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Disinfection and cleaning of heater–cooler units: suspension- and biofilm-killing

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SUMMARY

Background: Non-tuberculous mycobacteria (NTM) infections in cardiac surgery patients, caused by *Mycobacterium chimaera* or *Mycobacterium abscessus*, have been traced to NTM-aerosols produced by heater–cooler units of cardiopulmonary bypass equipment.
Aim: To develop a protocol to disinfect the water reservoir(s) of heater–coolers to reduce NTM numbers and thereby prevent potential NTM aerosolization; and to devise an approach to disrupt surface biofilms of heater–coolers to reduce reinoculation of the heater–cooler reservoir(s) after disinfection.
Methods: A laboratory-scale Centers for Disease Control and Prevention bioreactor and a heater–cooler were inoculated with *M. chimaera* or *M. abscessus* to measure the ability of different disinfection protocols to reduce NTM colony-forming units in water and biofilm samples and to delay the reappearance of NTM after disinfection.
Findings: The combination of an enzyme detergent cleaning agent and Clorox® were equivalent to Clorox alone in reducing *M. chimaera* cfu in heater–cooler water reservoir samples. However, reappearance of those bacteria was delayed by 12 weeks by the combination of enzyme detergent cleaning agent and Clorox exposure compared to Clorox disinfection alone.
Conclusion: A combination of an enzyme detergent and Clorox was an effective disinfection treatment and significantly delayed the reappearance of *M. chimaera* in the heater–cooler reservoir.

Introduction

Mycobacterium chimaera and *Mycobacterium abscessus* infections were reported in patients following cardiovascular surgery [1,2]. Although the infections were rare, they were associated with high mortality and both the US Food and Drug Administration and the European Centre for Disease Prevention and Control issued alerts [1–4]. The infections have been traced to the presence of *M. chimaera* or *M. abscessus* in the water reservoirs of heater–coolers used to control both blood and patient temperatures during cardiac surgery [5–7]. *M. chimaera* and *M. abscessus* are members of the group of environmental mycobacteria (non-tuberculous mycobacteria, NTM) that are widely distributed in the human environment [8,9]. Importantly for these outbreaks, the mycobacterial habitats include drinking-water distribution systems and premise plumbing, most notably hospitals and homes [8,9]. In the case of the *M. chimaera* infections, the source of the *M. chimaera* in the heater–coolers was the water supply of the manufacturer [7]. The source of the *M. abscessus* in the heater–cooler was the hospital's public water supply [2].

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NTM cells are resistant to the concentrations of disinfectants commonly used for drinking water treatment (e.g. chlorine, chloramine, and ozone). NTM are ~1000 times more resistant to chlorine than the bacterium used as the industry standard for disinfection, *Escherichia coli* [10]. For example, at a concentration of 1 ppm chlorine, it would take ~5 s to kill 99.9% of *E. coli* cells, but 2 h to kill *Mycobacterium avium*, a close relative of *M. chimaera* [10]. The hydrophobic mycobacterial cells preferentially attach to surfaces to form thick biofilms containing high numbers (e.g. 10,000/cm^2) of cells and the layers of cells and extracellular materials in biofilms substantially increase disinfectant resistance [11,12]. Standard disinfection regimens fail to kill even a small proportion of *Mycobacterium* spp. cells in biofilms and the surviving biofilm cells can recolonize the water circulating in the reservoirs where it can be aerosolized. The re-emergence of *M. chimaera* in heater–coolers in spite of cleaning and disinfection has been reported [13]. In practice, that means a heater–cooler must be disinfected often – as frequently as weekly, otherwise the reservoir becomes populated with NTM cells that can be aerosolized.

The objectives of this study were to measure the efficacy of killing *M. chimaera* employing a novel disinfection protocol. That protocol anticipated that as a majority of mycobacterial cells in heater–coolers are in biofilms, measurements were conducted to determine whether the addition of a biofilm-disruption step, mediated by commercial enzyme detergent solutions, would increase the disinfected-mediated killing of biofilm-associated mycobacterial cells. In addition to measuring killing of *M. chimaera* cells in laboratory biofilms, the time before reappearance of *M. chimaera* in reservoirs of heater–coolers was also measured. That second approach was based on the hypothesis that reduction of biofilm *M. chimaera* numbers would delay the reinoculation and reappearance of mycobacteria in reservoir water.

Methods

Mycobacterial strains

Two strains were used for the study: *Mycobacterium chimaera* NC-W-2-1 was isolated in the Virginia Tech laboratory from a water sample collected from a Sorin 3T heater–cooler and *M. abscessus* AAY-P-1 from a patient isolate.

Enzyme detergent cleaning agents

The enzyme detergent cleaning agents, Enzyclean® IV (Weiman Products, LLC, Gurnee, IL, USA) and Prolystica® 2× concentrate (Steris Corp., St Louis, MO, USA) were employed as biofilm-disrupting agents in disinfection measurements of *M. chimaera* or *M. abscessus* (alone or in combination with Clorox®; Clorox Co., Oakland, CA, USA) to measure their possible synergistic antimicrobial activity.

Media for growth, isolation and enumeration

M. chimaera and *M. abscessus* were grown to mid-log$_{10}$ phase growth (10^8 cfu/mL) in Middlebrook 7H9 broth (BD, Sparks, MD, USA) containing 0.5% (v/v) glycerol and 10% (v/v) oleic acid–albumin with aeration (120 rpm) at 37°C. Colonies of *M. chimaera* or *M. abscessus* were grown and enumerated on Middlebrook 7H10 agar medium containing 0.5% (v/v) glycerol and 10% (v/v) oleic acid–albumin.

Water acclimation

Rather than inoculate medium-grown cells of *M. chimaera* into the water reservoir of the heater–cooler and risk osmotic shock and stress as a result of adaptation from medium to water, cells of *M. chimaera* were collected and suspended in sterile local Blacksburg tap water. Specifically, following growth of the *M. chimaera* or *M. abscessus* strains to mid-exponential phase, cells were collected by centrifugation (5000 *g* for 20 min), washed twice in sterilized local Blacksburg tap water, and suspended in sterile Blacksburg tap water. The cells were then incubated in water for seven days at room temperature to acclimate them to drinking water. Samples measured before and after acclimatization showed that there was no decrease in colony-forming units (cfu)/mL. Water acclimation is essential for measuring mycobacterial susceptibility to any antimicrobial agent, as laboratory medium-grown cells are substantially more susceptible compared to water-acclimated cells [10].

NTM isolation, identification, and DNA fingerprinting

NTM in water samples were diluted 1:1 in D/E Broth and plated directly or dilutions prepared and 0.1 mL was spread on Middlebrook 7H10 agar medium (BD) in triplicate and incubated at 37°C for up to three weeks when colonies were counted [8]. To enumerate NTM in biofilms in the CDC Biofilm Reactor, coupons were removed aseptically, placed in 5 mL of 1:1 sterile Blacksburg tap water: D/E Broth and mycobacterial cells were suspended by 60 s vortexing. Samples (0.1 mL) of those suspensions were spread on M7H10 agar medium [11]. Identification and repetitive element sequence-based polymerase chain reaction (rep-PCR) fingerprinting of representative *M. chimaera* or *M. abscessus* isolates on M7H10 agar was performed as described [8]. To inactivate any enzyme detergent or disinfectant, all samples were diluted in 1:1 in D/E Neutralizing Broth (Acumedia, Lansing, MI, USA) [14]. Chlorine neutralization and absence (<1 ppm) was confirmed using chlorine test strips (Serim Research Corp., Elkhart, IN, USA).

CDC Biofilm Reactor

The CDC Biofilm Reactor (BioSurface Technologies Corp., Bozeman, MT, USA) consists of eight polypropylene coupon holders suspended from a polyethylene lid [15]. Each coupon holder carries three coupons (1.27 cm diamaeter). The lid with coupon holders is mounted on the top of a glass beaker (1 L volume) with side arm, such that the coupons are suspended in any liquid (350 mL), while shear forces are generated by a stir bar. The assembled reactor containing paddles, coupons, and Blacksburg tap water was sterilized by autoclaving. After cooling, the water was inoculated with a water-acclimates suspension of *M. abscessus* to a final density of 10^5 cfu/mL and incubated at room temperature with stirring (120 rpm) for one week to allow for biofilm formation [11].

CDC Biofilm Reactor disinfection measurements

Clorox-mediated killing of biofilm-grown cells of *M. abscessus* on stainless steel coupons in the CDC Biofilm Reactor was measured as decreases in cfu in the presence or absence of Enzyclean® IV as described [12].

Heater–cooler

A Hemotherm model 400 CE Dual Reservoir Cooler/Heater (Cincinnati Sub-Zero Products, Inc., Cincinnati, OH, USA) was employed for the measurements of disinfection efficacy. Only one patient infection involving the presence of a Hemotherm has been identified amongst a study of 339 US Food and Drug Administration Medical Device [16]. The Hemotherm provided by Cincinnati Sub-Zero had been returned to the manufacture after use in the field. It had been drained and left idle for approximately one year, but it still contained water in different traps and drains totalling 250 mL. Water and biofilm samples were collected and cultured for isolation and identification. The results provide an example of cultivable mycobacterial flora of a heater–cooler (Supplementary Table S1). After that sampling and analysis of the results (Supplementary Table S1), the Hemotherm was subjected to two cycles of Clorox disinfection and then used for the experiments reported here. None of the *Mycobacterium* spp. listed in Supplementary Table S1 were subsequently recovered from the heater–cooler.

Measurement of disinfection efficacy in heater–coolers

The procedures for disinfection of the heater–cooler followed that of the operation manual for the instrument of the manufacturer. In all experiments, the heater–cooler was inoculated and thereby heavily colonized with *M. chimaera*. To inoculate or sample the Hemotherm's Heat and Cool reservoirs, the hinged lid was opened and the separate reservoir lids below removed for access.

For the experiments described herein using the Hemotherm, each reservoir (i.e. Heat = 5.7 L and Cool = 7.6 L) was inoculated with $1–2 \times 10^{11}$ cfu of the water-acclimated suspension of *M. chimaera*. The instrument was turned on and water circulated on the Heat and Cool cycles for 5 min each to distribute the inoculum. Samples were then collected from the two reservoirs and plated for *M. chimaera* cfu/mL to establish the starting cell density. Samples were also collected immediately after the sequential 5 min enzyme detergent cleaning (if employed), the 5 min Clorox disinfection, and three-fold draining and refilling steps, following the manufacturer's operating manual. All samples were immediately mixed with an equal volume of sterile D/E Neutralizing Broth (Acumedia, Lansing, MI, USA) to neutralize both the detergents and Clorox [17]. Those samples and dilutions in D/E Neutralizing Broth were spread (0.1 mL in triplicate) on M7H10 agar medium to count surviving colonies and calculate the extent of killing. For experiments to measure the effect of adding a cleaning step, the enzyme detergent Prolystica 2× concentrate was added to each reservoir at a final concentration of 4 mL/L before Clorox and the water circulated through the instrument on both the Heat and Cool cycles for 5 min each. After circulation of Prolystica for 5 min and collection of samples from both reservoirs for cfu measurement, Clorox was added to the reservoirs to achieve a final concentration of 30 mL/L following the guidance of the manufacturer's protocol and water circulated through the reservoirs for 5 min. Please note that Prolystica and Clorox were present together in the heater–cooler reservoirs for the disinfection cycle. Following Clorox exposure, samples were collected from both reservoirs for immediate enumeration of surviving cells as described above. Finally, the instrument was drained and refilled three times with sterile distilled water and then filled with sterile distilled water and samples collected for enumeration of surviving cells. Following incubation of Parafilm® (Bemis Co., Neenah, WI, USA)-sealed M7H10 agar plates at 37°C for 14 days, numbers of *M. chimaera* colonies were counted and survival values calculated.

Triplicate plates were spread to ensure accurate and reproducible colony counts and allow calculation of robust averages and standard deviations. Triplicate counts also reduced the contribution of count variation due to the aggregation of mycobacterial cells (driven by hydrophobicity). Further, triplicate plates are more likely to allow identification of unusually high (artefact) counts due to the uneven dispersal of aggregates.

Reappearance of M. chimaera following disinfection

Following enzyme detergent cleaning, disinfection, and the three-fold draining and refilling, water samples were collected from the instrument's reservoirs at weekly intervals up to 12 weeks to identify when *M. chimaera* colonies reappeared. The heater–cooler was operated Monday to Friday, 4 h on Heat cycle and 2 h on Cool cycle to mimic operation in hospitals.

Results

Effect of cleaning agent on killing biofilm-grown M. abscessus cells in the CDC Biofilm Reactor

Although Enzyclean exposure alone failed to kill any *M. abscessus* cells in the biofilms, it increased killing in combination with 1 ppm Clorox (Table I). That result supported the hypothesis that enzyme detergents could increase killing of NTM cells in biofilms and encouraged their utilization in protocols for heater–cooler disinfection.

Table I
Clorox®-dependent killing of *Mycobacterium abscessus* cells in biofilms in the presence and absence of Enzyclean®

Treatment	Percent survival[a] (no. of measures)	Percent killing[a]	*P*-value (*t*-test)
Water suspension	100% (control)	0% (control)	
Enzyclean	106 ± 15% (9)	0% (9)	>0.05
Clorox	85 ± 22% (9)	15% (9)	>0.05
Enzyclean and Clorox	66 ± 21% (9)	34% (9)	<0.01

[a] Percent survival and killing calculated on the basis of cfu *M. abscessus*/cm^2 biofilm with number of replicates in parenthesis.

Isolation and identification of NTM in heater–cooler

Mycobacterium spp. isolates recovered from the Hemotherm 400CE heater–cooler are listed in Supplementary Table S1. No isolates of *M. chimaera* were recovered, consistent with the fact that the infection outbreaks have been associated with Sorin 3T heater–coolers that were colonized with *M. chimaera* at the manufacturing plant [7]. The *Mycobacterium* species and numbers are typical of water and biofilm samples collected from premise plumbing in the USA [8].

Loss of inoculated *M. chimaera* in the heater–cooler

Following inoculation of the Hemotherm 400CE and 5 min periods of water cycling in the Cool and Heat reservoirs, the cfu/mL of each reservoir water suspension was measured. Based on the volume of the reservoirs, the cfu/mL should have been 2.3×10^7 (Heat) and 1.7×10^7 (Cool). However, the average cfu/mL after inoculation and circulation in three independent measurements were 6.1×10^4 (Heat, 0.27%) and 6.1×10^4 (Cool, 0.36%). That means an average of 99.76% (Heat) and 99.64% (Cool) *M. chimaera* cells were lost from suspension. Following the twin 5 min circulations, the number of *M. chimaera* cfu/mL in reservoir water samples remained steady for up to four days.

Effect of the clorox to kill *M. chimaera* in the presence or absence of prolystica

Due to the lack of antimycobacterial activity of Enzyclean, another enzyme detergent, cleaning agent, Prolystica, available throughout the world, was employed in a disinfection protocol with Clorox in a Model CE 400 Hemotherm. Prolystica killed a substantial proportion of *M. chimaera* cells and in combination with Clorox reduced the number of *M. chimaera* cells below the level of detection (Table II). Killing by the combination was duplicated at two different starting densities (Experiments 1 and 2). Clorox alone failed to eradicate *M. chimaera* (Table II). The surviving colonies were identified as *M. chimaera* and shared the same rep-PCR fingerprint as that of the inoculated cells.

Reappearance of *M. chimaera* following Clorox-only versus Prolystica cleaning with Clorox disinfection

No *M. chimaera* cells (as cfu) appeared in the weekly Heat and Cool reservoir samples following the combined Prolystica and Clorox protocol for up to 12 weeks (Table III). By contrast, substantial numbers of *M. chimaera* colonies were recovered from the Cool reservoir after three weeks of incubation and more *M. chimaera* cells appeared in the following weeks (Table III). As the cfu/mL continued to increase after seven

Table II
Killing of *M. chimaera* by Clorox® in the presence and absence of Prolystica®

Protocol and sample	cfu/mL	Survival (%)	Kill (%)
Clorox alone: Heat reservoir[a]			
Post-inoculation/pre-disinfection	1.8×10^5	100	0
Post-Clorox	<1.0	<0.0006	>99.9994
Post-drain/refill	6	0.003	99.997
Clorox alone: Cool reservoir[a]			
Post-inoculation/pre-disinfection	1.7×10^5	100	0
Post-Clorox	<1.0	<0.0006	>99.9994
Post-drain/refill	10	<0.006	>99.994
Prolysica and Clorox: Heat reservoir, Experiment 1			
Post-inoculation/pre-disinfection	2.6×10^4	100	0
Post-Prolystica	4.0×10^1	0.15	99.85
Post-Clorox	<3.3	<0.013	>99.987
Post-drain/refill	<3.3	<0.013	>99.987
Prolystica and Clorox: Cool reservoir, Experiment 1			
Post-inoculation/pre-disinfection	2.3×10^4	100	0
Post-Prolystica	7.5×10^1	0.33	99.67
Post-Clorox	<3.3	<0.014	>99.986
Post-drain/refill	<3.3	<0.014	>99.986
Prolystica and Clorox: Heat reservoir, Experiment 2			
Post-inoculation/pre-disinfection	1.54×10^5	100	0
Post-Prolystica	1.6×10^3	1	99.0
Post-Clorox	1.4×10^2	0.09	99.91
Post-drain/refill	<3.3	< 0.002	>99.998
Prolystica and Clorox: Cool reservoir, Experiment 2			
Post-inoculation/pre-disinfection	1.34×10^5	100	0
Post-Prolystica	5.7×10^3	4	96
Post-Clorox	7.6×10^2	0.6	99.4
Post-drain/refill	<3.3	<0.025	>99.975

[a] Average of two independent experiments.

Table III
Reappearance as colony-forming units (cfu/mL) of *Mycobacterium chimaera* following Clorox®-only and the Prolystica® + Clorox® combined cleaning and disinfection.

Weeks after disinfection	Clorox-only		Prolystica + Clorox[a]	
	Heat	Cool	Heat	Cool
Immediate	6	<1.0	<1.0	<1.0
1	<1.0	<1.0	<1.0	<1.0
2	<1.0	<1.0	<1.0	<1.0
3	<1.0	53	<1.0	<1.0
4	144	156	<1.0	<1.0
5	210	200	<1.0	<1.0
6	120	150	<1.0	<1.0
7	440	320	<1.0	<1.0
8	Halted	Halted	<1.0	<1.0
9			<1.0	<1.0
10			<1.0	<1.0
11			<1.0	<1.0
12			<1.0	<1.0

[a] Heater–cooler from Prolystica–Clorox protocol, Experiment 1, Table II.

weeks, no further samples were collected. No other *Mycobacterium* spp., including those listed in Supplementary Table S1, were recovered from the heater–cooler. Nothing (e.g. H_2O_2 or preservative) other than sterile water was added to the reservoirs during the post-disinfection period.

Discussion

The results presented here offer a route to disinfecting heater–coolers and to ensure that the heater–cooler reservoir water will remain relatively free (i.e. <3.3 cfu/mL) of *M. chimaera* for up to 12 weeks. Their absence in the reservoir water will prevent the generation of mycobacteria-laden aerosols and patient infection. As an enzyme detergent formulation increased mycobacterial killing of biofilm-adherent cells (Table I), it is likely that it also prevented the reappearance of *M. chimaera* following disinfection. Based on the results presented here, disinfection with only Clorox would require repeated disinfection every three weeks, whereas disinfection with Prolystica and Clorox would require a repeated disinfection only after every 12 weeks. Inclusion of an enzyme detergent step may be a time-saving and cost-effective alternative to the alternative of disassembly, replacement of parts, and reassembly.

Although separate experiments to identify the reason for the loss of cells from suspension following inoculation of the heater–cooler were not performed, it is quite likely that the very hydrophobic *M. chimaera* cells were lost due to their adherence to the surfaces of the pipes, tubes, pumps, and reservoir surfaces in the heater–cooler. NTM – including the *M. avium* complex relatives of *M. chimaera*, *M. avium*, and *M. intracellulare* – adhere to a variety of surfaces (e.g. polyvinyl chloride (PVC), stainless steel, and copper) rapidly to reach high densities (e.g. 1000 *M. avium* cfu/cm^2) on a variety of coupons within the time required to process coupon surfaces [11]. Specifically, PVC and galvanized coupons yielded 1600 and 930 *M. avium* cfu/cm^2 immediately after exposure to a suspension of 10^5 *M. avium* cfu/mL [11]. Evidently, the mycobacteria adhered irreversibly upon exposure and remained on the surfaces during the initial washing steps [11]. Other factors that could have influenced the loss of *M. chimaera* cells from suspension were the instrument's age, prior exposure of the heater–cooler to repeated rounds of disinfection, and remnants of prior biofilms of different composition.

The measurement of the reappearance of *M. chimaera* cells after disinfection was directed towards identifying the required frequency of repeated disinfection, now shown to be 12 weeks. Further, it also confirmed evidence of increased killing of *M. abscessus* cells by Clorox in the CDC reactor upon exposure to another enzyme detergent, EnzyClean (Table I). That experiment was performed to test the hypothesis that an enzyme detergent treatment would release and kill more biofilm-associated cells. Based on the results of the combined enzyme detergent–Clorox disinfection protocol (Table III), it appears that protocol can delay or reduce the chance of re-inoculation of reservoir water by surviving biofilm cells. It has been shown that the enzyme detergent formulations may result in the release of cells in biofilms [18]. Release of cells would reduce the barriers to penetration of Clorox that are present in biofilms and increase killing as well as reduce bacterial biofilm numbers [18]. Further experiments are needed to determine the validity of that hypothesis.

The studies reported here do not represent a validation of a protocol. First, consideration should be given to the limit of detection of colony counts (3.3 cfu/mL). Given the large volume of the water reservoir (i.e. 13 L), there could be substantial numbers of *M. chimaera* remaining in the heater–cooler water system after the combined enzyme detergent and Clorox protocol; namely, 10,000 in total. Likely, those are in biofilms and can reinoculate the water. Another limitation of this study was that biofilms were not sampled directly to document the presence of *M. chimaera* cells after disinfection and draining and refilling. However, it would be impossible to sample all biofilms in a heater–cooler because of lack of access and the multiplicity of different surface compositions. Second, limited sampling would necessarily be biased (i.e. what surface to choose?) and possibly misdirect biofilm disinfection of specific surfaces. Third, in the absence of detectable numbers of *M. chimaera* cells in reservoir samples immediately after the disinfection, draining and refilling, the only source of *M. chimaera* cells as cfu, would have to be biofilms. Fourth, evidence of the rapid disappearance of *M. chimaera* cells upon inoculation is consistent with adherence to surfaces, the first step of biofilm formation. A further limitation is that the studies did not examine the efficacy of different dosages of the enzyme detergent or Clorox, nor was the efficacy of other enzyme detergents investigated. In addition, the exposure concentrations were limited to those recommended by the manufacturers of the enzyme detergent and the heater–cooler, and the effect of the combined enzyme detergent and Clorox exposure on the heater–coolers themselves has not been assessed; bearing in mind the strong chemical reactivity of both agents, such a future assessment is needed. Finally, the period of time for biofilm formation was brief (i.e. less than 20 min between heater–cooler inoculation and enzyme detergent and disinfectant challenge). It would be expected that an older, more mature biofilm would be more difficult to disrupt. However, Enzyclean was able to disrupt the 21-day biofilms of *M. abscessus* formed in the CDC bioreactor

(Table I). The efficacy of this protocol to kill and prevent the reappearance of *M. chimaera* cells in heater–coolers made by other manufacturers has been replicated.

M. chimaera and *M. abscessus* were chosen as the test bacteria based on the fact that they have been isolated from heater–coolers and linked to infections in patients who had undergone cardiac surgery [1,2]. As *M. chimaera* is a slowly growing *M. avium* complex (MAC) and *M. abscessus* a rapidly growing mycobacterial species, the results are likely to serve as accurate predictors of the behaviour of other *Mycobacterium* species [9]. As the mycobacteria are the most disinfectant-resistant bacteria and rapidly form biofilms of high density, the Prolystica and Clorox combination protocol will likely serve to disinfect heater–coolers and other medical equipment colonized by other opportunistic premise plumbing pathogens, such as *Pseudomonas aeruginosa* and *Legionella pneumophila* [10,11].

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Conflict of interest statement
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Appendix A. Supplementary data

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