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PROJECT REPORT

Evaluation of decontamination products used for bacterial spores at BSL-3 laboratories

Alternatives to the decontamination product Nu-Cidex

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TABLE OF CONTENTS

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SAMMANFATTNING PÅ SVENSKA

Vid en avsiktlig eller naturlig spridning av riskklass 3 bakterier och relevanta virus är en fungerande krisberedskap, med avseende på medicinska motåtgärder och dekontaminationsförfaranden, beroende av snabba preliminära laboratorieresultat. Till skillnad från odlingsdiagnostik, som ofta ses som en gyllene standard, erbjuder molekylär diagnostik metoder som snabbare kan analysera en större mängd prover med frågeställning om riskklass 3 bakterier eller virus.

Nu-Cidex har under flera år använts som saneringsmedel för Bacillussporer vid säkerhetslaboratorier. Eftersom Nu-Cidex inte längre är möjligt att beställa i Sverige är det angeläget att hitta ett fullgott alternativ.

I den här studien valdes tre produkter för att studera saneringseffektiviteten på *Bacillus* sporer. Under de förhållanden som försöken utfördes så visades att alla studerade produkter är fullgoda alternativ till Nu-Cidex med avseende på saneringseffektivitet på *Bacillus thuringiensis* sporer medan *Bacillus anthracis* sporer visade sig vara mera resistenta. Studierna på *B. anthracis* sporer var begränsad till ett preparat och utfallet blev att en något högre koncentration av ett specifikt preparat har god saneringseffekt på sporerna.

Den här studien bekräftar också att det är av yttersta vikt att det preparat som man avser använda är testat under just de förhållanden man har på sitt eget laboratorium samt på de specifika agens man avser sanera mot. Denna rapport kan ses som en vägledning vid val av produkt för sanering av sporer på säkerhetslaboratorium.

CONCLUSION IN ENGLISH

Nu-Cidex has been routinely used as a decontamination agent for *Bacillus* spores at high containment (BSL-3) laboratories. However, as of 2014 Nu-Cidex is no longer available in Sweden, and it became urgent to find an acceptable alternative.

In this study, three different products were tested for their decontamination efficacy on *Bacillus* spores. All three products are good alternatives to Nu-Cidex with respect to decontamination effectiveness of *Bacillus thuringiensis* spores. The study against *Bacillus anthracis* was limited to only one decontamination product, and it was found that *B. anthracis* spores were more resistant against this product. When a higher concentration of this product was used, the decontamination effect on the spores was good.

This study confirms that it is of utmost importance that the decontamination product is tested under normal conditions in each laboratory and on the specific agents that are to be reduced. This report can guide choice of spore decontamination products in a BSL-3 laboratory.

1. BACKGROUND

The aim of this project was to evaluate alternative products for decontamination of bacterial spores at BSL-3 laboratories. In December 2014, Johnson&Johnson ceased manufacture of the product Nu-Cidex and it was no longer available in Sweden. Nu-Cidex was routinely used in laboratories at the Public Health Agency of Sweden (Folkhälsomyndigheten), the Swedish Defence Research Agency (Totalförsvarets forskningsinstitut, FOI, and some of the laboratories at the National Veterinary Institute (Statens veterinärmedicinska Anstalt, SVA). As an alternative to Nu-Cidex, these laboratories substituted an inhouse recipe of acidified chlorine made by adding acetic acid to chlorine. This formulation reduces spores to an acceptable level[i]. However, the formulation is corrosive to surfaces and requires special protective equipment, for example, an air-filtering mask equipped with a chemical filter. Therefore a call for an alternative product to Nu-Cidex was highly prioritized.

One of the reasons that Nu-Cidex is no longer available, was difficulties to fulfill the new EU Biocidal Products Regulation (No. 529/2012[ii]). This regulation aims to harmonise rules on the supply and use of biocidal products while ensuring a high level of protection of human, animal and environmental health. The active compound(s) in the Nu-Cidex alternatives had to be approved by the European Chemicals Agency (ECHA). However, it was difficult to verify the "ECHA-approval" for the active compounds in each product due to incomplete list of contents in some products. Therefore, our list of candidate products was expanded from two to four in the event that one of the products was removed from the market for some reason or other.

The products compared in this study where chosen to have different active substances. Two of them contain peracetic acid like Nu-Cidex (Perasafe and Terralin) and two generate chlorine dioxide (DK-DOX and CleanWaterTec). The inhouse chlorine formulation was also included as a control. Parameters like user-friendliness, stability, and price were also evaluated. The products and parameters are listed in the Appendix.

2. GOAL

The goal of the project was to find an appropriate alternative to Nu-Cidex for decontamination of *bacterial* spores in BSL-3 laboratories. *Bacillus thuringiensis* spores were used as a proxy for *Bacillus anthracis* spores in testing the compounds, followed by a more limited comparison using *Bacillus anthracis* spores with one of the compounds (DK-DOX).

3. MATERIAL AND METHOD

3.1 MATERIAL

- *Bacillus thuringiensis* ATCC 35646 .
- *Bacillus anthracis* ATCC 14578.
- Stainless steel discs (Ø 20 mm) as test surfaces (Anton Johansson, Rostfria Verkstads AB, Uppsala Sweden).
- Decon 90 (Decon Laboratories Limited)
- Sephadex G-25 in PD-10 columns (GE Healthcare)
- NA (Nutrition Agar) plates and BAB (Blood Agar Base) plates
- Disinfection products: Perasafe, acidified Chlorine (Klorin) <pH7 (acetic acid added), DK-DOX surface 750 ppm, DK-DOX surface 1500 ppm, DK-DOX surface 3000 ppm, CleanWaterTech

3.2 METHOD

The modified protocol in this study was based on three standards for testing disinfectant effectiveness [iv].

In brief, microorganism suspensions were air dried on a stainless steel surface and exposed for 10 minutes to the disinfectants listed in the Appendix. The effectiveness was calculated by comparing the number of live/active spores on the surface after decontamination with the non-decontaminated control.

All experiments were performed at room temperature.

3.2.1 Spore preparation

Bacillus thuringiensis spores were prepared as described by Delafield [v]. *Bacillus anthracis* spores were prepared by using BBLTM AK agar #2 Sporulating plates. Spore batches were kept in 0.45 % NaCl at 4° C until use.

Prior to each experiment, the spore batches were suspended by vigorous vortexing. The number of spores was adjusted to approximately 10^7 colony forming units (CFU/ml), maintained at room temperature, and used within 2 hours. Numbers were verified by viable count.

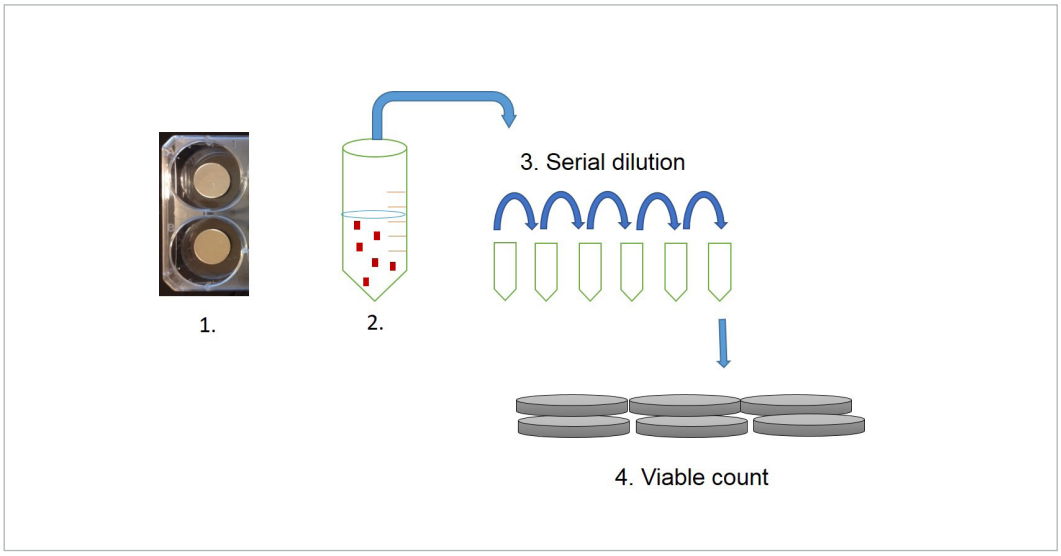
3.2.2 Preparation of test organisms on test surface

Stainless steel discs (Ø 20 mm) were used as the test surface. The discs were cleaned in Decon 90, rinsed in distilled water and 70 % ethanol, and dried by evaporation (SS-EN 14349:2007).

The discs were placed in sterile 6-well plates and an aliquot of 100 µl spore solution was spread uniformly over them. The solutions were air-dried at room temperature for a minimum of 60 minutes until visible dryness. *B. thuringiensis* experiments were evaluated by preparation of two duplicate discs for each experiment, while the analysis of *B. anthracis* used a single disc in each experiment. The tests were repeated at three different time points.

3.2.3 Decontamination of test organisms

The commercial disinfectant solutions were prepared and used according to the manufacturer's instructions except that all products were allowed to be active on the surface for 10 min even if the recommended time was shorter (5 min was recommended for two of the four products). The *B. anthracis* spores were incubated for 5 min with DK-DOX.



3.2.4 Analysis of spores

Figure 1: Overview of the analysis procedure.

1. Two stainless discs placed in a 6-well plate.
2. Falcon tube containing spores recovered from the stainless steel discs dissolved in NaCl.
3. Sufficient amount of 10-fold serial dilutions.
4. Spores are spread on sufficient number of agar plates.

The spores were recovered from the stainless steel disc by applying 2.5 ml of diluent (0.9 % NaCl) and then pipetting it up. Thereafter, the diluent was applied to a PD-10 column to disrupt the decontamination process after the given impact lag, and to exclude any eventual toxic effect from the decontamination solution. Due to limited working area in the BSL-3 laboratory, the gel filtration step was omitted in the experiments with the *B. anthracis* spores. The recovered diluents containing the spores were ten-fold serial diluted in duplicate and spread on NA agar plates. After 16 hours incubation at 37°C, the colonies were counted.

Bacillus spores were expressed in $\log_{10}$ as mean CFU ml^{-1} for the decontaminated and control (non-decontaminated) discs.

4. RESULTS

4.1 COMPILATION OF PRODUCTS FOR DECONTAMINATION

The table in the Appendix specifies the parameters for each product. These are the active substance(s), administration/management, duration of action, corrosivity, stability of the reconstituted and stock solutions, and price. The table was compiled in 2015 and is partially incomplete as not all information was available at the time.

4.2 REDUCTION OF *B. THURINGIENSIS* SPORES

All products reduced the amount of spores with 6 $\log_{10}$ or more (Figure 2). This is more than sufficient as an acceptable reduction of bacterial spores is considered to be ≥ 3$\log_{10}$ [vi].

The very low residuals after decontamination using the inhouse chlorine solution and CleanWaterTec, were only found in one of the three experiments and the reduction level for that specific experiment was still more than 5 $\log_{10}$.

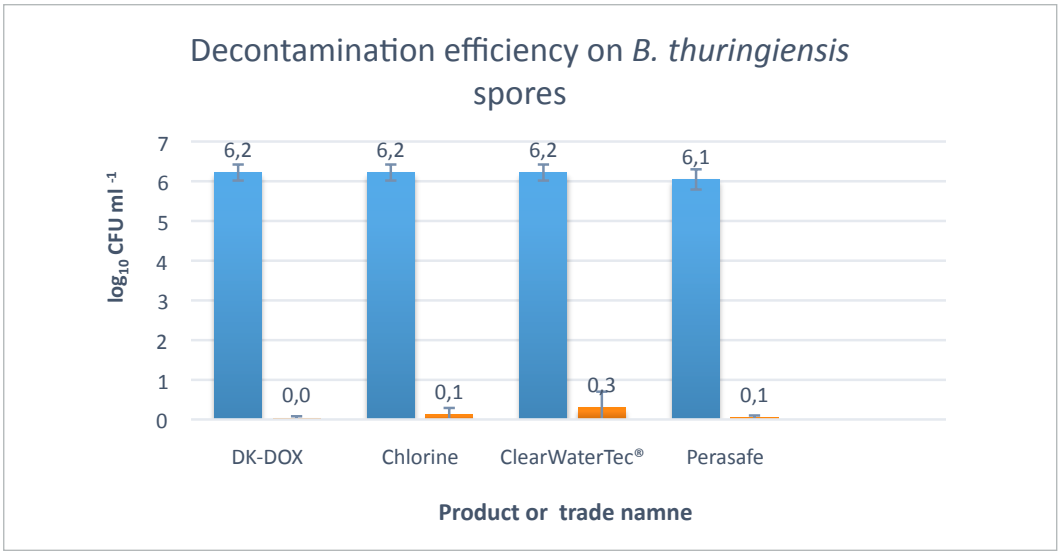


Figure 2. Blue bars represent the log number of recovered spores without decontamination and orange bars the log numbers after decontamination.

4.3 REDUCTION OF *B. ANTHRACIS* SPORES

Due to limitations in time and space at the BSL-3 laboratory, only one product (DK-DOX) was tested with the *B. anthracis* spores. The initial experiment with 750 ppm DK-DOX showed insufficient decontamination efficiency (Figure 3). Therefore, the following experiment was expanded to include two higher concentrations of DK-DOX. All spores were inactivated when exposed to 1500 ppm or 3000 ppm, indicating that a spore concentration of at least 6 $\log_{10}$ was eradicated.

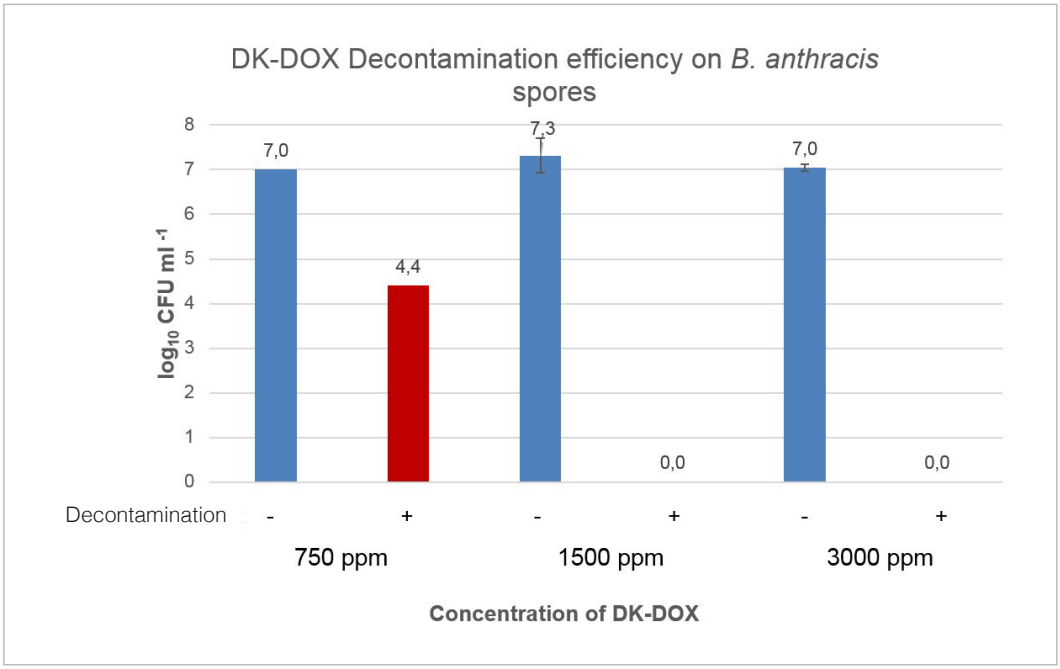


Figure 3. Blue bars represent the log number of spores recovered without decontamination and red bars the log number after decontamination. The experiment using 750 ppm was performed once while the experiment with 1500 ppm and 3000 ppm represents the mean of three separate experiments.

5. DISCUSSION

Four decontaminants were tested in this study: Perasafe, DK-DOX, CleanWaterTec and an inhouse chlorine formulation. The BSL-3 laboratory in Bundeswehr, Germany, had recently switched from Perasafe to Terraline because it was easy to handle (no dust at the activation step of the product) and was better documented (personal communication). Terraline was therefore one of the initial candidate products in our study, but had to be excluded due to delivery problems. Today it is possible to order this product in Sweden, if there is a demand for testing it in the future.

As shown in the Appendix, there are multiple parameters to take into account when deciding what product to use. The most important ones are the stability of the reconstituted and stock solutions. All stock solutions in our comparison are stable for at least one year, while the stability of reconstituted solution varies. For example, reconstituted DK-DOX is stable for 4-6 weeks, which is much longer than for the other products. On the other hand, it requires a 25-hour activation, which is relatively long. Given that the activation time is important for preparedness, we advise that DK-DOX be routinely prepared every month. Due to limitations in time and space at the BSL-3 laboratory this study was limited to test only one product against *B. anthracis* spores. In this study DK-DOX was chosen as the stability of the reconstituted product was relatively long – a quality that is convenient for work at BSL-3 laboratories.

B. thuringiensis is often used as model organism for *B. anthracis* in decontamination studies All four tested products reduced the number of active *B. thuringiensis* spores with 6 log10 or more, which makes them eligible replacements for Nu Cidex. However, the DK-DOX results for *B. anthracis* spores showed that survival after decontamination can differ between related species. Nevertheless, satisfactory decontamination efficiency was obtained after increasing the concentration to 1500 ppm. However, as only one product was tested against *B. anthracis* spores it should not be excluded that the other three products have a similar efficacy. The decision on what product to use must therefore be based on additional data and information.

6. CONCLUSIONS

All four products (DK-DOX, Perasafe, CleanWaterTec, and the inhouse chlorine solution) can be used for decontamination of *Bacillus thuringiensis* spores, which is the standard microorganism proxy for decontamination studies. One of the products, DK-DOX, was also tested on *B. anthracis* spores. A concentration of 1500 ppm of DK-DOX, was necessary to decontaminate *B. anthracis* spores on a steel surface.

6.1 BENEFITS OF COOPERATION

All the authorities in FBD will benefit from this study as the decontamination products tested can be used to reduce bacterial spores at BSL-3 laboratories. The results from this study may guide the choice of decontamination product.

7. APPENDIX. SPECIFICATIONS OF DECONTAMINATION PRODUCTS

Product	Active substaces	Administration Management	Duration of action	Corrosive	Stability of recons-tituted solution	Stability of stock solution	Price
DK-DOX Surface	Chlorine dioxide pH neutral	Spray Spray flasks with tablet(s) to add for activation. 24 hours for activation in 30 °C	5 min	Not corrosive	4-6 weeks	1 year	162 SEK/ liter
Chlorine solution (inhouse)	Sodium hypo-chlorite Acetic acid	Not spray Chlorine is mixed with acetic acid and water	10 min	Corrosive		Years	50 SEK/ several liters
Clean WaterTec	Chlorine dioxide pH neutral	Spray In-house reconstituted solution	10 min	Not corrosive	One week	1 year	110 SEK/ 100 ml 230 SEK/ 500 ml

Product	Active substaces	Administration Management	Duration of action	Corrosive	Stability of recon-stituted solution	Stability of stock solution	Price
Nu Cidex	**Peroxide** Hydrogen peroxide 1.6%, acetic acid, peracetic acid	Not spray Two components are mixed	5 min	Not corrosive	24 hours	?	?
Perasafe	**Peroxide** Dinatriumcarbonate 30-50%, Hydrogen peroxide 10%, Acetic acid 0.26 %, Citric acid 10-20%, sodium carbonate 10%, Peracetic acid 15%	Spray Dusty when mixed Activated at 30-35°C for 3-5 min	10 min	Not corrosive	24 hours	2 years	50 SEK/ liter
Terralin	**Peroxide** Peracetic acid, hydro-gen peroxide, acetic acid.	Spray Two components are mixed	5 min	Not corrosive	8 hours (at least)	1.5 years	100 SEK/ 4 liter

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