Evaluation of mycobactericidal activity of selected chemical disinfectants and antiseptics according to European standards

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Source of support: Departmental sources

Background: The history of the investigation of standardized mycobactericidal activity of disinfectants and antiseptics is not very long. There is growing interest among the manufacturers of disinfectants in carrying out research on the antimicrobial activities in accordance with European standards (EN). This research could facilitate the introduction of high-quality disinfectants to the market. The aim of this study was to evaluate the mycobactericidal activity of selected chemical disinfectants and antiseptics used in the medical and veterinary fields.

Material/Methods: This study included 19 products submitted to the National Medicines Institute in Poland for evaluation of mycobactericidal activity. These products contain in their composition active substances belonging to different chemical groups, including aldehydes, alcohols, amines, quaternary ammonium compounds, phenols, guanidine, and oxidizing compounds. This study, conducted according to the manufacturers’ description of the preparations, was carried out in accordance with European standards, which also met the Polish standards: PN-EN 14204: 2013, PN-EN 14348: 2006, and PN-EN 14563: 2012.

Results: Tested products for disinfection and antiseptics containing active substances from different chemical groups showed high mycobactericidal activity and met the requirements of the appropriate European standards in most cases. In the case of products containing guanidine and amine compounds, the concentration of active ingredients used in the test and the test conditions specified by the manufacturer did not provide the mycobactericidal activity required by the standards.

Conclusions: Prior to the launch of a new product on the market, it is important to establish the appropriate usage and testing conditions of the preparation, such as its practical concentration, contact time, and environment condition (clean or dirty).

MeSH Keywords: European Union • Anti-Bacterial Agents • Tuberculosis • Antisepsis • Disinfection

Full-text PDF: http://www.medscimonit.com/download/index/idArt/890175
Background

The investigation of the mycobactericidal activity of disinfectants and antiseptics has been standardized for several years. The first European standard EN 14204 for testing the mycobactericidal activity of products used in the veterinary field was implemented by the CEN (European Committee for Standardization) in 2004. The standard EN 14563, dedicated to mycobactericidal products used in the medical area, was established in 2008. The laboratory research performed used mycobacterial strains derived from the American Type Culture Collection (ATCC), except for Mycobacterium tuberculosis, due to the risk of infection in the laboratory and the slow growth of microorganisms [1]. In addition, the mycobacteria that cause tuberculosis are classified by the US Centers for Disease Control and Prevention (CDC) in Atlanta as category C, and this pathogen can be used as a biological weapon.

According to World Health Organization (WHO), tuberculosis, HIV, and malaria are the most common infectious diseases in the world (one-third of the world population is infected with Mycobacterium tuberculosis) [2]. In this context, effective disinfection, in addition to regular immunization, is a significant method for the limitation of mycobacterial infections.

Mycobactericidal activity testing was performed in accordance with European standards (EN) based on the culture of mycobacteria, which means that the test results are known after 21 days. However, the use of the Mycobacterium terrae strain, which contains the gfp in 2+ gene, causes fluorescence of living mycobacteria cells and can reduce the detection time to 15 days [3,4].

There is growing interest among manufacturers of disinfectants and antiseptics in conducting tests of mycobactericidal activity in accordance with ENs, which will make the presence of high-quality products on the market possible.

The aim of this study was to evaluate the mycobactericidal activity against 2 mycobacterial species, Mycobacterium avium and Mycobacterium terrae, or the activity only against Mycobacterium tuberculosis, of selected chemical disinfectants and antiseptics used in the medical and veterinary areas in accordance with relevant European standards [5–7].

The Department of Antibiotics and Microbiology at the National Institutes of Health, Warsaw, Poland, has investigated the antimicrobial effectiveness of antiseptic and disinfectant products for many years. Recently, we compared selected commercial mouthwash and disinfection products [8]. Our department is certified by the European Directorate for the Quality of Medicines (EDQM) for microbiological tests carried out in accordance with ISO/EN 17025 and possesses the accreditation of the Polish Centre for Accreditation (No. AB 774) for microbiological testing of disinfectants and antiseptics, according to several ENs.

Material and Methods

Products

These studies included 19 products submitted to the National Medicines Institute in Poland for the evaluation of mycobactericidal activity. These products contain active substances that belong to different chemical groups, including aldehydes, alcohols, amines, quaternary ammonium compounds, guanidine, phenols, and oxidizing compounds (Table 1).

Standards

Investigations, according to the manufacturers’ description of the products, were carried out in accordance with European standards, which also met the Polish standards: PN-EN 14204: 2013 (EN 14204: 2012): Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of mycobactericidal activity of chemical disinfectants used in the veterinary area – Test method and requirements (phase 2, step 1) [5]; PN-EN 14348: 2006 (EN 14348: 2005): Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of mycobactericidal activity of chemical disinfectants in the medical area including instrument disinfectants – Test method and requirements (phase 2, step 1) [6]; and PN-EN 14563: 2012 (EN 14563: 2008): Chemical disinfectants and antiseptics – Quantitative carrier test for the evaluation of mycobactericidal or tuberculocidal activity of chemical disinfectants used for instruments in the medical area – Test method and requirements (phase 2, step 2) [7].

Products recognized as mycobactericidal should be active against both Mycobacterium avium and Mycobacterium terrae mycobacterial species. Product activity against Mycobacterium tuberculosis is determined in accordance with the recommendations of the European Committee for Standardization (CEN), only to strain M. terrae.

The product meets the requirements of the PN-EN 14563: 2012 when after 60 min of contact time of the product and mycobacteria suspension in the appropriate temperature (10°C or 20°C), it demonstrates at least a 1% reduction in counts of 4 (reduction ≥4 log cfu/mL) of both organisms (mycobactericidal activity) or only against M. terrae (tuberculocidal activity).

Depending on the application of the products, interfering substance were used: clean conditions were simulated by 0.3 g/L BSA 3.0 g/L (according to EN 14204)

Keywords: Mycobactericidal activity, disinfectant, antiseptic, Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium terrae, mycobacterial strains
and dirty conditions, 3.0 g/L BSA, 3.0 mL/L sheep erythrocytes (according to EN 14348 and EN 14563) or yeast extract 10 g/L, BSA 10 g/L (according to EN 14204).

### Strains

In the studies performed according to the PN-EN 14348 and PN-EN 14563, 2 reference strains of mycobacteria – *Mycobacterium avium* ATCC 15769 and *Mycobacterium terrae* ATCC 15755 – were used. In tests performed according to PN-EN 14204, only the strain *M. avium* ATCC 15769 was used.

### Results

The majority of products (17/18) for use in the medical field were tested in accordance with EN 14348. One product designed for use in the veterinary field was evaluated according to EN 14204. Both of these standards are to be tests of phase 2 step 1 (suspension method) in which the products are tested under simulated conditions by the introduction of inorganic loads (hard water to dilute the product) and organic loads (albumin, albumin + erythrocytes, or albumin + yeast extract). Only 4 studies were conducted in accordance with EN 14563.

### Table 1. Characteristic of disinfectants and antiseptics tested in this study.

| Name of product, form | Manufacturer | Composition of active substances |
|----------------------|--------------|----------------------------------|
| Aldesan E, liquid    | Septoma, Poland | Glutaraldehyde 2%, ethanol 25% |
| Aldizol, liquid      | Septoma, Poland | o-phenylphenol 7.0%, 4-chloro-3-methylphenol 4.5%, glutaraldehyde 4.0% |
| Chlor-Clean, tablets | Guest Medical Ltd., Great Britain | Sodium dichloroisocyanurate |
| Desisoft Ytdeinfektion, liquid | Rekal Svenska AB, Sweden | Polyhexamethylene biguanidine hydrochloride 0.35% w/w |
| Lysoformin Plus-Schaum, liquid | Lysoform, Germany | N-(3-aminopropyl)-N-dodecylpropan-1,3-diamine 0.46 g, didecyl dimethyl ammonium chloride 0.10 g, polyhexamethylene biguanidine 0.18 g/100 g |
| OneMed Easydes, liquid | Farmos Ltd., Finland | 55–60% ethanol, t-butanol <2%, ammonium chloride <0.5%, alkylamine <0.5% |
| Rafasept, liquid | Septoma, Poland | o-phenylphenol 12.5%, 4-chloro-3-methylphenol 3.0% |
| Septyl Amyco, powder | Septoma, Poland | o-phenylphenol 12.0 g, of 4-chloro-3-methylphenol 4.0 g, 2-benzyl-4-chlorofenol 1 g/100 g |
| Septyl R, liquid | Septoma, Poland | Chlorocresol (4-chloro-3-methylphenol) 1.0 g /100g |
| Soft Care Des E Spray, liquid | JohnsonDiversey UK Ltd. | Ethanol 715 g/kg |
| ST4, powder | Steril-4 S.R.L., Italy | Sodium percarbonate >40 g, TAED >25 g/100 g |
| Steril C, powder | Steril-4 S.R.L., Italy | Sodium perborate, TAED |
| Steril-Ser, powder | Steril-4 S.R.L., Italy | TAED > 25 g/100 g |
| Synröl PAA10, liquid | Synpeko, Poland | Peracetic acid 10%, hydrogen peroxide 13%, acetic acid 10% |
| Synsept AG, liquid | Synpeko, Poland | Glutaraldehyde 12%, benzalkonium chloride 3%, didecyl dimethyl ammonium chloride 6% |
| Synsept BOR, powder | Synpeko, Poland | Sodium perborate 24%, TAED – 12% |
| Synsept PAA, liquid | Synpeko, Poland | A: peracetic acid 3.5%; B: hydrogen peroxide 20% (5% A + 5% B) |
| Virusolve + EDS, liquid | Amity International, Great Britain | Alkilotriamine 12 g, 2-aminoethanol 8 g |
Disinfection of surgical instruments and other medical devices. Amine-based disinfectants have become quite popular for the disinfection procedure. Because of the toxicity of aldehydes and phenols, new disinfectant formulations are being developed. The antimicrobial activity of amine derivatives has been studied for some time. Much work has been devoted to the study of the mycobactericidal activity of products containing ortho-phthalaldehyde (OPA), glutaraldehyde (GTA), and other dialdehydes.

Fraud et al. [11] showed high mycobactericidal activity of aldehydes (OPA) at 0.5% (v/v) unadjusted pH 6.5 and pH 8, under both clean and dirty conditions. Test organisms consisted of glutaraldehyde (GTA)-sensitive strains of Mycobacterium chelonae NCTC 946, M. abscessus NCTC 10882, two GTA-resistant M. chelonae strains, and M. terrae NCTC 10856 (a proposed M. tuberculosis surrogate). All mycobacterial reference strains used in the suspension test under clean conditions were very sensitive to 0.5% alkaline OPA, resulting in a log reduction factor of 5 after 1 min exposure or after 10 min with M. abscessus NCTC 10882.

Discussion

Mycobactericidal activity of disinfectants and antiseptics depends on chemical compositions, concentrations of use, durations of contact time, and organic loads. Aldehydes, phenols, quaternary ammonium compounds, and polyalkylamines are commonly used as detergents/disinfectants in the disinfection procedure. Because of the toxicity of aldehydes and phenols, new disinfectant formulations are being developed. The antimicrobial activity of amine derivatives has been studied for some time.

Amine-based disinfectants have become quite popular for the disinfection of surgical instruments and other medical devices. Korsolex® AF (15.6% dodecyl-bis-propylene triamine and 5.1% lauryl propylene diamine) was studied by Hernandez et al. [9]. The mycobactericidal and tuberculocidal activities of Korsolex® AF against M. tuberculosis H37 Rv ATCC 25618, Mycobacterium kansasii ATCC 12478, M. chelonae ATCC 35752, and a MAI (M. avium – M. intracellulare) clinical strain were determined using quantitative bacteria suspension and carrier tests. The effects of organic load and hard water were also considered. Korsolex® AF had acceptable efficacy against several species of mycobacteria in quantitative suspension and carrier tests at a concentration of 2% with an exposure time of 30 min. One of the 4 strains – the MAI clinical strain – was the least sensitive: the reduction in number of mycobacteria was about 4.6 log, while for the other strains the reduction rate was more than 5 log.

Glutaraldehyde is widely used as the active ingredient in high-level disinfectants for heat-sensitive, semi-critical medical instruments, including soft endoscopes or bronchoscopes, which may be contaminated with different strains of bacteria, including mycobacteria, after usage. Flexible fiber-optic bronchoscopy has become accepted as a safe tuberculosis diagnostic and therapeutic procedure and it is typically well tolerated by the patient. However, the transmission of Mycobacterium tuberculosis, atypical mycobacteria, and other pathogens between patients undergoing bronchoscopy has been reported due to improper cleaning and disinfection procedures [9].

Miner et al. [10] demonstrated that products containing concentrations of ≤20% w/w isopropanol and ≤8% potassium acetate in combination with ≤3.5% w/w glutaraldehyde at alkaline pH values killed 6 log of mycobacteria – Mycobacterium bovis var BCG, M. terrae ATCC 15755, and glutaraldehyde-resistant M. chelonae var abscessus ATCC 14472 – within 10 min at 20°C.

In our study, products containing glutaraldehyde (Aldesan E, Aldizol, and Synsept AG) have also been found to be effective against mycobacteria (i.e., they meet the requirements of EN 14348).
Table 2. Results of mycobactericidal activity of tested disinfectants and antiseptics; assays carried out in accordance with European Standards.

| Product             | Standard   | Test conditions – according to manufacturers order | Result (log of mycobacteria reduction) |
|---------------------|------------|----------------------------------------------------|----------------------------------------|
| Aldesan E           | EN 14348   | Undiluted, 60 min, dirty condition                 | M.a. >6.27; M.t. >6.22                 |
| Aidizol             | EN 14348   | 1.75%, 15 min, clean condition, 2.5%, 15 min dirty condition | M.a. >6.54; M.t. >6.21                 |
| Chlor-Clean         | EN 14348   | 1000 ppm, 15 min, clean condition                  | M.a. = 4.59; M.t. = 4.40               |
| Desisoft Ytdesinfektion | EN 14348 | Undiluted, 5, 30 i 60 min, clean and dirty condition | M.a. <2.66; M.t. <2.42                 |
| Lysoformin Plus-Schaum | EN 14348 | Undiluted, 15 min, dirty condition                 | M.a. >6.65; M.t. >6.21                 |
| OneMed Easydes      | EN 14348   | Undiluted, 1 min, dirty condition                  | M.a. <4.19; M.t. <3.09                 |
| Rafasept            | EN 14348   | 1.5%, 15 min, dirty condition                      | M.a. >6.55; M.t. >6.26                 |
| Septyl Amyco        | EN 14348   | 2%, 30 min; 2.25%, 15 min; 2.75% 15 min, clean and dirty condition | M.a. >6.55; M.t. >6.03                 |
| Septyl R            | EN 14348   | 1.5%, 15 min, clean and dirty condition            | M.a. >6.54; M.t. >6.13                 |
| Soft Care Des E Spray | EN 14348 | Undiluted, 30 s, clean condition                   | M.a. = 5.81; M.t. = 5.71               |
| ST4                 | EN 14204   | 0.5%, 10 min, high levels soiling                  | M.a. >2.89                             |
| Steril C            | EN 14348   | 1%, 20 min, dirty condition                        | M.a. <3.17; M.t. = 4.02                |
| Steril-Ser          | EN 14348   | 1%, 10 and 15 min, dirty condition                 | M.a. >6.43; M.t. >6.07                 |
| Synrol ALC          | EN 14563   | Undiluted, 1 min, clean condition                  | M.a. >6.48; M.t. >6.13                 |
| Synrol PAA10        | EN 14348   | 2%, 15 min, dirty condition                        | M.a. >4.59; M.t. <2.87                 |
| Synsept AG          | EN 14348   | 4%, 60 min, dirty condition                        | M.a. >4.58; M.t. >4.02                 |
| Synsept BOR         | EN 14348   | 2%, 15 min, dirty condition                        | M.a. >4.92; M.t. >6.13                 |
| Synsept PAA         | EN 14348   | 5%A + 5%B, 10 min, dirty condition                | M.a. >3.17; M.t. <2.96                 |

Test conditions: concentration, contact time, clean or dirty conditions, M.a. – *M. avium*, M.t. – *M. terrae*.
In the carrier test, in which discs of polypropylene (the material of which endoscopes are constructed) were covered with biofilm formed from a mixture of mycobacteria with sodium alginate, a high degree of bacterial reduction was achieved (≥5 log) for a 0.5% w/v solution of OPA, but after a longer contact time (30–60 min) and only under clean conditions [12].

Mycobactericidal activity of Cidex (2% glutaraldehyde) and Cidex OPA (0.55% ortho-phthalaldehyde) was evaluated with *M. smegmatis* ATCC 19420 and with clinical isolates of *M. fortuitum*, *M. abscessus*, and *M. tuberculosis* H37Rv (apart from the clinical strain of *M. chelonae*). Cidex and Cidex OPA were effective against tested organisms, showing greater than a 5-log reduction in colony-forming units (CFU) after 5 min of exposure under both clean and dirty conditions [13].

In the past decade, high-level disinfectants based on glutaraldehyde have become widely used in disinfection because of their mycobactericidal activity. However, disinfection with glutaraldehyde has recently been of some concern because of its toxicicity, skin and respiratory sensitizing of hospital staff, and the emergence of resistant mycobacteria. This agent also selects for anti-glutaraldehyde.

Several studies have been performed to assess possible alternatives to glutaraldehyde.

The *in vitro* and *in vivo* studies conducted by Hernandez et al. [14,15] showed the equivalency of products for disinfection of bronchoscopes, containing 2% glutaraldehyde (Cidex) and 0.26% peracetic acid (Perasafe). Both preparations caused a reduction in the number of mycobacteria of more than 5 log after 20 to 30 min, under both clean and dirty conditions.

In our studies, products containing peracetic acid also caused high reduction ratios (over 6 log) of both mycobacterial strains of *M. terrae* and *M. avium* after 10 to 15 min of contact time in a study conducted in dirty conditions.

Chlorine dioxide may be an alternative to aldehydes in disinfection of soft endoscopes and other medical equipment.

In the carrier test according to the modified method of prEN 14563: 2005, Hernandez et al. [16] studied the activity of the product ‘Tristel Sporicidal Wipes’. This system is composed of 2 components: a wipe that is saturated with a mixture of organic acids, preservatives, buffers, and corrosion inhibitors, and a bottle containing a sodium chlorite-based foam. Prior to testing, the wipes were prepared by squirting foam onto the wipe and then scrunching it by hand to mix the 2 components of the product to activate the disinfectant. The chlorine dioxide concentration in the activated wipe was 200 ppm. The results showed that the chlorine dioxide wipes were mycobactericidally active against *M. avium* ATCC 15769 with 30 s contact time with mechanical action and in 60 s without mechanical action, under both clean and dirty conditions.

In our suspension studies (EN 14348), chlorine-based disinfectant (Chlor-Clean) showed activity against both strains of mycobacteria *M. avium* and *M. terrae*, compatible with the standard. Increasing the chlorine concentration of use from 1000 ppm to 10 000 ppm, despite the changing conditions from clean to dirty at the same contact time; 15 min resulted in a significant increase in the degree of reduction of bacteria, from approximately 4 log to over 6 log.

An example of a different, new biocidal substance from the oxidizing agents group is 2-butaneone peroxide, which is proposed in Table 3. Detail results of mycobactericidal activity of Virusolve + EDS product carried out in accordance with EN 14563.

**Table 3.** Detail results of mycobactericidal activity of Virusolve + EDS product carried out in accordance with EN 14563.

| Microorganism     | Contact time (min) | Logarithm of reduction in number of mycobacterial cells in the samples of 2.5% concentration | Dirty condition 3.0 g/L bovine albumin | Clean condition 0.3 g/L bovine albumin |
|-------------------|--------------------|------------------------------------------------------------------------------------------------|--------------------------------------|
|                   | I test             | II test                                                                                      | I test                               |
| ATCC 15769        |                    |                                                                                             |                                      |
| *M. avium*        | 5                  | >4.89                                                                                       | 4.39                                 |
|                   | 15                 | >4.89                                                                                       | 4.39                                 |
|                   | 30                 | >4.89                                                                                       | 4.39                                 |
|                   | 60                 | >4.89                                                                                       | 4.39                                 |
| ATCC 15755        | 5                  | <1.37                                                                                       | <1.37                                |
| *M. terrae*       | 15                 | <1.37                                                                                       | <1.37                                |
|                   | 30                 | <1.37                                                                                       | <1.37                                |
|                   | 60                 | <1.37                                                                                       | <1.37                                |

After 20 to 30 min, under both clean and dirty conditions.
for use in antiseptic and disinfectant products dedicated for skin disinfection and for the disinfection of instruments and surfaces in a hospital environment. García-de-Lomas et al. [17] tested the biocidal activity of different concentrations of 2-butaneone peroxide against different microorganisms: bacteria, spores, fungi, viruses, and also mycobacteria, including *M. terrae* ATCC 15755. Mycobactericidal activity was assessed following the suspension method described in EN 14348. It showed a degree of reduction of approximately 7 log at a 0.5% solution, after 60 min of contact time at 20°C. Parallel toxicity tests were conducted. Toxicity assessment showed negative results in the acute dermal irritation test, acute eye irritation test, and acute oral toxicity test [17]. The results allow for the recognition of 2-butaneone peroxide as an active ingredient suitable for use in the new formulation of antiseptics and disinfectants.

Taking into account the biocidal efficacy of disinfectants, as well as security to users and the environment and compatibility with disinfected materials, a new formulation based on hydrogen peroxide (accelerated hydrogen peroxide, AHD) was developed [18,19]. It contains very low levels of certain food-grade anionic and non-ionic surfactants, which act in synergy with hydrogen peroxide to produce the desired microbicidal activity. Omidbakhsh and Sattar [18] investigated the activity of such a product containing 0.5% hydrogen peroxide against mycobacteria: *M. bovis*, BCG strain and *M. terrae* ATCC 15755. The resulting degree of reduction after a contact time only 5 min at 20°C for these strains was over 6 log in the presence of 5% serum.

Our results confirmed the efficacy of products containing hydrogen peroxide (Synrol PAA10 and Synsept PAA) against mycobacteria *M. avium* and *M. terrae*. The complex composition of the products should also be considered, as they also include other active substances.

**Disinfecting products that contain substances from the group of quaternary ammonium compounds do not exhibit effective biocidal activity against mycobacteria. Bello et al. [13] conducted studies with 2 disinfectants – Gerdex and K-ller – which both contain the quaternary ammonium compound dimethyl benzyl lauryl ammonium bromide in concentrations of 10% and 0.16%, respectively. Gerdex and K-ller caused only a 2-log cell reduction of *M. tuberculosis* H37Rv, *M. abscessus*, and *M. chelonae*, even after 60 min of contact time under both clean and dirty conditions. The products tested in our study (Lysosorfin Plus-Schaum, OneMed Easylsy and Synsept AG), in addition to quaternary ammonium compounds, also contain other active ingredients. Only the product Lysosorfin Plus Schaum showed no reduction in accordance with EN 14348 of *M. terrae* cells, even under clean conditions after 15 min of contact time (<3.1 log), but in the case of a *M. avium* the degree of reduction was on the border of the requirements according to standard (approximately 4.1 log).

**Conclusions**

The tested products for disinfection and antisepsis contain active substances from different chemical groups: aldehydes, alcohols, quaternary ammonium compounds, phenolic compounds, and oxidizing agents. In most cases they showed mycobactericidal activity that complied with European standards. In the case of products that contain guanidines and amine compounds, the concentration of active ingredients used and the test conditions specified by the client that ordered the test did not show the mycobactericidal activity required by the standards. Prior to the introduction of the product to the market, it is important to establish the appropriate conditions for the use of the product, such as the concentration to use, contact time, and clean or dirty conditions.

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