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Evaluation of temperature, drying time and other determinants for the recovery of Gram-negative bacterial pathogens in disinfectant efficacy testing

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SUMMARY

Background: In the clinical setting, surface disinfection is an important measure to reduce the risk of cross transmission of micro-organisms and the risk of nosocomial infections. Standardized methods can be used to evaluate disinfection procedures, as well as the effectiveness of the active ingredients used for disinfection. However, despite standardization, the results of such methodologies are still determined by several factors, and incorrect results may lead to invalid assumptions about the effectiveness of a disinfectant, posing significant health risks for patients and health personnel.

Aim: The objective of this study was to evaluate several determinants for the recovery of *Pseudomonas aeruginosa* and other test organisms to establish their influence on the results of standardized disinfection methodologies, and to find Gram-negative strains that can be used as suitable replacements for *P. aeruginosa*.

Methods: The effects of inoculum application method, drying time, temperature and carrier material on the survival and recovery of the test organisms were evaluated using Student's *t*-test, one-way analysis of variance and Tukey's multiple comparison test.

Findings and conclusions: Temperature, drying time, application method and carrier material were found to affect the recovery of *P. aeruginosa* cells significantly, and therefore influence the outcome of the methodologies used. This study also showed that *P. aeruginosa* could be replaced with the Gram-negative species *Acinetobacter baumannii*, a test organism used in many standardized methodologies, which responds better under the same circumstances and has a behaviour similar to that of *P. aeruginosa* in disinfectant efficacy tests.

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Introduction

In hospitals and clinical environments, micro-organisms from colonized patients and staff are shed into areas in the vicinity of patients and on to surfaces, turning them into important contributors to the spread of nosocomial pathogens [1]. To curb the increase of nosocomial infections [2], there has been a surge in the use of compounds for environmental disinfection of high-touch surfaces [1,3].

The efficacy of the disinfectants used for these decontamination approaches must be quantified using specific standardized procedures [1]. Despite standardization, the effectiveness of a disinfectant remains dependent on a variety of factors [1,2], including aspects inherent to the product (e.g. formulation, concentration), application method (e.g. surface, temperature) and target micro-organisms. Moreover, depending on the application area or the specific conditions of the methodology, the standardized norms list the test organisms with which the procedure is meant to be carried out. For efficacy testing of bactericidal products, a variety of Gram-positive and Gram-negative organisms are used, and among the latter, *Pseudomonas aeruginosa* is one of the most frequently listed.

Within the methodologies for efficacy testing of disinfectants under practical conditions, such as EN 13697:2019 or EN 16615:2015, it is required that the suspension of each test micro-organism should be inoculated on to the germ carrier and left to evaporate until 'visually dry' [4,5]. However, the physiological stress imposed by the removal of water from cells is not tolerated equally by all organisms [6]. Although susceptibility to desiccation is difficult to compare between studies, lower viability among Gram-negative species is often clear [6], leading to lower bacterial recovery and, consequently, inaccurate test results [7].

This study evaluated the influence of several determinants (temperature, carrier material, drying time, inoculum application method) on the recovery rate of *P. aeruginosa, Acinetobacter baumannii and Salmonella enterica* in comparison with representative Gram-positive species (*Staphylococcus aureus* and *Enterococcus hirae*).

Methods

Bacterial strains

The following Gram-positive and Gram-negative reference strains were used in this study: *S. aureus* subsp. *aureus* ATCC 6538, *E. hirae* ATCC 10541, *P. aeruginosa* (Schroeter) ATCC 15442, *S. enterica* subsp. *enterica* ATCC 13076, *A. baumannii* ATCC 19606 and *A. baumannii* ATCC 19568.

Germ carriers

Three types of carriers were used: (a) polyvinyl chloride (PVC): PUR-treated, 12.1 x 8.6 x 8.6 cm; 2.5 mm thick (Verbund für Angewandte Hygiene e.V., Bonn, Germany); (b) stainless steel: 100 x 500 x 200 mm; 0.5 mm thick (BAM Maschinenbau GmbH, Altenstadt an der Waldnaab, Germany); and (c) high-density polyethylene (HDPE): polyethylene PE 300 — natural white, 500 x 200 x 2 mm (ONLINE-PLAST, Koblenz, Germany).

The carriers were placed in 5% Decon-90 solution (Fisher Scientific, Schwerte, Germany) for 60 min, rinsed for 10 s under fresh running tap water, rinsed for 10 s with distilled water or ultrapure water, submerged in 70% isopropanol (Carl Roth GmbH, Karlsruhe, Germany) for 15 min, and air-dried immediately before use. Each carrier, regardless of the material, was only used once.

Preparation of test suspensions according to EN 13697:2019

Bacteria from inoculated 24—36 h tryptic soy agar (TSA) plates (Oxoid, Basingstoke, UK) were suspended in NaCl-peptone broth adjusted to pH 7.0 ± 0.2 (3.56 g/L KH_2PO_4; 5.77 g/L Na_2HPO_4; 4.3 g/L NaCl and 1.0 g/L meat peptone; all reagents from Carl Roth GmbH). The density of the suspension was adjusted with a DEN-1B densitometer (Grant Instruments, Royston, UK) to 1.5—5 x 10^8 colony-forming units (CFU)/mL for all test micro-organisms, except *P. aeruginosa* (1.5—5 x 10^9 CFU/mL). Afterwards, 1 mL of the suspension was combined with 1 mL of 0.06% bovine serum albumin (BSA) for a final concentration of the organic load in the test procedures of 0.03%.

Preparation of test suspension according to EN 16115:2015

Bacteria from inoculated 24—36 h TSA plates were suspended in NaCl-peptone broth (described above). The suspension was adjusted to 1.5—5 x 10^9 CFU/mL, and 0.9 mL was combined with 0.1 mL of 0.3% BSA for a final concentration of the organic load in the test procedures of 0.03%.

Evaluation of the effect of inoculum application method and drying temperature on the survival of test organisms

Test suspensions of *S. aureus* and *P. aeruginosa* were prepared according to EN 16615:2015 (CEN, 2015). The suspension (50 µL) was applied to the PVC surface with a pipette and (a) spread out in a 25-cm^2 area with a sterile metal spatula; or (b) distributed as 10 equally distanced droplets of 5 µL each without spreading. The inocula were dried at 20 °C or 37 °C until the surface was visibly free of humidity using a forced-air UVP incubator (Analytik Jena, Jena, Germany) set to either temperature. A minimum of six parallel PVC surfaces were inoculated per bacterium, per application method and per drying temperature in three separate test runs. The bacteria were recovered using nylon swabs soaked previously in a sterile TLSH neutralization solution [3% Tween 80 (Merck KGaA, Darmstadt, Germany), 3% saponin (AppliChem GmbH, Darmstadt, Germany), 0.3% lecithin and 0.1% L-histidine (both Carl Roth GmbH)] and then used for 1 min in different directions in the test field. They were mixed by vortexing for 25 s in reaction tubes containing 5 mL of TLSH. Serial dilutions of these suspensions were plated in TSA (Oxoid) and incubated aerobically at 37 °C for 36—48 h to obtain the bacterial counts. The percentage recovery rates were calculated by comparing the number of living cells recovered after full drying (N_t) with the initial number of living cells (N_0) sampled before drying commenced, using the following formula:

$$\text{Recovery rate } (\%) = \frac{100}{10^{(logNo - logNt)}}$$

To complement this experiment, the cell counts of bacterial suspensions were determined at two different drying temperatures (20 °C and 37 °C) at several time points, prepared in accordance with the methods described in EN 16615:2015 (see online supplementary material).

Evaluation of the recovery rate of P. aeruginosa on PVC vs stainless steel germ carriers

To determine the effect of the carrier material on the recovery rate of the bacteria, a test based on the methodology of EN 16615:2015 [4] was performed. A suspension of *P. aeruginosa* was prepared, and 50 µL was applied to four PVC carriers and four stainless steel carriers. All carriers were then incubated with ventilation at either 20 °C or 37 °C in a forced-air UVP incubator (Analytik Jena) set to either temperature. Starting with a carrier with t=0, they were removed from the incubator at 5-min intervals up to a maximum incubation time of 25 min. The test organisms were recovered from the carriers using the swabbing method described previously. Dilutions of 10^{-3}, 10^{-4} and 10^{-5} were plated in duplicate and incubated aerobically at 37 °C for 36–48 h. Percentage recovery rates were determined using the formula shown above.

Drying tests according to EN 13697 with several Gram-negative organisms

Strains of *A. baumannii* ATCC 19568 from ASTM E2967 and *A. baumannii* ATCC 19606 from EN 17272:2020, as well as *S. enterica* and the standard test organism of European norms *P. aeruginosa*, were used to compare their sensitivity in drying tests according to EN 13697:2019. Fifty microlitres of 10^8 CFU/mL of each micro-organism was pipetted on to a steel carrier and dried at 37 °C with ventilation over a period of 40 min in a forced-air UVP incubator (Analytik Jena). The test was performed with nine parallel carriers for each organism.

Every 5 min, the test organisms were recovered from the germ carriers. Dilutions of 10^{-3}, 10^{-4} and 10^{-5} were plated out and incubated, counted and analysed according to EN 13697:2019 [5]. The suspensions were recovered from the carriers at 5-min intervals up to 40 min.

As reference, the survival of *P. aeruginosa* was correlated with water loss of the test suspension by weighing the stainless steel carriers after the respective drying times had elapsed.

Drying tests according to EN 16615 with several Gram-negative organisms

The drying resistances of *A. baumannii* ATCC 19606 and *P. aeruginosa* were determined by drying test suspensions applied to PVC surfaces at 20 °C without air circulation in a room in which relative humidity was maintained at 45 ± 5 %. The test organisms were recovered after three drying times: (a) when the surface was half-dry (determined visually as half the size of the original inoculum); (b) when the surface was 'optically dry'; and (c) 5 min after the surface was 'optically dry'. Each test organism had nine parallel carriers, and the inoculum was recovered from each carrier using a nylon swab as described previously. The percentage recovery of the micro-organisms in relation to the initial count was determined by comparing the number of living cells recovered after each of the three drying endpoints (N_t) with the initial number of living cells (N_0) sampled before drying commenced, using the following formula:

$$\textit{Recovery rate}\ (\%) = \frac{100}{10^{(logNo - logNt)}}$$

In addition, to evaluate the behaviour of alternative germ carriers and their effect on the survival of test organisms, drying tests in accordance with EN 16615:2015 [4] were also carried out on stainless steel and HDPE with *A. baumannii* ATCC 19606 and *P. aeruginosa*, in the same conditions as described above.

Statistical evaluation

To evaluate the effect of inoculum application method and drying temperature on the survival of test organisms, Student's *t*-test was used with an error probability of 5% [8]. In addition, to evaluate the recovery rate of *P. aeruginosa* on PVC vs stainless steel germ carriers, as well as for the drying tests according to EN 13697 with several Gram-negative organisms, one-way analysis of variance was performed to compare surface materials and temperatures at each drying point, followed by Tukey's multiple comparison test to determine any significant differences between the groups. Kruskal–Wallis test was used when the data were non-normally distributed.

Results

Effect of inoculum application method and drying temperature on the survival of test organisms

PVC surfaces were inoculated with bacterial suspensions in two different manners: (a) spreading with a spatula; and (b) in the form of droplets without spreading. The drying times for the suspensions and mean recovery rate of the bacteria are presented in Figure 1. Regardless of the application method and temperature, mean drying times of *S. aureus* were not significantly different to those of *P. aeruginosa*. Significant differences were found, however, for the percentage recovery rate of both test organisms at 20 °C when the test suspension was applied in droplets compared with application with a spatula. In general, recovery rates were lower than 50% for *P. aeruginosa*, with the highest achieved at 20 °C with application as droplets (47.79 ± 9.24%), and the lowest achieved at 37 °C with application with a spatula (4.30 ± 0.72%).

Evaluation of the recovery rate of P. aeruginosa on PVC vs stainless steel germ carriers

The influence of drying on the recovery of *P. aeruginosa* from different carriers was examined according to EN 16615:2015 on PVC and stainless steel test surfaces (Table I). The inoculum was distributed with a sterile spatula. After the inoculum had been deposited on the PVC carriers, different-sized droplets formed which could not be distributed evenly after repeated attempts. In comparison, the inoculum on the steel carriers could be spread out evenly with the spatula with a single attempt.

As shown in Table I, the recovery from both surfaces decreased over time, and significant differences were found between drying temperatures (P=0.037) and carrier material

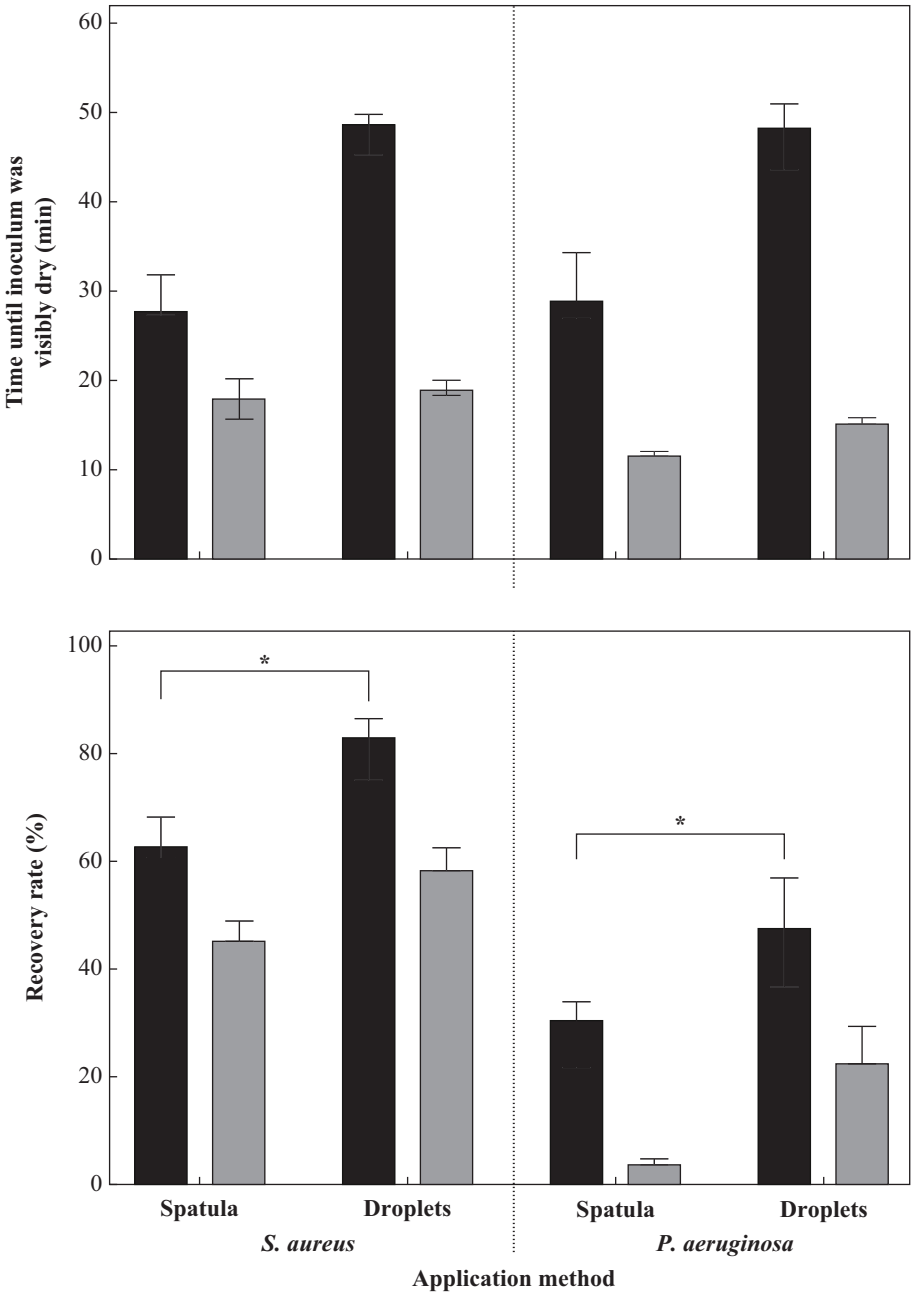

Figure 1. Comparison of drying times (upper panel) and percentage recovery rates (lower panel) of 50 μL of a suspension of *Staphylococcus aureus* (left) and *Pseudomonas aeruginosa* (right) at 20 °C (black bars) and 37 °C (grey bars) after application of inoculum as droplets or with a spatula on polyvinyl chloride carriers. Results shown are for an average of $N=6$ for each organism. *Significant difference.

($P=0.029$). Moreover, significant differences were observed after 10 ($P=0.0012$) and 15 ($P=0.0192$) min of drying for both carrier types.

Drying tests according to EN 13697 with several Gram-negative organisms

In drying tests according to EN 13697:2019, the recovery cell counts for the *Acinetobacter* strains had only slight differences, and after 15 min had passed, they remained consistently higher than those for *P. aeruginosa* (Figure 2). After 20 min of drying, *S. enterica* and *P. aeruginosa* had recovery cell counts below the minimum cell count of $10^{6.27}$ prescribed in EN 13697:2019. After 15 and 20 min, the drying behaviour of *P. aeruginosa* was significantly different to that of *A. baumannii* ATCC19568 alone ($P=0.0059$). After 25 min of drying, the drying behaviour of all alternative organisms was significantly different compared with the drying behaviour of *P. aeruginosa*, in particular for A*cinetobacter* spp. ($P\leq0.0001$).

Drying tests according to EN 16615 with several Gram-negative organisms

Considering the previous results, new drying tests according to EN 16615:2015 were carried out with strains used in EN 17272:2020: *A. baumannii* ATCC 19606 and *P. aeruginosa*.

Table I
Recovery rates (%) of a suspension of *Pseudomonas aeruginosa* on stainless steel and polyvinyl chloride (PVC) carriers, dried at 20 °C and 37 °C

Time (min)	20 °C		37 °C	
	PVC	Steel	PVC	Steel
0	98.7	96.6	97.8	96.9
5	98.3	96.9	98.0	93.9
10	97.8	93.0	97.3	82.6
15	97.0	92.2	94.4	14.1
20	89.1	88.0	90.9	2.5
25	86.1	84.1	75.8	0.7

Results shown are for an average of $N=4$ for each organism.

Figure 3 shows the percentage recovery rate at three time points and on different materials. After each drying time, a significantly higher number of cells was recovered for *A. baumannii* compared with *P. aeruginosa*, except for drying on HDPE. For all materials, the lowest recovery cell counts for both organisms remained mostly above the minimum cell count of the drying control ($10^{6.88}$).

Discussion

Surface disinfection is crucial to reduce the risk of cross-transmission of micro-organisms and the risk of nosocomial infections in clinical environments [3]. Disinfecting procedures as well as the effectiveness of active compounds used for disinfection can be evaluated with standardized methodologies, the outcomes of which vary greatly depending on a number of factors [1,2]. One of the most important factors is the recovery of test organisms after drying on the test surfaces. As potential germ reduction is calculated by the difference between the number of bacterial cells before and after application of the disinfectant, sufficient and reproducible recovery of the test organisms from the test surfaces is a prerequisite for carrying out a valid test [9]. If the number of bacteria decreases as a result of dehydration death during disinfection, the disinfectant cannot be assessed correctly and this could lead to incorrect assumptions about its effectiveness, posing significant health risks [10–12].

In this study, a number of these determinant factors and their influence on the recovery rate of *P. aeruginosa* were evaluated. Experiments carried out with bacterial suspensions of *P. aeruginosa* and *S. aureus* at 20 °C and 37 °C (Figure 1 and Figure S1, see online supplementary material) showed that, regardless of temperature, *S. aureus* tolerates the drying process better than *P. aeruginosa*, for which there was a significantly higher recovery when the inoculum was left to dry in the form of droplets compared with spreading it with a spatula. It was also clear that the recovery of *P. aeruginosa* decreased with increasing drying time. The drying process and recovery rate depended greatly on the drying temperature. For instance, recovery was lower after 30 min at 37 °C than after 70 min at 20 °C, demonstrating that time and temperature are both key factors to consider during drying.

Bacterial recovery was also dependent on the interaction between temperature and carrier material. This study showed that recovery rates were significantly lower for stainless steel carriers, particularly at 37 °C (Table I). The difference in recovery rates was probably observed because the inoculum was spread out with a spatula on the steel surface, but not on PVC. The formation of droplets on the latter probably

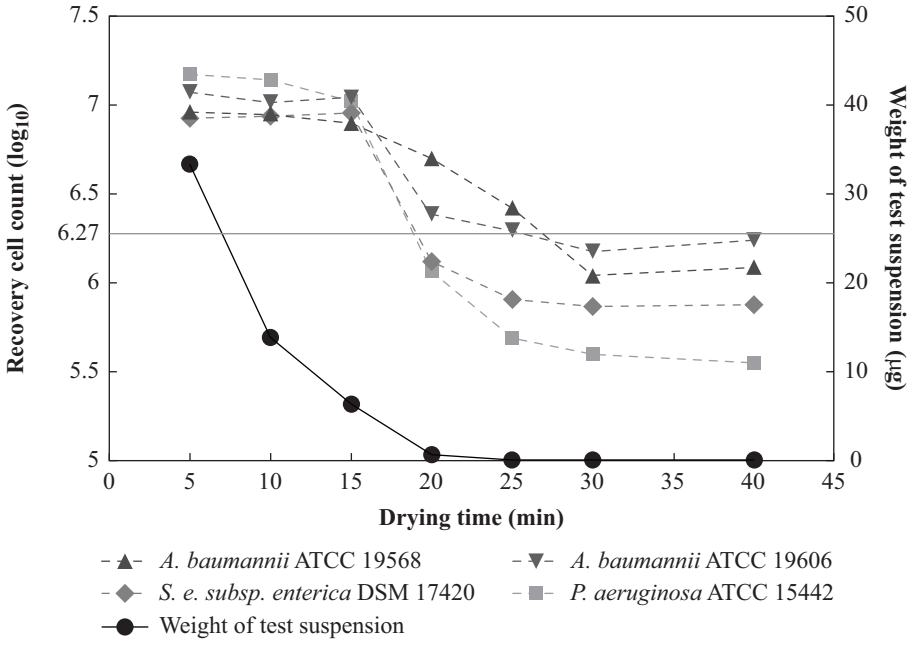

Figure 2. Drying curve at 37 °C of *Pseudomonas aeruginosa* and alternative Gram-negative test organisms according to EN 13697 on stainless steel carriers. The line at $10^{6.27}$ ($6.27\log_{10}$) represents the minimum recovery of the drying control as stipulated in EN 13697. The weight (water) loss (in µg) of the test suspension of *P. aeruginosa* is shown with the continuous line. Results shown are for an average of $N=9$ for each organism.

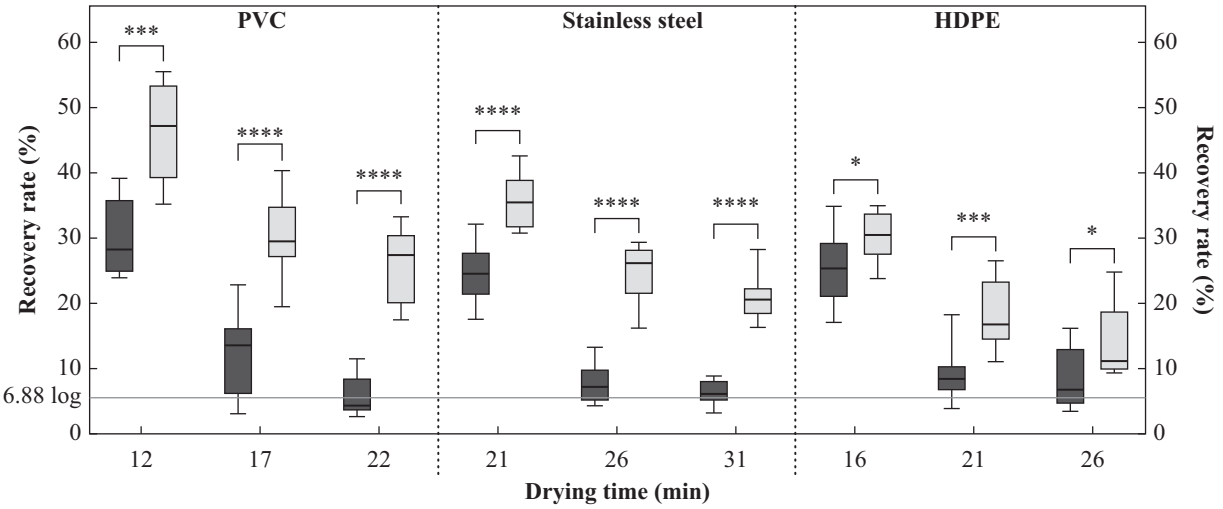

Figure 3. Percentage recovery (in relation to the respective initial cell count) of *Acinetobacter baumannii* (light grey bars) and *Pseudomonas aeruginosa* (dark grey bars) at three drying points at 20 °C according to EN 16615 on polyvinyl chloride (PVC), stainless steel and high-density polyethylene (HDPE) carriers. The minimum cell count of $10^{6.88}$ (6.88 $\log_{10}$) corresponding to the drying control according to EN 16115 is shown as a continuous line. Results shown are for an average of N=9 for each organism.

prevented desiccation of the cells at 20 °C but not at 37 °C, where the larger surface of the inoculum meant earlier death for *P. aeruginosa*. These observations highlight the many ways in which material, inoculum application method and temperature interact and determine the recovery of bacterial cells in efficacy tests. From the analysis of the study results, the optimal drying time for *P. aeruginosa* at 20 °C was established as 17—20 min on PVC, 20 min but no more than 26 min on stainless steel, and 16—21 min on HDPE. The most notable difference was seen on stainless steel, where the optimal drying time was reduced to a maximum of 10 min when drying occurred at 37 °C.

Differences in recovery rates are largely explained by the intrinsic desiccation resistance of the test organisms. For *S. aureus*, resistance to drying has been reported extensively on a variety of surfaces such as cotton, plastic packaging and bottles [11,13].

The lower recovery rates of *P. aeruginosa* result from the desiccation of its thin, sensitive cell walls [14]. However, despite its drying sensitivity, *P. aeruginosa* is used in tests such as EN 16615:2015 as a standardized test organism because it a typical inhabitant of wet surfaces and puddles in hospital environments, and is thus considered to be clinically relevant [14,15].

Some actions have been suggested to overcome the issues presented by drying sensitivity of Gram-negative bacteria. Ramm *et al.* [12] suggested, for instance, using a higher cell concentration in the starting inoculum, or combining it with proteins that may help with stabilization. If the necessary recovery rates are still not achieved, a suitable replacement organism for *P. aeruginosa* should be considered.

Accordingly, this study explored the possibility of using *A. baumannii* and comparing it with *P. aeruginosa*. Two strains of *A. baumannii* used previously as test organisms in standardized tests were used to this effect: *A. baumannii* ATCC 19568, used in the American standard ASTM E2967 — 15, a surface test with the automatic wiping device 'Wiperator'; and *A. baumannii* ATCC 19606, used in EN 17272:2020, a methodology for room disinfection by automated process. The results showed that recovery rates and drying times for *A. baumannii* were significantly and consistently higher than those for *P. aeruginosa*, regardless of carrier material (Figures 2 and 3): an optimal drying time of up to 22 min on PVC, 25 min on stainless steel and up to 28 min on HDPE.

In a study that evaluated several environmental surfaces, Katzenberger *et al.* [16] reported that, along with *E. faecium*, *A. baumannii* shows high survival capability regardless of surface material. In fact, *A. baumannii* outlives *P. aeruginosa* and it can even outlive *S. aureus* [16—18]. Additionally, disinfection tests according to EN 13727:2019 performed with *A. baumannii* and several Gram-negative bacteria against 2-propanol showed that *A. baumannii* did not resist concentrations >25%, a comparable behaviour to *P. aeruginosa*, the standard test organism for EN 13727:2019 [19].

A. baumannii is considered to have drying resistance comparable to that of *S. aureus* [20], and it has been reported to be especially prone to spreading in hospitals [16,18], where it can be recovered easily [21]. This is highly relevant considering that the World Health Organization classifies *A. baumannii* as an organism of critical priority among antibiotic-resistant micro-organisms [22]. The frequency of reported multi-drug-resistant *Acinetobacter* spp. has been increasing steadily in recent years, and the strains may become endemic once outbreaks occur [18]. Research has also shown that the transmission potential of *A. baumanii* is significantly higher than that of meticillin-resistant *S. aureus* [23].

This study showed that temperature, drying time, application method and carrier material affect the recovery of *P. aeruginosa* cells, and therefore influence the outcome of the methodology used. This study showed that *P. aeruginosa* can be replaced by another Gram-negative species, *A. baumannii*, which responds better under the same circumstances and has a similar behaviour to *P. aeruginosa* in disinfectant efficacy tests. Evidently, the replacement of *P. aeruginosa* is not always practical, and institutions that specialize in efficacy testing should establish a drying curve with any possible replacement

bacterium according to their own laboratory conditions. The end goal is to perform tests that reflect practical situations accurately while ensuring sufficient bacterial recovery. Valid outcomes are important for increasing the availability of effective disinfectants, which are vital to prevent and reduce the spread of important pathogenic and opportunistic bacteria in the medical area.

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Author contributions

Leonie Ruffert: Investigation, methodology, formal analysis, writing — review and editing.
Bendix Klarczyk: Investigation, methodology, formal analysis, writing — review and editing.
Anna Ulatowski: Investigation, methodology, formal analysis, visualization, writing — review and editing.
Diana C. Mogrovejo: Conceptualization, formal analysis, visualization, writing — original draft, writing — review and editing.
Florian H. H. Brill: Conceptualization, resources, supervision, project administration, funding acquisition, writing — review and editing.
Eike Steinmann: Supervision, project administration, funding acquisition, writing — review and editing.
Jörg Steinmann: Supervision, project administration, funding acquisition, writing — review and editing.

Conflict of interest statement

The following authors declare a current relationship with Dr. Brill und Partner GmbH Institut für Hygiene und Mikrobiologie, Hamburg, Germany: Diana C. Mogrovejo and Anna Ulatowski (current employees), Florian H.H. Brill (Owner and Managing Director), Jörg Steinmann (Deputy Chairman) and Eike Steinmann (Chairman of the Advisory Board). The following authors declare a previous relationship with Dr. Brill und Partner GmbH Institut für Hygiene und Mikrobiologie, Hamburg, Germany: Leonie Ruffert (employed during her Master's work and received statutory minimum wage), Anna Ulatowski (employed during her Master's work but received no financial compensation for it specifically), and Riadh Bendix Klarczyk (employed as a Master's student during his Master's work).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2023.08.006.

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