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Influence of Drying Condition and Other Aspects on Recovery of *Pseudomonas aeruginosa* in the PN-EN 14561 Carrier Test

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Abstract

Pseudomonas aeruginosa is a bacterium sensitive to desiccation. Its use in disinfectant testing methods is important given its association with hospital-acquired infections. According to PNEN 14561:2008, obtaining a sufficient number of *P. aeruginosa* cells on a glass test surface under clean conditions after the drying process is essential for evaluating the bactericidal effectiveness of disinfectants. Does reducing the drying temperature of glass carriers contaminated with *P. aeruginosa* under clean conditions from the standard-recommended 37°C ± 1°C to 24°C ± 1°C improve bacterial recovery after drying? The recovery of *P. aeruginosa* from glass surfaces after drying was performed by the procedure specified for the water control, as outlined in PN-EN 14561:2008. Additionally, in the 24°C ± 1°C, the glass carriers were inoculated onto TSA medium, whereas at 37 °C ± 1°C the number of *P. aeruginosa* was determined in hard water following the contact time. The mean recovery of *P. aeruginosa* (6.37 log ± 0.12 log) was significantly higher in the 24°C ± 1°C variant compared to the 37°C ± 1°C variant (5.60 log ± 0.24 log). At 24°C ± 1°C, *P. aeruginosa* remained on the glass carrier after recovery, whereas at 37°C ± 1°C, the bacteria were detected in hard water. Lowering the drying temperature did not allow recovery of *P. aeruginosa* at the level required by PN-EN 14561:2008. The reduction in *P. aeruginosa* recovery resulted from bacterial detachment into hard water and retention on the carrier surface.

Keywords: *P. aeruginosa*, drying conditions, recovery, glass surface, PN-EN 14561:2008

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium considered sensitive to temperatures above 42°C. Its optimal growth temperature is 37°C, but it can survive across a wide range of temperatures from 4°C to 42°C, allowing it to inhabit diverse ecological niches (Diggle and Whiteley 2020; Tribelli and López 2022). *P. aeruginosa* is commonly found in water, soil, and on surfaces within hospital environments (Couchoud et al. 2023). Although it is classified as a desiccation-sensitive bacterium, it can survive in dry conditions through various defense mechanisms (Skaliy and Eagon 1972; Karash and Yahr 2022). Nevertheless, it primarily occupies humid environments where it forms biofilms. In medical settings, *P. aeruginosa* can contaminate taps, shower drains, sink traps, as well as catheters, prostheses, respiratory equipment, and endoscopes. It is recognized as one of the most frequent pathogens responsible for hospital-acquired infections, particularly those associated with mechanical ventilation in patients (Tribelli and López 2022; Couchoud et al. 2023). Volling et al. (2024) estimated the transmission rate of *P. aeruginosa* from a sink to a patient in intensive care unit to be between 5 and 7% (Volling et al. 2024). The presence of *P. aeruginosa* on environmental surfaces, combined

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with its high ability to acquire resistance to antibiotics and other antimicrobial substances, including certain disinfectants (Rozman et al. 2021), highlights the need to control this pathogen, particularly due to the biological threat it poses to immunosuppressed patients (Pang et al. 2019). For these reasons, *P. aeruginosa* is used as one of the test organisms to evaluate the bactericidal effect of disinfectants in suspension methods (phase 2, step 1) and carrier methods (phase 2, step 2) (PN-EN 14885:2022-09). While suspension methods allow for sufficient initial bacterial counts to test disinfectant efficacy, carrier methods face challenges in obtaining an adequate number of bacteria on the glass surface after drying under clean conditions, i.e., with an organic load of 0.3 g/l of bovine albumin. This limitation prevents meeting the recovery criteria specified in the PN-EN 14561:2008 standard, which governs testing the bactericidal effect of disinfectants used for the immersion disinfection of surgical instruments (PN-EN 14561:2008).

The objective of this study was to evaluate whether lowering the drying temperature of *P. aeruginosa*-contaminated glass carriers under clean conditions from the PN-EN 14561:2008 standard recommendation of 37°C ± 1°C to 24°C ± 1°C would enhance the recovery of *P. aeruginosa* after drying. Additionally, the study aimed to identify the key factors affecting the recovery rate of *P. aeruginosa* from the carrier surface in our laboratory.

Experimental

Materials and Methods

Preparation of test suspension. The test organism, *P. aeruginosa* ATCC® 15442™, was stored at –80°C on beads in microbanks. The strain was streaked onto TSA (Tryptic Soy Agar) and incubated at 37°C for 24 hours. The next passage obtained after 24 hours of incubation at 37°C was used to prepare the test suspension in accordance with the PN-EN 14561:2008 standard. The density of the *P. aeruginosa* suspension was estimated spectrophotometrically (λ = 650 nm) and confirmed by streaking on TSA medium (incubation 37°C; 48 h). The test suspension was combined with an organic load (clean conditions: 0,3 g/l albumin solution in diluent) in a ratio of 9 ml suspension to 1 ml organic load. A volume of 50 µl of this mixture was applied to the surface of a glass carrier measuring 1 cm × 1 cm. The carrier was a one-sided frosted glass slide with dimensions of 75 mm × 25 mm × 1 mm, manufactured by the Superior Paul Marienfeld GmbH & Co. KG (Germany). The surface was prepared for testing in accordance with the PN-EN 14561:2008 standard.

Drying conditions and test procedure. The carriers containing the test suspension and organic load were dried in an incubator without a fan at two temperature conditions: i) 24°C ± 1°C, ii) 37°C ± 1°C. When drying at 24°C ± 1°C, calcium chloride for desiccators was placed in the incubator to reduce humidity. Temperature and humidity were monitored throughout the drying process and drying time was measured until the carrier surfaces appeared visually dry. Tests at 24°C ± 1°C were performed during the summer, while those at 37°C ± 1°C were performed in winter, which affected the humidity levels inside the incubator. The number of *P. aeruginosa* in suspension (N) and the number of *P. aeruginosa* on a glass carrier after drying were measured, following the hard water control procedure (Nw) as specified in PN-EN 14561:2008. For carriers dried at 24°C ± 1°C, the contact time with hard water was 60 minutes, while for those dried at 37°C ± 1°C the contact time with hard water was 15 minutes. After the contact time, each carrier was transferred to a neutralizer with glass beads and shaken for 60 seconds (modification compared to the standard, which specifies the shaking time at 15 seconds). The neutralization time was 5 min ± 10 s. After this time, a series of dilutions was prepared in the neutralizer. Two 1 ml samples from each dilution were inoculated using the pour plate technique and incubated on TSA medium at 37°C for 48 hours. Additionally, to identify other limitations in the recovery of *P. aeruginosa*, for the 24°C ± 1°C condition, the glass carrier was rinsed with 10 ml of diluent and was inoculated onto TSA medium (retention on the carrier surface). For the 37°C ± 1°C condition, after transferring the carrier to the neutralizer, 0.5 ml of hard water in which the carrier had remained was used to prepare a series of tenfold dilutions (detachment into hard water). Two 1 ml samples from dilutions ranging from 10^{-1} to 10^{-4} were inoculated using the pour plate technique. Each variant was tested in six independent replicates. Recovery results of *P. aeruginosa* from the carrier surface are presented in decimal logarithmic scale [log].

Statistical analysis. The mean values and standard deviations were calculated for the obtained results. To determine whether the recovery of *P. aeruginosa* differed significantly between 24°C ± 1°C and 37°C ± 1°C,

the 95% confidence interval (CI) was calculated using a two-tailed test at a significance level of 0.05, with degrees of freedom $df = 5$.

Results

Effect of temperature and humidity on the recovery of *P. aeruginosa* from glass carriers under clean conditions. Given the notable differences in humidity observed between the two temperature settings, the influence of this parameter was included in the analysis and considered alongside temperature. In the studies on the recovery of *P. aeruginosa* from the surface of glass carriers under clean conditions, in the 24°C ± 1°C variant, the recorded temperature ranged from 24.6°C to 24.8°C and remained within the acceptable range. Relative humidity varied from 37% to 40% (Fig. 1). The highest recovery of *P. aeruginosa* from the surface after drying was observed at 24.8°C ± 0.4°C and 40% ± 2% humidity (6.50 log). The lowest recovery values of *P. aeruginosa* were recorded at 24.6°C ± 0.2°C with 37% ± 1% humidity (6.22 log) and at 24.7°C ± 0.1°C with 40% ± 2% humidity (6.23 log). However, these temperature

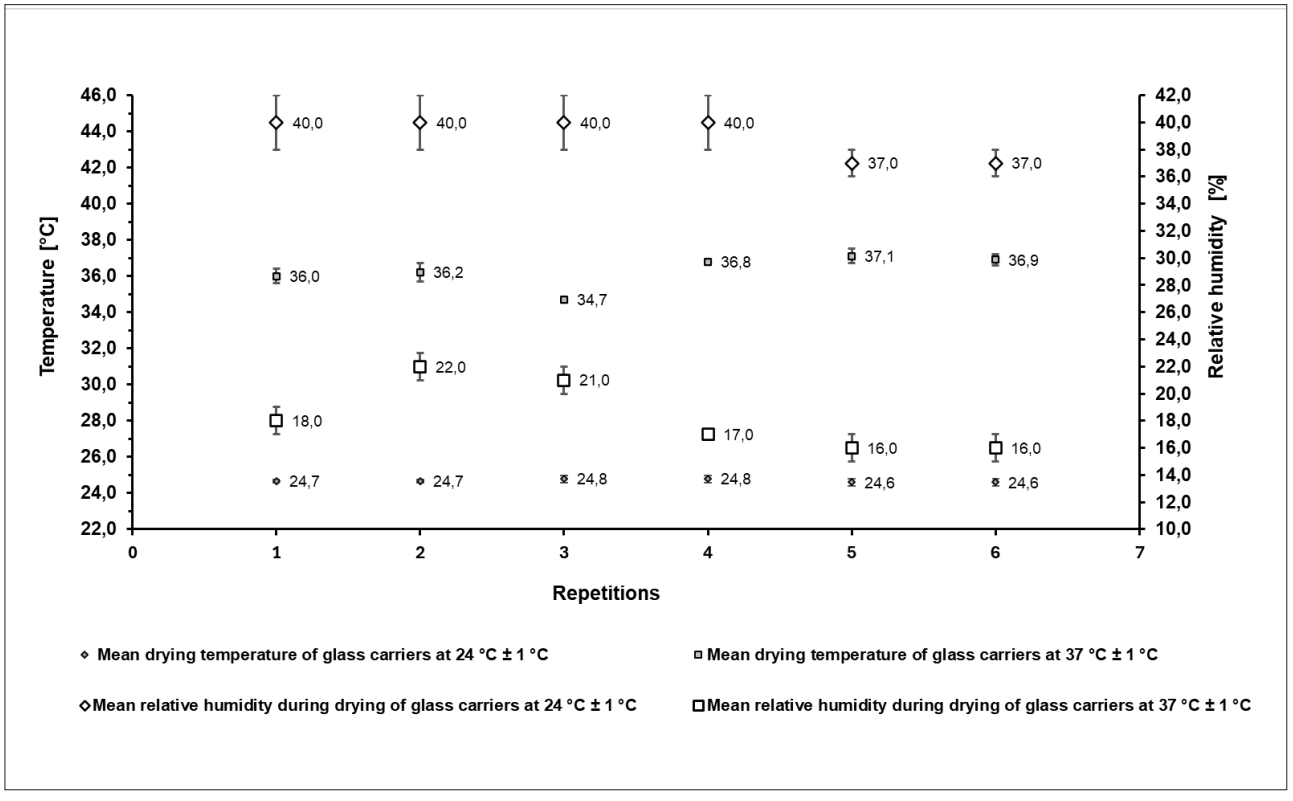


Fig. 1. Mean changes in temperature and relative humidity (± standard deviation) during the drying of glass carriers contaminated with *Pseudomonas aeruginosa*, recorded across individual replicates under two temperature conditions: 24°C ± 1°C and 37°C ± 1°C.

and humidity variations did not result in significant differences in *P. aeruginosa* recovery between replicates in this drying variant (Fig. 2). In the 37°C ± 1°C variant, temperatures ranged from 34.7°C to 36.9°C. In replicate 3, the temperature fell below the target range (34.7°C ± 0.1°C), but this did not significantly affect *P. aeruginosa* recovery compared to other replicates. Relative humidity ranged from 16% to 22%, with replicate 3 reaching a relatively high value of 21% ± 1% (Fig. 1). However, this did not significantly impact the bacterial recovery. The highest recovery of *P. aeruginosa* (5.95 log) occurred at 36.0°C ± 0.4°C and 18% ± 1% relative humidity (replicate 1), while the lowest recovery of *P. aeruginosa* (5.30 log) was recorded at 37.1°C ± 0.4°C and 16% ± 1% humidity (Fig. 2). As with the 24°C ± 1°C variant, the drying parameters applied at 37°C ± 1°C did not lead to significant differences in *P. aeruginosa* recovery between replicates.

No significant differences were observed in the density of *P. aeruginosa* suspensions used to prepare suspensions in organic load (under clean conditions) for drying on glass carriers. However, a significant difference was found in the mean recovery of *P. aeruginosa* after drying at two temperature variants: 24°C ± 1

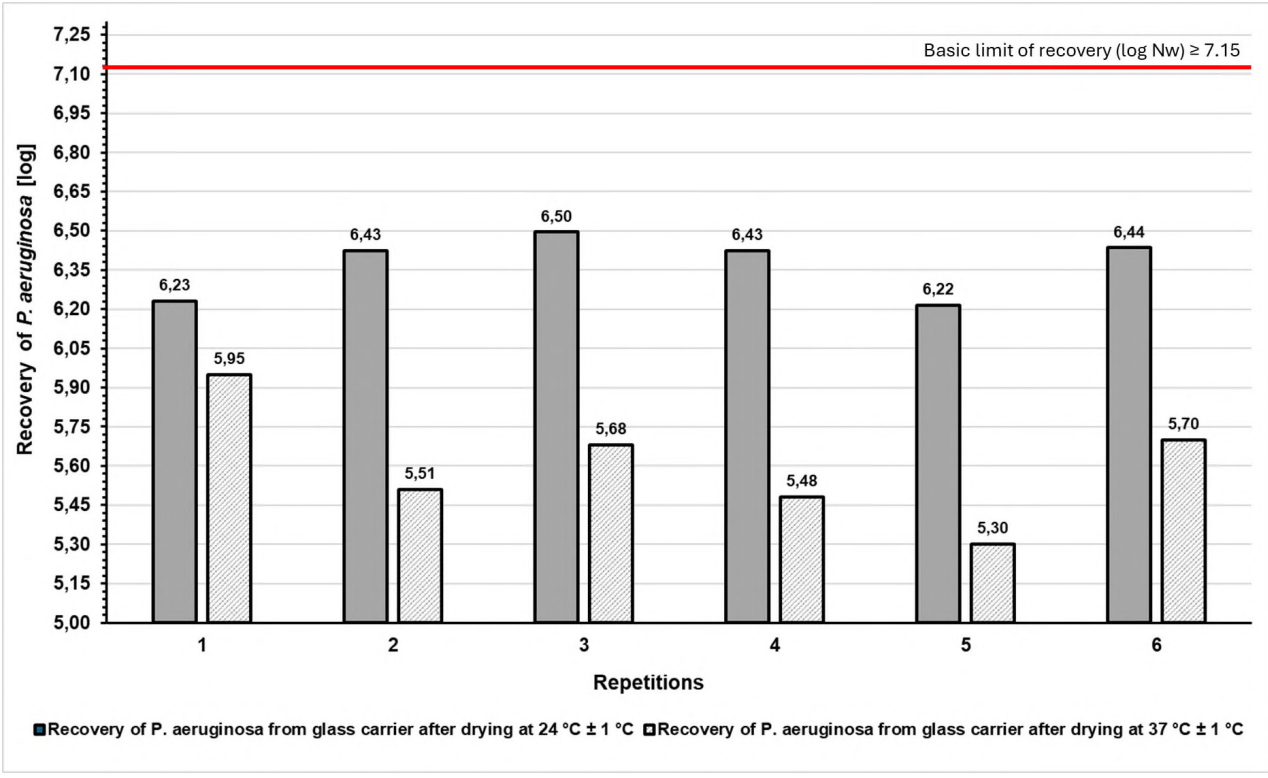


Fig. 2. Recovery of *Pseudomonas aeruginosa* from glass carriers [log] after drying in individual replicates (n = 6) under two different temperatures: 24°C ± 1°C and 37°C ± 1°C (clean conditions).

°C and 37°C ± 1°C. The 95% confidence interval (CI) at a significance level of 0.05 for a two-sided test and degrees of freedom *df* = 5 was 6.37 log ± 0.12 log at 24°C ± 1°C and 5.60 log ± 0.24 log at 37°C ± 1°C. The mean recovery of *P. aeruginosa* under clean conditions after drying at the lower temperature was higher than that obtained at the higher temperature. However, this higher recovery at 24°C ± 1°C was achieved with double the drying time of the *P. aeruginosa* suspensions (Table I). In neither drying condition, 24°C ± 1°C nor 37°C ± 1°C variants, was the *P. aeruginosa* recovery threshold required by the PN-EN 14561:2008 standard, i.e., ≥ 7.15 log, achieved. In studies evaluating the retention of *P. aeruginosa* on glass surfaces after recovery at 24°C ± 1°C, bacterial growth was observed on the glass carrier in quantities ranging from 194 CFU to >330 CFU. In the 37°C ± 1°C variant, *P. aeruginosa* growth was observed in hard water after removal of the contaminated carrier, with the average count of 6.17 log ± 0.37 log. These findings suggest that the procedure used resulted in incomplete recovery of *P. aeruginosa* from the carrier surface. Additionally, there was partial detachment of the microorganism during immersion, which led to the formation of a suspension in hard water within the 15-minute contact time.

Table I

Mean values (MN) and standard deviations (SD) of suspension density (N), recovery of *Pseudomonas aeruginosa* from glass carrier (Nw) and drying time (t) of contaminated glass carriers (n = 6) in two different temperatures: 24°C ± 1°C and 37°C ± 1°C.

N [log]				Nw [log]				t [min]			
24°C		37°C		24°C		37°C		24°C		37°C	
MN	SD	MN	SD	MN	SD	MN	SD	MN	SD	MN	SD
9,63	0,09	9,62	0,05	6,37	0,12	5,60	0,22	122	9	59	10

Discussion

The study indicated that *P. aeruginosa* recovery was greater at lower temperatures and higher humidity levels; however, the modified drying conditions were still inadequate to meet the recovery level required by the PN-EN 14561:2008 standard.

According to the literature, *P. aeruginosa* can survive at temperatures up to 42°C (Diggle and Whiteley 2020). For this reason, the temperature conditions used should not have adversely affected its viability on the glass surface. The significantly higher recovery of *P. aeruginosa* from glass surfaces dried at 24°C ± 1°C, compared to those dried at 37°C ± 1°C, may be attributed to the extended drying time and higher relative humidity at the lower temperature. According to Karash and Yahr (2022), *P. aeruginosa* survival increases when the drying period is extended, and the rehydration rate is slower. These conditions allow cells to activate protective mechanisms that mitigate osmotic and oxidative stress. Moreover, *P. aeruginosa* shows greater survival in moist environments, while survival in dry environments is possible but requires *P. aeruginosa* to achieve a certain degree of desiccation tolerance (Karash and Yahr 2022). In light of above literature data, the use of milder drying conditions and higher humidity may have contributed to the higher recovery of *P. aeruginosa* at 24 °C ± 1 °C, as the cells were not exposed to environmental changes that could lead to rapid cell dehydration.

Additionally, in the 37°C ± 1°C variant, the use of *P. aeruginosa* glass carriers immediately after drying in the test with hard water at a temperature of 20°C ± 1°C, was associated with a larger temperature difference compared to the 24°C ± 1°C variant. This more pronounced thermal shift may have potentially reduced *P. aeruginosa* recovery at 37°C ± 1°C. Considering the unfavourable conditions in the 37°C ± 1°C variant, the change in drying temperature from 37°C ± 1°C to 24°C ± 1°C, as used in research, did not contribute to achieving the recovery of *P. aeruginosa* required by the PN-EN 14561:2008 standard. The difference in recovery observed was too small to justify recommending a reduction in the drying temperature for *P. aeruginosa* carriers. Although drying the carriers at a lower temperature did not decrease the recovery of *P. aeruginosa*, it significantly increased both the drying time and the overall duration of the test. Moreover, the lower recovery of *P. aeruginosa* in the 37°C ± 1°C variant could be caused by much lower humidity compared to humidity in the 24°C ± 1°C variant. The author suggests that the primary reason for the loss in recovery is the retention of microorganisms on the carrier surface, and their detachment from the surface when exposed to hard water. This is supported by results showing the presence of *P. aeruginosa* on the carriers after neutralization at 24°C ± 1°C, as well as in hard water following the immersion of the carrier at 37°C ± 1°C. Although it is difficult to determine, changes in the recovery of *P. aeruginosa* may also result from different contact times used in the tests with hard water in the 37°C ± 1°C variant and in the 24°C ± 1°C variant. It was assumed that microorganism detachment in hard water would be tested at a shorter contact time than that used in the 24°C ± 1°C variant, which may have contributed to increased recovery from the carriers in the 37°C ± 1°C variant. The use of an extended recovery time of *P. aeruginosa* in the neutralizer with glass beads (60 s instead of 15 s) compared to the standard requirements did not enable complete bacterial recovery. In the 24°C ± 1°C variant, *P. aeruginosa* was grown in uncountable numbers (> 330 CFU). The identification of bacteria that detach in hard water at a temperature of 37°C ± 1°C indicates the need to enhance bacterial adhesion to the carrier. This could potentially be achieved by using a glass surface with improved adhesive properties or by increasing the viscosity of the suspension. Although this area of research was not the focus of our study, existing literature indicates that *P. aeruginosa* produces higher amounts of exopolysaccharides at lower temperatures, with the peak production observed at 20°C. However, as the temperature rises to 25°C, 30°C, and 37°C, the quantity of exopolysaccharide produced tends to decrease. This phenomenon may weaken the protective mechanisms of the cells against stress factors, reducing the survival of *P. aeruginosa* on surfaces (Kim et al. 2020). Reduced exopolysaccharides production may also decrease *P. aeruginosa's* adhesion to surfaces (Boyd and Chakrabarty 1995). It also limits the functions of exopolysaccharides in *P. aeruginosa* cells, such as protection against desiccation, maintaining hydration, and early biofilm formation (Gheorghita AA et al. 2023). Although bovine albumin solution plays a similar role to exopolysaccharides in the carrier test, the concentration of bovine albumin, used under clean conditions, does not provide adequate suspension viscosity or increase its adhesion to the carrier surface, in contrast to dirty conditions, where the albumin concentration is ten times higher (PN-EN 14561:2008).

It has been suggested that a higher initial microbial count may enhance recovery and survival after drying (Morgan et al. 2006). However, in this case, it was not feasible to improve the recovery of *P. aeruginosa* after drying by increasing its concentration in the initial suspension, due to the maximum inoculum density specified in the PN-EN 14561:2008 standard. The bacterial load in the initial suspensions was high and possibly underestimated due to the high number of *P. aeruginosa* obtained from hard water following carrier immersion in the 37°C ± 1°C variant. The findings of our study indicate that the survival of *P. aeruginosa* is enhanced in humid environments. Therefore, it may be beneficial to incorporate relative humidity control into the drying conditions specified in PN-EN 14561:2008 or to carry out the drying process under controlled humidity conditions. In our study, while we monitored humidity during drying, we did not conduct the experiments under controlled humidity. Further research is necessary, as the recovery of microorganisms after drying is influenced by various factors, including the conditions of the growth medium, the growth phase, and the physiological state of the microorganisms prior to drying (Morgan et al. 2006).

The studies conducted indicate that the low recovery of *P. aeruginosa* from glass surfaces is primarily due to losses that occur during mechanical activities at different stages of the test involving hard water, rather than just drying conditions or a lower concentration of the protective substance (i.e., albumin solution). The mechanical activities include immersing the carrier for the specified contact time, detaching microorganisms, and mechanically recovering them by shaking the carrier with glass beads in the neutralizing solution.

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Conflict of interest

The author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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