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Effect of airborne hydrogen peroxide on spores of *Clostridium difficile*

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Summary
Background Contamination of surfaces by spores of *Clostridium difficile* is a major factor influencing the spread of healthcare-associated *C. difficile* infection. The aim of this study was to test the effect of an automated room disinfection system that provides an aerosol of 7.5 % hydrogen peroxide (H_2O_2) disinfectant, on spores of two different strains of *C. difficile*, and to evaluate the impact of biological soiling on the efficacy of H_2O_2 disinfection.

Material and method The strains used were a *C. difficile* PCR ribotype 027 and a *C. difficile* ATCC 9689. Spore suspensions of each strain were applied to ceramic tiles and exposed to aerosolized H_2O_2 at different locations in a test room. Biological soiling was simulated by bovine serum albumin and sheep erythrocytes. At set time points spores were recovered, plated onto Columbia 5 % sheep blood agar, and surviving bacteria were counted as colony-forming units (cfu).

Results No viable spores of either strain were recovered after a 3 h exposure to gaseous H_2O_2. Spores located inside a drawer showed recovery of approximately 1E5 cfu/ml for *C. difficile* ribotype 027 after 1 h. In the presence of organic matter, a more than fivefold log reduction compared with not exposed controls could be observed for spores of either strain tested.

Conclusion Appropriate decontamination of surfaces exposed to spores of *C. difficile* is challenging for conventional cleaning methods. Aerosolized H_2O_2 delivered by automated room disinfection systems could possibly improve surface decontamination and thereby reduce transmission of healthcare-associated *C. difficile* infection. Also in the presence of organic matter H_2O_2 disinfection appears to be an effective adjunct for decontamination of environmental surfaces.

Keywords *C. difficile* · Spores · Airborne hydrogen peroxide · Surface · Decontamination

Effekt von gasförmigem Wasserstoffperoxid auf Sporen von *Clostridium difficile*

Zusammenfassung
Hintergrund Kontamination von Oberflächen mit Sporen von *Clostridium difficile* ist ein wesentlicher Faktor für die Entstehung nosokomialer *C. difficile* Infektionen. Zum Zwecke der Raumdekontamination stehen automatisierte Systeme zur Verfügung, welche Lösungen von Wasserstoffperoxid vernebeln. Das Desinfektionspotential eines solchen Systems wurde hinsichtlich seiner Wirkung auf unterschiedliche *C. difficile* Stämme, in An- und Abwesenheit organischer Verunreinigung, untersucht.

Material und Methode Es wurden Sporensuspensionen zweier *C. difficile* Stämme, Ribotyp 027 sowie ATCC 9689, auf Keramikkacheln aufgebracht und an verschiedenen Positionen eines Versuchsraumes platziert. Die anschließende Begasung erfolgte mit einem Aerosol einer 7,5 %igen H_2O_2 Lösung. Zur Simulation organischer Verunreinigung wurden den Sporensuspensionen Rinderalbumin sowie Schaferythrozyten beigemengt. Zu definierten Zeitpunkten erfolgten eine Resuspendierung der Sporen und die Anzüchtung rekultivierbarer Bakterien.

Ergebnisse Nach dreistündiger Begasung konnten keine lebensfähigen Sporen beider *C. difficile* Stämme resuspendiert werden. Der sporizide Effekt war geringer auf Sporen, die im Inneren einer Lade platziert waren; hier ließen sich nach einer Stunde Begasung noch 1E5 KBE/ml *C. difficile* RT 027 rekultivieren. Bei Simula-

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tion organischer Verunreinigung konnte eine über fünffache log-Reduktion im Vergleich zu nicht exponierten Kontrollen beobachtet werden.

Schlussfolgerung Die Vernebelung eines Aerosols von Wasserstoffperoxid hat das Potential, die Oberflächenkontamination mit *C. diffile* zu reduzieren und somit die Übertragung nosokomialer *C. difficile* Infektionen hintanzuhalten. Auch bei organischer Verunreinigung scheint die Begasung mit H_2O_2 eine wirksame Ergänzung zur herkömmlichen Oberflächendekontamination zu sein.

Schlüsselwörter *C. difficile* · Sporen · Wasserstoffperoxid · Oberflächen · Dekontamination

Introduction

Clostridium difficile infection (CDI) is considered to be the main cause of bacterial infectious gastroenteritis in healthcare settings and a frequent cause of large hospital outbreaks [1]. Its clinical manifestations can range from mild diarrhea to severe pseudomembranous colitis with a 30-day mortality rate of up to 30 % [2]. CDI shows a considerable propensity to recur with recurrence rates of approximately 30 % [3]. While vegetative cells of *C. difficile* can survive in the environment on moist surfaces for up to 6 h, its spores are capable to persist for months and confer resistance to heating, drying, and chemical agents, including many commonly used disinfectants [4, 5].

CDI incidence has been rising worldwide, with approximately five episodes per 10,000 days of hospital stay in Europe [3]. In most healthcare facilities, CDI has become a more common infection than the classic nosocomial infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) [4].

The impact of CDI on healthcare settings and the associated economic burden are considerable: additional resources have to be expended on patient isolation, rigorous hygiene measures and specific therapy to eradicate *C. difficile* [6]. Excess costs through management of CDI are estimated to range from an extra 4067 to € 9276 per case [4].

Since 2000, highly virulent variants of toxigenic *C. difficile* have caused epidemics of CDI characterized by greater incidence, severity, and fatality [6]. These hypervirulent strains cluster into a distinct phylogenetic group and can be classified by polymerase chain reaction (PCR) ribotyping as ribotype 027 (RT 027) [7]. First reported responsible for major epidemics in North America, *C. difficile* RT 027 was soon detected in an increasing number of European countries including Austria [8].

Besides appropriate contact precautions and strict hand hygiene, environmental cleaning and surface decontamination are considered cornerstones of CDI prevention.

Environmental surfaces are a likely reservoir for pathogenic microorganisms, and play a role in the acquisition of healthcare-associated infections [9]. Multiple studies have demonstrated that surfaces can be contaminated with risk pathogens such as MRSA, vancomycin-resistant *enterococci*, *Acinetobacter* sp. as well as with spores of *C. difficile*, which can remain viable for several weeks to months [5, 10]. Regarding CDI, symptomatic patients as well as asymptomatic carriers contribute significantly to environmental contamination and disease transmission [11]. *Clostridium difficile* was recovered from a variety of sites in hospital rooms long after patient discharge [12], and there is evidence that contamination of patient rooms from previous occupants is associated with hospital-acquired CDI [13, 14]. Thus, routine hospital terminal disinfection does not reduce contamination sufficiently to fully prevent transmission.

Effective disinfection with conventional methods is challenging, as it relies on a human operator to correctly select and formulate an appropriate agent, and distribute that agent to all target surfaces for the specified contact time.

One novel method to improve the effectiveness of methods that significantly reduce surface contamination by nosocomial pathogens is the use of automated room disinfection systems (RDS). Different types of RDS are currently used in clinical settings, the most common being aerosolized hydrogen peroxide (H_2O_2) systems and H_2O_2 vapor systems. Most aerosolized H_2O_2 RDS deliver a pressure-generated aerosol, using a solution containing < 10 % H_2O_2 and < 50 ppm silver [13]. After exposure, the aerosol is left to decompose passively into oxygen and water.

H_2O_2 is the simplest peroxide and a biocide that, like other oxidative biocides such as chlorine and peracetic acid, is proposed to lead to peroxidation and disruption of membrane layers, enzyme inhibition, disruption of protein synthesis and, finally, cell death [15]. Such oxidizing agents are usually compounds of low-molecular weight and are considered to pass easily through bacterial cell walls and membranes [15].

Recent studies attributed RDS delivering H_2O_2 a great potential to aid to prevent environment-borne transmission through improving terminal disinfection of clinical surfaces [13, 15]. The aim of the present study was to test the antimicrobial efficacy of an RDS that provides an aerosol of 7.5 % H_2O_2 disinfectant, on spores of two different strains of *C. difficile*, as well as the impact of biological soiling on the efficacy of H_2O_2 disinfection.

Material and methods

Bacterial strains and growth conditions

The strains used in this study were a clinical strain (*C. difficile* PCR ribotype 027), isolated from a patient with severe diarrhea in 2013, and a commercially available reference strain (*C. difficile* ATCC 9689), both showing resistance to fluoroquinolones. The strains were stored at −80 °C in cryobank vials (Mast Diagnostics, Bootle Mer-

seyside, UK) until testing. Reculturing was performed on Columbia Blood Agar plates (BioMérieux, Marcy-l'Etoile, France).

Preparing of spore suspensions and organic matter

Clostridium difficile strains were inoculated onto Columbia 5% sheep blood agar plates (BioMérieux) and incubated for 7–10 days at 36 ± 1 °C under anaerobic conditions. Spores were harvested when ≥ 90% of the cells had converted into spores as determined by observation using Gram staining and microscopy. The spores were washed three times with distilled water, resuspended in distilled water, treated with ethanol 96% for 30 min, and evaluated microscopically by enumeration to ensure a purity of ≥ 95%. Until use, suspensions were stored at +4 °C. To assess the colony-forming efficiency of *C. difficile* spores, aliquots of serial dilutions of spore suspensions were plated onto Columbia 5% sheep blood agar plates (BioMérieux). Plates were incubated anaerobically at 37 °C for 24 h, and colonies were enumerated to determine initial counts.

To investigate the impact of biological soiling, bovine serum albumin (SERVA GmbH, Germany) and sheep erythrocytes (2% sheep red blood cells, AGES, Austria) were added to the spore suspension to obtain a final concentration of 0.3% each. The $\log_{10}$ reduction was calculated as $\log_{10}(N_0/N_{10})$, where N_0 = the number of spores in the positive control and N_{10} = the number of viable spores recovered from the test after 1, 2, and 3 h of exposure to H_2O_2.

The initial spore concentration used corresponded to approximately 10^5 cfu/ml for testing in absence of organic matter, and to approximately 10^6 cfu/ml for the test in presence of organic matter.

Test surfaces and application of spore suspension

Rectangular ceramic tiles, five times 5 cm in size, served as test surfaces. To standardize surface properties, tiles were washed with anionic tensides (Baktolin Basic Pure, Bode Chemie GmbH, Germany), rinsed with distilled water and with 70% ethanol. Subsequent sterilization was done with dry heat at 121 °C for 15 min. Tiles were stored in sterile bags until use.

An amount of 100 µl spore suspension was applied onto each ceramic tile and spread evenly with a sterile bacterial spatula. Spore suspensions were dried at room temperature for 1 h in a safety cabinet under laminar flow conditions.

Test room and hydrogen peroxide fumigation

An 18.9 sq. m-sized outpatient examination room, with a cubage of 54.7 m^3, was chosen as test area for H_2O_2 fumigation. Furniture included one examination couch, two desks, a mobile chest of drawers, located in-between the desks, and a washbasin. Tiles with spore suspensions without organic matter were placed in two positions (on a table top and inside a chest's drawer kept half open during evaporation). To investigate the impact of biological soiling, tiles with spore suspensions comprising organic matter were positioned in one location (on a table top).

The RDS used in this work was a mobile device (DCXpert; Braincon Technologies®, Vienna, Austria) that delivers 7.5% H_2O_2. The device was set up in the center of the room, at a distance of approximately 80 cm to the test surfaces. A gas detector (Draeger, Vienna, Austria) was placed next to the samples to log H_2O_2 concentration during and after termination of fumigation.

Prior to decontamination, the entrance door was sealed with adhesive tape and room ventilation was shut down. The test room was closed throughout the decontamination process, and no people were present.

H_2O_2 fumigation was performed for 3 h. At set time points, tiles were removed from the room by lab staff wearing protective clothing, and spore suspensions were resuspended immediately as described below. Controls were conducted with spore suspensions on tiles left unexposed to H_2O_2 vapor. After termination of fumigation, H_2O_2 was left to decompose until a concentration of below 1.0 ppm was reached.

Resuspension of spores and assessment of surviving bacteria

To resuspend surviving spores, tiles were placed upside down in sterile plastic containers with approximately 40 glass beads (VWR International, Pennsylvania, USA) and 10 ml resuspension fluid comprising distilled water (8.5 ml) and neutralization medium (1.5 ml) (Dey-Engley Neutralizing Broth; Sigma-Aldrich, USA). Upon shaking vigorously on a horizontal shaker (IKA Labortechnik, Staufen, Germany) for 5 min, the gained samples were serially diluted in distilled water to 10^{-5} and an aliquot of 1 ml was plated onto Columbia Blood Agar plates (BioMérieux). After incubating the agar anaerobically for 72 h, colony-forming units (cfu) were counted manually and log-reduction compared with unexposed controls was calculated.

Spore controls

In addition to spores of *C. difficile*, commercially available spore discs containing spores of *Geobacillus stearothermophilus* ATCC 12980 (Apex Discs, Tri-Scale, Apex Labs®) were simultaneously exposed to H_2O_2 vapor, serving as bioindicators to control the effect of decontamination. The spore strips were marked by numbers and placed aside the actual test surfaces. Exposure to H_2O_2 was performed equally, and time of exposure was identical with that of tiles inoculated with spores of *C. difficile*. Bioindicators were cultured blindly in a 10 ml tryptic soy

broth (CASO-Bouillon; Carl Roth GmbH, Germany) for 7 days at 55–60 °C. Growth or lack of growth was read in terms of turbidity; a clear broth was defined as negative, a turbid broth as positive.

Results

Spores of Clostridium difficile in the absence of organic matter

For the test surfaces positioned on the table top, a 1 h exposure of spores of *C. difficile* RT 027 to gaseous H_2O_2 revealed survival of 1 cfu/ml. Subsequent sampling at time points 2 and 3 h showed no recovery of any viable spores.

Recovery of spores of *C. difficile* RT 027 from tiles located inside the drawer showed survival of 10^5 cfu/ml after 1 h of H_2O_2 exposure displaying no reduction compared with unexposed controls. Sampling after 2 h of evaporation resulted in recovery of 3 cfu/ml. No viable spores of *C. difficile* RT 027 could be recovered from tiles located inside the drawer after 3 h of H_2O_2 exposure. After a 1 h exposure of spores of *C. difficile* ATCC 9689 to gaseous H_2O_2 no viable spores could be recovered from the test surfaces located on the table top. No survival of any spores was detected at subsequent sampling points. Spores of *C. difficile* ATCC 9689 that were located inside the drawer were less affected by fumigation. Exposure to H_2O_2 allowed for recovery of approximately 5×10^4, 2×10^3, and 11 cfu/ml at sampling points 1, 2, and 3 h, respectively (Fig. 1).

Spores of Clostridium difficile in the presence of organic matter

Sampling after a 1 h exposure of *C. difficile* RT 027 spores to gaseous H_2O_2 showed no recovery of any viable spores. No viable spores were recovered at sampling points 2 and 3 h.

After a 1 h exposure of spores of *C. difficile* ATCC 9689 to gaseous H_2O_2, recovery of spores revealed growth of 1 cfu/ml. Subsequent sampling at time points 2 and 3 h showed growth of 15 and 3 cfu/ml, respectively (Fig. 2).

Spore controls

Spore discs of *G. stearothermophilus* positioned on the table top displayed a fivefold log-reduction after 1 h of fumigation and a sixfold log-reduction at sampling points 2 and 3 h, compared with not exposed controls.

As for spore discs located inside a drawer, a fivefold log-reduction of *G. stearothermophilus* spores could be seen after 1 and 2 h of fumigation. Further exposure to H_2O_2 resulted in a sixfold log-reduction at the 3 h sampling point.

Discussion

Hospital acquired infections and particularly nosocomial outbreaks are associated with substantial morbidity and mortality, with subsequent increase in the duration of hospital stay and in healthcare costs [16].

Broad evidence exists that surface contamination with important nosocomial pathogens poses a risk for transmission of these organisms, and that decontamination of surfaces often stays behind demands. Contact with the environment, for instance, was shown to result as likely in the contamination of the hands of healthcare workers, as direct patient contact did [17].

Liquid preparations of oxidative biocides like H_2O_2 and peracetic acid are well-established surface disinfectants. However, their application relies on a human operator to correctly select and formulate an appropriate agent, and distribute that agent to all target surfaces for a specified contact time; a process implying considerable sources of error and uncertainty.

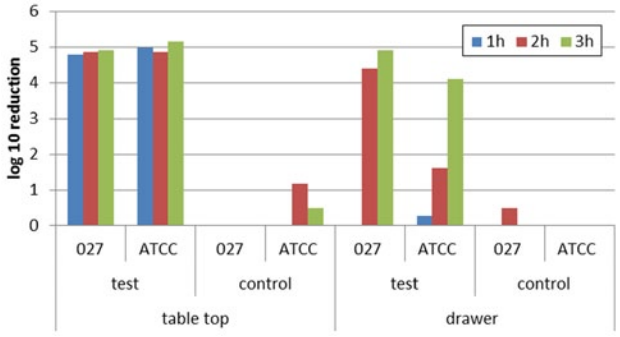

Fig. 1 Log_{10} reduction under hydrogen peroxide (H_2O_2) exposure (as compared with not exposed controls) of two *Clostridium difficile* strains (without organic soiling) placed at a desk or in a drawer; initial spore concentration: approximately 10^5 cfu/ml

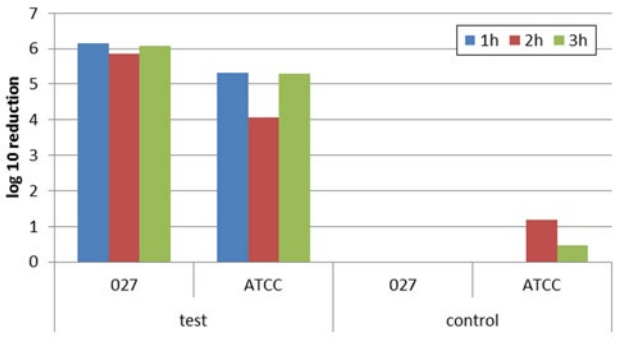

Fig. 2 Log_{10} reduction under hydrogen peroxide (H_2O_2) exposure (as compared with not exposed controls) of two *Clostridium difficile* strains (with organic soiling) placed at a desk; initial spore concentration: approximately 10^6 CFU/ml

RDS delivering gaseous H_2O_2 might contribute to thwart this discomfort, and are increasingly used at healthcare institutions in Europe and elsewhere [18]. Besides a possible benefit through repeatable applicability, and a reduction of reliance on an operator, RDS delivering gaseous H_2O_2 could pose additional advantage over conventional cleaning methods, since gaseous H_2O_2 interacts stronger with cellular macromolecules, causing greater oxidation of amino acids and proteins, compared with liquid formulations [15].

In the present study, we investigated the effect of 7.5 % gaseous H_2O_2, delivered by a programmable device, on spores of two different strains of *C. difficile*. Our findings revealed a more than 4.9-fold log-reduction of both, spores of *C. difficile* RT027 and *C. difficile* ATCC 9689 after 3 h of exposure to gaseous H_2O_2. After 1 h of H_2O_2 exposure, a more than 4.7-fold log-reduction could yet be observed for both, spores of *C. difficile* RT 027 and *C. difficile* ATCC 9689 located on a table top. This is in accordance with previous studies displaying an approximately fourfold log-reduction of *C. difficile* spores in similar decontamination protocols [13], and suggests a reasonable reduction given that the concentration of contamination on hospital surfaces is usually in the 2-$\log_{10}$ range [19].

Different types of RDS delivering gaseous H_2O_2 are currently used in healthcare settings; systems delivering an aerosol of H_2O_2—most frequently in concentrations below 10 %—and devices producing a heat-generated H_2O_2 vapor of typically 30–35 % aqueous H_2O_2 [13].

Aerosolized H_2O_2 systems have been repeatedly shown to effectively reduce *C. difficile* environmental contamination. Shapey et al. [20] investigated the effect of an RDS utilizing a H_2O_2 concentration of ~ 5 % on *C. difficile* environmental contamination in elderly care wards. After a single cycle of H_2O_2 decontamination a 94 % reduction of positive samples for *C.difficile* was observed (48/203 positive samples before H_2O_2 treatment, 7/203 positive samples after H_2O_2 treatment). This is comparable to a 91 % reduction of samples positive for *C. difficile* observed in a study by Barbut et al. [21], conducted with an RDS delivering an aerosol of 5 % H_2O_2. The in vitro part of the respective work, which assessed the sporicidal effect by use of a spore-carrier test, yielded a mean reduction of 4-$\log_{10}$ for spores of *C. difficile* after an approximately 1-h decontamination cycle (similar to our data showing a 4.7-fold log-reduction after 1 h of H_2O_2 exposure).

RDS producing H_2O_2 vapor have also been shown to exert strong sporicidal effects.

Havill et al. [22] reported a sixfold log-reduction of *C. difficile* spores after H_2O_2 decontamination (mean cycle time 153 min.), utilizing vaporized 30 % H_2O_2. Barbut et al. [21] used the same concentration of H_2O_2 for vapor decontamination of a hospital room and achieved comparable log-reductions of *C. difficile* spores (~ 6$\log_{10}$) [23].

H_2O_2 vapor systems operate with higher concentrations of H_2O_2 compared with aerosolized H_2O_2 RDS, which may contribute to the more pronounced sporicidal effects attributed to these systems [23]. However, the relationship between the level of residual contamination and infection is currently unclear [13], and further work is needed to determine the level of reduction required to interrupt transmission in various settings.

In the present study, the sporicidal effect of gaseous H_2O_2 was less pronounced on spores located inside a drawer, which might have resulted from ventilation deficiencies in this location. Andersen et al. [14] showed that decontamination of the internal parts of medical equipment by aerosolized H_2O_2 is less pronounced when the respective device was switched off and ventilation thereby disabled. Though, proper distribution of gaseous disinfectants via RDS must not be underestimated and validation of RDS should be mandatory, to ensure that such automated processes are effective and repeatable [13].

To avoid time-consuming microbiological sampling, the use of bioindicators can provide a cheap and easy semiquantitative measure of decontamination efficacy; 6-$\log_{10}$ bioindicators are considered an appropriate test for validating RDS [13]. In the present study, we could show a 6-$\log_{10}$ inactivation of the bioindicator *G. stearothermophilus* located on a table top and inside a drawer after 3 h of fumigation. This inactivation is consistent with previously published data on this topic, and has been shown to correlate well with the elimination of pathogens from surfaces [13].

Biological soiling influences the activity of biocides against bacterial spores and thus, appropriate cleaning of target surfaces should be mandatory prior to disinfection. Organic matter can reduce the effectiveness of RDS [13]. As H_2O_2 disinfection is applied after cleaning, levels of soiling encountered in the field should be low; nevertheless, H_2O_2 disinfection has also been shown to be effective in rooms that had not been cleaned [10]. Also in the present study, we could show considerable reduction of spores of *C. difficile* in the presence of organic matter, that was even more pronounced for spores of hypervirulent *C. difficile* RT027 (6-$\log_{10}$ after 3 h of H_2O_2 fumigation) than for *C. difficile* ATCC 9689 (5-$\log_{10}$). We do not know, why killing kinetics differed between the two strains tested, and we are aware of our study's limitation due to lack of multiplicate experiments. However, since sensitivity to H_2O_2 vapor in presence of organic matter could possibly be strain dependent, the influence of biological soiling on H_2O_2 resistance of important nosocomial pathogens should be addressed in future studies.

Contamination of surfaces by spores of *C. difficile* is a major factor influencing the spread of healthcare-associated CDI, and appropriate decontamination of surfaces exposed to spores of *C. difficile* is challenging for conventional cleaning methods. We could attest an RDS providing gaseous H_2O_2 in an automated and controlled way, a good sporicidal effect on both, spores of *C. difficile* RT 027 and *C. difficile* ATCC 9689, as well as a strong antimicrobial effect (of more than 3-$\log_{10}$) on further important nosocomial pathogens including MRSA, extended-spectrum beta-lactamase-producing *Escherichia coli*, vancomycin-resistant enterococci, and exten-

sively drug-resistant *Acinetobacter baumannii* (data not shown).

The use of RDS delivering gaseous H_2O_2 could constitute an effective adjunct to terminal room disinfection protocols comprising liquid formulations, and possibly aid to control important nosocomial pathogens. However, further studies are needed to determine parameters affecting decontamination of inner parts of furniture and medical equipment that are less ventilated during fumigation, as well as to further examine the impact of biological soiling on H_2O_2 fumigation resistance.

Conflict of interest

All authors declare that they have no conflict of interest.

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