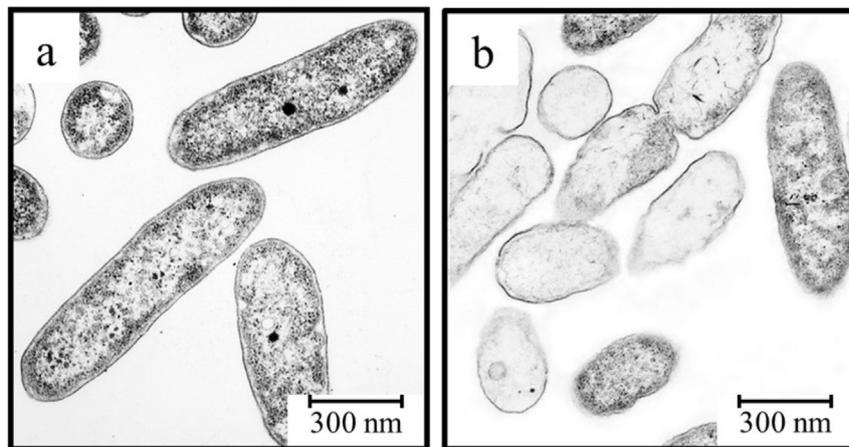


Fig. 4. Image of the internal structure of the PAO1 strain by transmission electron microscopy. (a) Bacterial cells before biofilm formation. (b) Bacterial cells treated with 15 ppm SAEW after biofilm formation. Dark cells are normal bacterial cell with content of inner material, while light cells are bacterial cells that had lost the inner material after the immersion to SAEW.

effective chlorine concentration of 50 ppm, and the removal ability is not influenced by pH [17]. However, SAEW at pH 5.7 removed 85.4% of the biofilm and 99.9% of alginate after immersion for 10 min at 30 ppm. This result suggests that SAEW has a superior biofilm removal ability to that of SHS.

Antimicrobial activity against *P. aeruginosa* is far more difficult with biofilm formation than without biofilm formation. Although the mechanism underlying the bactericidal activity of HOCl is not completely understood, it is thought to inhibit the membrane transport capacity, damage membranes and DNA, and inhibit enzyme activity indispensable for proliferation [18,19]. The chlorine concentrations required to disinfect the biofilm-forming *P. aeruginosa* in 10 min were 3000 ppm for chlorhexidine, 5000 ppm for benzalkonium chloride, and 1000 ppm for SHS [20]. However, these concentrations are highly toxic to the human body and the procedure is highly complicated, which is not practical. For

SAEW, the effective chlorine concentration required to disinfect biofilm-forming *P. aeruginosa* in 10 min was only 30 ppm; additionally, it was effective at a much lower concentration than that for the conventional disinfectant. Furthermore, when the temperature of SAEW was 45 °C, the bactericidal effect was observed, even for an effective chlorine concentration of 10 ppm. These results suggest that SAEW has a much higher bactericidal effect on biofilm-forming *P. aeruginosa* than conventional antiseptics.

Device-related infections involving biofilms are a serious problem, but existing cleaning methods with disinfectants are not sufficiently effective [21]. Strongly acidic electrolyzed water with a similar bactericidal mechanism to that of SAEW is expected to be an effective disinfectant for endoscopes [22]. However, endoscope manufacturers does not recommend to use because strongly acidic electrolyzed water tends to be inactivated and corrosive [23]. On the other hand, SAEW is highly convenient and stable because is

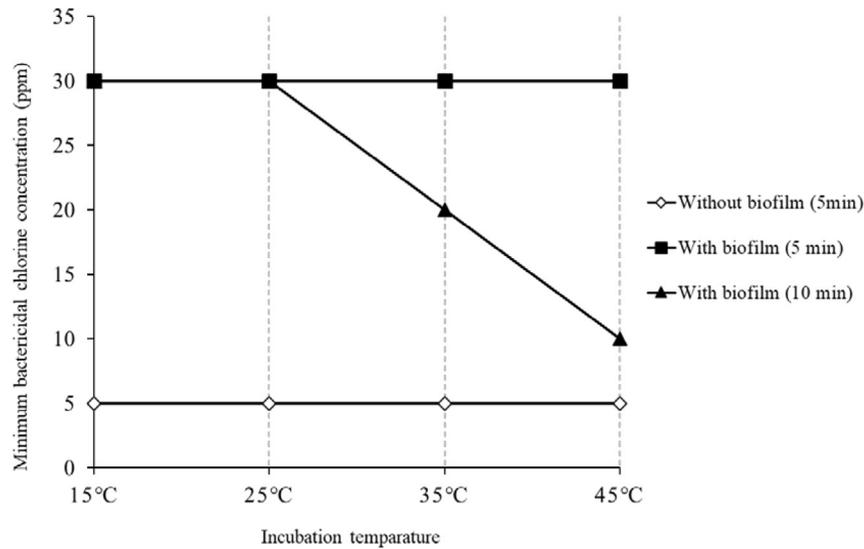


Fig. 5. Minimum bactericidal chlorine concentration (MBC) of SAEW against PaM6 strains. MBC results are shown according to the incubation time (5 or 10 min) and with/without biofilms. ◇: Without biofilm (5 min), ■: With biofilm (5 min), ▲: With biofilm (10 min).

produced simply by directly connecting a generator to a water supply and can be used immediately after it is generated [24,25]. Therefore, we expect to be able to use the endoscope more safely by disinfecting by SAEW (30 ppm, 45 °C, 10 min) before and after the endoscope is used, in addition to the conventional washing process. In addition, SAEW may be useful for disinfection of biofilms adhering to instruments for oral contact such as baby-bottles and tableware. Because, SAEW has been approved as a food additive by the Japanese Ministry of Health, Labour and Welfare based on the lack of residual chlorine after disinfection and its high safety [10,21,26]. Our results indicate that SAEW may become to safe and reliable disinfectant for various devices. However, since the actual biofilm adheres to the surface of the instrument, the evaluation of the effectiveness due to the difference in material, shape and washing method is also an item to be studied in the future.

In conclusion, this study showed that SAEW is an effective disinfectant for biofilm-forming *P. aeruginosa*. These results also suggest that SAEW is a useful tool for disinfecting medical devices contaminated with biofilms.

Ethics approval and consent to participate

Not applicable.

Funding

No funding.

Authors' contributions

All authors read and approved the final manuscript.

Conflicts of interest

There is no particular conflict of interest to disclose.

Availability of data and materials

Not applicable.

Acknowledgments

I wish to thank Morinaga Milk Industry Co., Ltd. for lending the SAEW generator (Purester μ -Clean). We would like to thank Editage (<https://www.editage.com/>) for English language editing.

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