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EFFECT OF DIFFERENT DRYING CONDITIONS FOR THE VIABILITY OF *CANDIDA ALBICANS* PRESENT ON CARRIERS

Ewa Röhm-Rodowald, Agnieszka Chojecka, Bożenna Jakimiak, Olga Wiercińska, Beata Ziemba, Krzysztof Kanclerski*

Department of Biological Contamination Control, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland

ABSTRACT

Background. Problems in substantial under recovery of *Pseudomonas aeruginosa* and *Candida albicans* from carriers have been demonstrated for laboratories performing phase 2, step 2 efficacy tests on disinfectants relative to levels required by the EN 13697 standard. It was thus necessary to determine recoveries of these microorganisms following procedural losses incurred during drying and to optimise drying conditions such that recoveries then complied with the standard.

Objectives. The aim of the study was to establish optimal drying conditions for the recovery of *Candida albicans* ATCC 10231 from carriers.

Materials and Methods. The evaluation was performed according to the EN 13697:2001 standard procedure. A test suspension of *Candida albicans* and interfering substance were inoculated onto the surface of carriers (2 cm diameter stainless steel discs) and then dried under different conditions consisting of: a 37°C incubation with and without an incubator fan as well as at 23°C (room temperature) in a laminar air flow cabinet. Carriers were dried until the surfaces appeared visibly dry and the number of surviving organisms then recovered from the surface were quantified. The following were calculated for colony forming units (cfu); N ($\log_{10}$ cfu in a 0.05 ml test suspension), NC (the control $\log_{10}$ cfu in neutralizing medium), Nts (cfu numbers remaining on the surface) and the N-NC difference which should not exceed 2 $\log_{10}$ when microorganism recoveries are adequate and without any toxicity effects of the neutralising medium. Experiments was conducted using validating procedure (NC) which is performed with distilled water.

Results. Drying at 37°C adversely affected the survival of *Candida albicans* and prevented the levels of microbial recovery from carriers to reach those specified by the EN 13697 standard. However, drying at around room temperatures of 23°C reduced *Candida albicans* mortality and increased recoveries from the carrier to levels compliant with the standard, where the N-NC differences were not greater than 2 $\log_{10}$.

Conclusions. The viability of *Candida albicans* ATCC 10231 is sufficiently improved when carriers are dried at 23°C, even if the drying time exceeds 60 minutes. The density of the initial test suspension (N) should also be increased.

Key words*: chemical disinfectants, quantitative carrier test, fungicidal activity, drying conditions, Candida albicans ATCC 10231*

STRESZCZENIE

Wprowadzenie. Laboratoria wykonujące badania skuteczności środków dezynfekcyjnych fazy 2 etapu 2 (metody nośnikowe), przede wszystkim według normy EN 13697, zasygnalizowały problemy z odzyskiwaniem z nośników *Pseudomonas aeruginosa* i *Candida albicans* na poziomie wymaganym w normie. Konieczne było podjęcie badań dotyczących wpływu warunków suszenia zaszczepionych nośników na odzysk oraz wyznaczenia optymalnych warunków odzyskiwania drobnoustrojów po suszeniu inoculum.

Cel. Celem niniejszych badań była ocena wpływu różnych warunków suszenia nośników z naniesioną zawiesiną *Candida albicans* ATCC 10231 na odzysk tych drobnoustrojów.

Materiał i metody. Badania wykonano zgodnie z procedurą stosowaną w normie EN 13697: 2001. Zawiesinę testową *Candida albicans* oraz substancje obciążające nanoszono na powierzchnie testowe (dyski stalowe o średnicy 2 cm) i suszono w różnych warunkach: 37°C w cieplarce bez nawiewu, 37°C w cieplarce z nawiewem, 23°C w komorze laminarnej z nawiewem. Nośniki suszono do momentu uzyskania powierzchni wizualnie suchej.

Po suszeniu liczba żywych organizmów odzyskanych z powierzchni była określana ilościowo. Z liczby jednostek tworzących kolonie (jkt) obliczano: N ($\log_{10}$ jtk w 0,05 ml zawiesiny testowej), NC ($\log_{10}$jtk na nośniku w kontroli neutralizowania),

***Corresponding author:** Ewa Röhm-Rodowald, Department of Biological Contamination Control, National Institute of Public Health – National Institute of Hygiene, Chocimska street 24, 00-791 Warsaw, Poland, phone: +48 22 54 21 330, fax +48 22 54 21 305, e-mail: erodowald@pzh.gov.pl

Nts (liczba jkt, która pozostała na powierzchni nośnika) oraz różnicę N-NC, która w przypadku prawidłowego odzysku drobnoustrojów i braku toksycznego działania neutralizatora nie powinna przekraczać 2.
Badanie wykonano, jako badanie walidacyjne NC, z użyciem wody destylowanej.
Wyniki. Suszenie w temperaturze 37°C niekorzystnie wpływało na przeżywalność *Candida albicans* i uniemożliwiało uzyskanie odzysku drobnoustrojów z nośnika na poziomie wymaganym w normie EN 13697. Suszenie w temperaturze pokojowej (około 23°C) zmniejszało śmiertelność *Candida albicans* i zwiększało odzysk drobnoustrojów z nośnika, pozwalając na spełnienie wymagania normy, wg którego różnica N-NC jest nie większa niż 2 w dziesiętnej skali logarytmicznej.
Wnioski. W celu zwiększenia przeżywalności *Candida albicans* ATCC 10231 nośniki należy suszyć w temperaturze 23°C, nawet, jeżeli czas suszenia przekracza 60 min. Wskazane jest również zwiększenie gęstości testowej zawiesiny wyjściowej (N).

Słowa kluczowe*: chemiczne środki dezynfekcyjne, ilościowa metoda nośnikowa, działanie grzybobójcze, warunki suszenia, Candida albicans ATCC 10231*

INTRODUCTION

A fundamental property of disinfectants is their efficacy of anti-microbial action. The principles governing the assessment of such actions are laid down in the European Committee for Standardisation as part of the Technical Committee 216 (CEN/TC 216) and so defined in the European Standards. Studies of chemical disinfectants' activity are divided into the following categories: suspension tests -phase 1 and phase 2 step 1; carriers' tests- phase 2 step 2. Disinfectant tests carried out in the laboratory conditions allow the standardization and control of the test conditions and the elimination of accidental factors that may affect their activities.

Standardised methods for the evaluation of bactericidal activity and fungicidal activity of disinfectants are quantitative methods where reductions in the numbers of viable microorganisms are defined after using the disinfectant in the proper parameters eg. concentrations, time etc.

The carrier methods of phase 2 step 2 are based on inoculation of the surface of a carrier with test microorganisms, drying and then applying the disinfectant solution under defined conditions.

The carrier then undergoes neutralisation and after a suitable interval, the numbers of viable microorganisms are determined by measurement of recoveries on the carrier surface [12]. The method also includes measuring the numbers of viable microorganisms left on the surface in the presence of only hard water and comparing these amounts with the reduced numbers when the test disinfectants are applied.

According to European requirements, standardisation includes the type of test microorganism, preparing solutions, presence of loading substances and the methods of neutralisation. It is recommended that carriers are dried at 37°C until the surface is visualised as being dry. How long the carriers are dried after the suspension of microorganisms has been applied, has not been precisely set because such drying occurs at different rates according to laboratory conditions and the type of drying device (eg. with or without a fan). The minimum time required to achieve the appearance of dryness must be determined by a given laboratory but should not exceed 60 minutes [2]. It is seen that the shorter the time of drying then the smaller is the reduction of the test organism's viability. The *Pseudomonas aeruginosa* and *Candida albicans* strains are particularly sensitive to such drying. The problem of recovering these bacteria was reported in 2012 at the meeting of CEN/TC 216/WG 3 concerning revising the EN 13697 standards [1]. It was proposed that studies be performed at various laboratories to;

- check the numbers of bacteria/fungi lost during drying,
- assess the effect of drying on microorganism recovery,
- improve the recoveries of microorganisms after the inoculum is dried (especially for *Pseudomonas aeruginosa* and *Candida albicans*),
- prepare a common proposition for the CEN/TC 216 regarding drying conditions for normally used carriers.

The aim of the presented study was to evaluate the effect of various drying conditions for a suspension of *Candida albicans* ATCC 10231 on microorganism recovery and establish the optimal drying conditions.

MATERIALS AND METHODS

Studies were performed in accordance with EN 13697:2001 standard procedures [2]. A suspension of the test bacteria *Candida albicans* ATCC 10231 was prepared in solutions of Bovine Serum Albumin (BSA) at concentrations of 0.3 g/L and 3.0 g/L of which 50 µl were placed onto the carrier surface (stainless steel discs; 2 cm in diameter). These were then dried under different conditions until the appearance of dryness was visualised. Carriers were then transferred into the 10 ml of neutralisers with 100 µl of distilled water, followed by agitation with 5g of glass balls. The liquid phase containing the test mixture was then diluted 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A 1ml aliquot was taken from each dilution

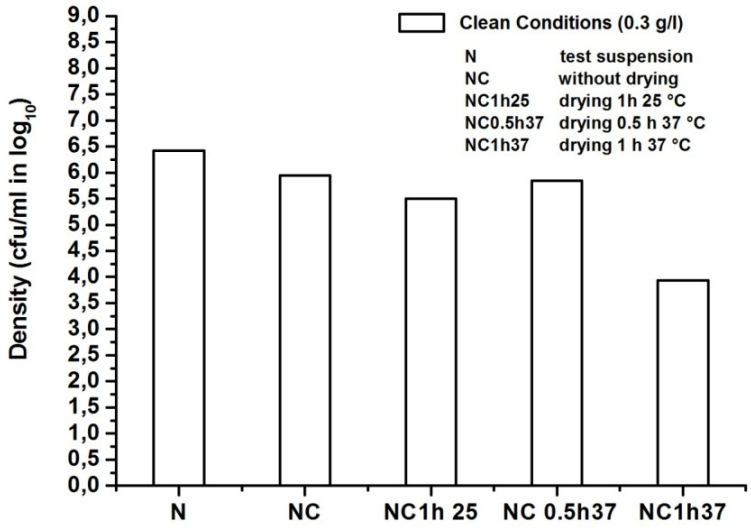

Figure 1. Values for test suspensions (N) and test surface recoveries (NC) under different temperatures and drying times.

into a *Petri* dish and 15 to 20 ml of a MEA solution cooled to 45°C. The NC value (the control $\log_{10}$ cfu in neutralizing medium) was calculated by:

$$NC = \log_{10} \left[\frac{(x + x')}{2} \times \frac{10}{d} \right]$$

Where:
d - the dilution factor used in the calculation
x, x' – number of cells per ml.

The procedure using distilled water (NC) was also used for carriers that had not been dried. In order to determine numbers of viable microorganisms in the initial suspension, a dilution of 10^{-6} was made (ie. $\log_{10}$ cfu in a 0.05 ml test suspension).

For the last two dilutions, 1ml aliquots were taken into *Petri* dishes and 15 to 20 ml of MEA solutions cooled to 45°C were added. The dishes were then incubated 30°C ± 10°C for 48 hours after which cells were counted; those with colonies that could not be counted were rejected. Pairs were chosen (*x, x'*) whose average values ranged between 50 - 300 colonies where N values were calculated as $\log_{10}$ cfu in a 0.05 ml test suspension according to the formula:

$$N = \log_{10} \left[\frac{(x + x')}{2} \times \frac{0{,}05}{d} \right]$$

The cfu were also counted which remained on the carrier surfaces (Nts). In such cases the carriers were transferred into Petri dishes containing around 10 ml of solidified MEA and placed on the bottom of the study surface, uppersidemost. 0.1 ml water was then added and the dried residue of the suspension present on the carrier was scraped off for one minute using the end of a sterile pipette. The surface was rinsed with 10 ml of MEA solution cooled to 45°C. Initial studies on carrier recoveries were then performed with or without drying in incubators under the following 3 conditions; (1) 1 hour at 25°C; (2) 0.5 hours at 37°C; (3) 1 hour at 37°C. Recoveries after drying the carriers were performed under selected conditions using the following; 37°C incubation with and without an incubator fan and at 23°C (room temperature) in a laminar air flow cabinet. The drying times for any given temperature were defined at the moment when a dry carrier surface could be visually identified. Studies were done on 3 suspensions each loaded at 2 BSA concentrations of 0.3 g/L (termed clean conditions) and 3 g/L (termed unclean conditions). Each measurements was done in quadruplicate (n = 4).

RESULTS

These studies demonstrated that recoveries of *Candida albicans* from carriers without drying (NC) are comparable to those of the test suspension (N). Carriers that were dried for 1 hour at 25°C or for half an hour at 37°C were not visually dry but partly dry. The recoveries from these carriers was insignificantly lower from those that were wet that were not dried. The lowest recoveries of *Candida albicans* were observed in carriers that appeared to be visually dry after 1 hour of drying at 37°C (Figure 1).

Using the various methods for drying and different temperature conditions, the following drying times were recorded; 60 - 75 minutes for an incubator without a fan, 50 minutes for an incubator with a fan and 65 - 110 minutes for the laminar air flow cabinet. Recoveries of *Candida albicans* from carriers treated with both of the organic loadings showed that they were slightly higher under unclean conditions, where carriers were dried in laminar air flow cabinet conditions at 23 °C (NC23), or

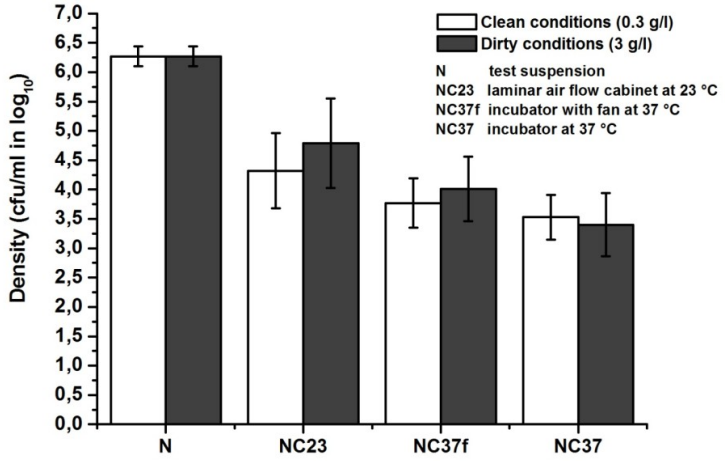

Figure 2. Values for test suspensions (N) and test surface recoveries (NC) in clean and unclean conditions using different equipment. Error bars represent the standard deviation.

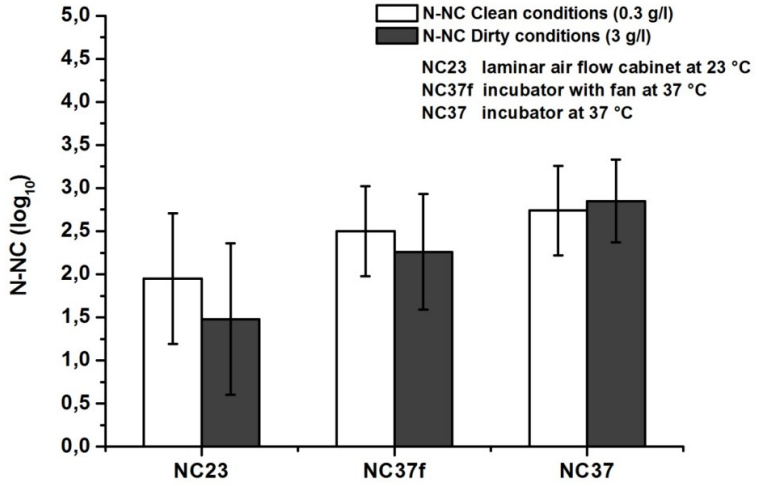

Figure 3. Values for test suspensions (N) and test surface recoveries (NC). Error bars represent the standard deviation.

in incubator with fan at 37 °C (NC37f). The recoveries from carriers dried in incubator without fan at 37 °C (NC37) were however, insignificantly lower compared to those under clean conditions. Indeed, both organic loadings demonstrated highest recoveries when subjected to drying in laminar air flow cabinet conditions at 23 °C (Figure 2).

In accordance with the EN 13697 standard, the differences between the previously defined (N) and (NC) values should be equal or less than 2 $\log_{10}$, thus indicating an adequate recovery of microorganisms together without any toxic effects resulting from neutralisation. This condition was fulfilled only in those carriers dried under laminar air flow cabinet conditions (NC23), at both organic loadings; taking into account the result means obtained from n = 4 carriers. In the case of other conditions (ie. NC37f or NC37), the average N-NC differences were higher than those of the set standards (Figure 3). Cell numbers forming colonies which remained on the carrier surfaces (Nts), were found to be less than 100 in all tests thereby being compliant with the EN 13697:2001 standard.

DISCUSSION

Results indicate that drying at 37°C has a large effect on *Candida albicans* viability resulting in a significant reduction as well as recoveries which were well below those specified in the EN 13697 standard. Drying at room temperature (ie. 23°C) reduces *Candida albicans* mortality and increases the carrier recovery of microorganisms which fulfils the requirements that the N-NC difference should not exceed 2 $\log_{10}$. The influence of drying on microorganism viability has been the subject of many studies, where the sensitivity of Gram-negative bacteria is greater than for the Gram positives [3, 4, 6, 8]. Very few studies have however been focused on the effect of drying on fungal viability [5].

Variations observed in viability after drying, in part arise due to different species and strains as well as in the differing experimental conditions adopted which include the numbers and colony density of the test suspension together with the moisture in the environment [5, 9]. Carrier drying studies performed on *Aspergillus*

flavus and *Penicillium spp*. at 55°C over 30 minutes, (using various suspension dilutions 10^2, 10^4 and 10^6 of spores/g), demonstrated that the drying deactivated respectively 32% and 36.6% of the *Aspergillus flavus* and *Penicillium spp* spores. The deactivation levels decreased the more concentrated the suspension was ie. the greatest recoveries were achieved at 10^6 spores/g [5]. The drying time also affects microorganism viability [4, 5, 6]. European standards state that the shorter the drying time then the test organism viability decreases less, particularly for *Pseudomonas aeruginosa*, therefore it is recommended that drying times should not exceed 60 minutes [2].

Studies by *Fuster-Valls et al.* [3] compared the drying at 22 °C of Gram-Positive and Gram-Negative bacterial suspensions on steel carriers under conditions of slow or fast air flow generated in a cabinet fitted with fans. It was found that the numbers of *Pseudomonas aeruginosa* and *Enterobacter cloacae* fell below detection limits using a long drying period of 4-6 hours. However in the case of *Staphylococcus aureus*, some cells remained viable for at least 3 days. When the drying was rapid, there were dramatic falls in *Pseudomonas aeruginosa* after only 2 hours, down to below the detection limits, but single cells of *Staphylococcus aureus* could be detected even after 72 hours [3].

Drying inoculum at 22°C is also recommended in methods used to define the efficacy of disinfectants intended for eliminating microorganism from the surface of tomatoes [7]. The latest drafts of the European Standards show a tendency to avoid drying at 37°C, but to instead use room temperature in laminar air flow cabinets [10, 11]. In such conditions, a visual appearance of dryness would be difficult to obtain in under 60 minutes and thus a drying period of 65 – 75 minutes was used in the current study. Other ways for increasing microorganism viability have been proposed in the draft standards such as increasing the initial suspension density (N) up to 10^8 cfu/ml in the case of *Pseudomonas aeruginosa* together with using glycerol as a thinner when preparing the suspensions [10, 11].

The presented study show that the protective effect of organic loading on the viability of microorganisms is confirmed when carriers are dried at 37°C in an incubator or at 23°C under laminar air flow conditions inside a cabinet.

CONCLUSIONS

An increase in the viability of *Candida albicans* ATCC 10231 on carriers and compliance with the EN 13697 standard can be achieved by the following means:
(1) Drying the carrier at 23°C, even if the visual appearance of dryness occurs after 60 minutes.
(2) Increasing the initial suspension concentrations (N).

Conflict of interest

The authors declare no conflict of interest.

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