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**DEVELOPMENT OF A COMBINED CARRIER TEST
FOR DISINFECTANT EFFICACY**

A Thesis submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of the Requirement for
the Degree of Master of Science
Department of Microbiology and Immunology
Faculty of Medicine

by

Maureen Best

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UNIVERSITÉ D'OTTAWA
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ABSTRACT

There is mounting concern on the efficacy of many germicides on the market because officially recognized germicidal tests for various classes of microorganisms vary widely and lack reproducibility and proper quantitation. This project outlines a novel carrier method for simultaneously and quantitatively assessing the efficacy of liquid chemical germicides against a mixture of microorganisms of varying degrees of resistance.

In the test, glass cups were contaminated with 10 μ l of a standardized mixture of *Staphylococcus aureus*, *Mycobacterium bovis* BCG, *Trichophyton mentagrophytes* spores, Sabin poliovirus type 1 and *Bacillus stearothermophilus* spores in 5% fetal bovine serum. The inoculum was dried for 60 min at room temperature ($22 \pm 2^{\circ}\text{C}$), covered with 60 μ l of the disinfectant under test or a balanced salt solution for the desired contact time. The carrier was then placed in 2,940 μ l of an eluent and eluates assayed separately for the five microorganisms.

Of the products tested, 2% alkaline glutaraldehyde, 0.6% sodium hypochlorite (about 5000 ppm free chlorine) and a 0.4% quaternary ammonium compound (QAC) containing 23% HCl were effective against all five challenge organisms; a hard surface spray containing 0.1% o-phenylphenol with 79% ethanol was effective against all but bacterial spores; 70% ethanol alone and povidone-iodine (1% available iodine)

were effective against *S. aureus*, the mycobacterium and the fungus; a 3% solution of peroxygen compounds was effective only against *S. aureus* and the poliovirus; 1.5% chlorhexidine gluconate, 0.06% QAC, and 0.03% *o*-phenylphenol + 0.03% *p*-tertiary amylphenol could inactivate nothing but *S. aureus*; 3% hydrogen peroxide was ineffective in all tests.

Further tests with a second organic load, 7.6 g/L Tryptose Phosphate Broth, yielded similar results. Water of a specified hardness (200 ppm CaCO₃) adversely affected two products (3% mixture of peroxygens and 0.03% *o*-phenylphenol + 0.03% *p*-tertiary amylphenol).

Comparative resistance testing demonstrated that the bacterial spores used in this study (their requirement for an elevated growth temperature allowed for simple separation from the combined inoculum), were at least as resistant as those of *Bacillus subtilis*, the species recommended for evaluating sporicides. *S. aureus* exhibited an intermediate resistance pattern between that of *Salmonella choleraesuis* and *Pseudomonas aeruginosa*, the two other bacterial species recommended for evaluating bactericidal claims. A drug-resistant strain of *M. tuberculosis* (MDR-TB) and an isolate of *M. avium-intracellulare* from an AIDS patient were more resistant than *M. bovis* BCG and *M. gordonae*. *M. terrae* exhibited a resistance pattern similar to MDR-TB. When compared with

the poliovirus, hepatitis A virus was more readily inactivated by 1% iodine and was more resistant than a 3% mixture of peroxygens and 0.1% o-phenylphenol containing 79% ethanol.

The findings demonstrate the feasibility of a novel approach to the testing of chemical germicides. The combined carrier test allows for concurrent evaluation of efficacy claims and demonstrates the resistance patterns of various classes of microorganisms. This method also permits a more reliable means of classifying germicides based on their spectrum of activity.

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1. REVIEW OF THE LITERATURE

1.1 Disinfectants and infection control

Individual cases of infections and several outbreaks in health-care institutions have been linked to inadequately disinfected endoscopes and other medical instruments. A recent review on the transmission of infections by flexible gastrointestinal endoscopy and bronchoscopy reported the transmission of 281 and 96 infections, respectively (Spatch et al. 1993). The severity of these cases ranged from asymptomatic colonization to death with the causative agents including *Salmonella*, *Pseudomonas*, and *Mycobacteria* species. In all cases, reasons for transmission were inadequate disinfection. In 1991, Health and Welfare Canada reported an outbreak of *Mycobacterium chelonii* among patients undergoing bronchoscopy and of *Pseudomonas aeruginosa* in 9% of patients undergoing upper gastrointestinal endoscopic procedures (HWC 1991). More recently an outbreak of *Serratia marcescens* was reported in a surgical intensive care unit (Vandenbroucke-Grauls et al. 1993). Both outbreaks were linked to inadequate disinfection of bronchoscopes.

Failure of disinfection to eliminate fungal and parasitic contamination of endoscopes has also been noted. For example, pseudoepidemics of *Trichosporon* sp. and

Rhodotorula sp. were traced to contaminated bronchoscopes (Hoffman et al. 1989; Schleupner and Hamilton 1980). In an outbreak of *Strongyloides* spp. esophagitis, strong evidence suggested cross-infection of the parasite from a single endoscope (Mandelstam et al. 1976).

Documented transmission of viral infections between patients undergoing endoscopy has been limited to one confirmed case of hepatitis B virus, transmitted through gastrointestinal endoscopy (Hanson et al. 1991). However, most studies have not addressed viral agents, and the frequency of viral transmission in this manner remains unknown.

Such iatrogenic spread becomes even more serious as increasing numbers of tuberculosis cases are reported, including those caused by multiple drug-resistant strains of *Mycobacterium tuberculosis* (MDR-TB). Large institutional outbreaks of more than 200 nosocomially acquired cases have occurred in hospitals, outpatient clinics and prison facilities (Beck-Sague et al. 1992; CDC 1990; CDC 1991; CDC 1992; Eldin et al. 1992; Fischl et al. 1992; Pearson et al. 1992; Perri et al. 1989; Uttley and Pozniak 1993; Villarino et al. 1992). Efforts to reduce the risk of nosocomial transmission of MDR-TB include the intensification of infection control practices in health-care settings, in which, disinfectants play a significant role.

To date, MDR-TB outbreaks have not been linked to

improper disinfection practices. However, since the transmission of tuberculosis by inadequately disinfected instruments is well documented, there is concern of possible spread of MDR-TB in this manner (Bezel et al. 1985; Leers 1980; Nelson et al. 1983; Spatch et al. 1993; Wheeler et al. 1989). The relative resistance of MDR-TB to disinfectants as compared to other virulent strains of tuberculosis and the species of mycobacteria used to evaluated tuberculocidal claims (*M. bovis* BCG) is not known.

Inadequate disinfection of bronchoscopes and other medical instruments has, in addition to transmission of infections between patients, resulted in the contamination of diagnostic specimens. Recently, contamination of bronchoscopes with *M. tuberculosis* resulted in reporting of false-positive mycobacterial cultures in a Canadian hospital laboratory (Bryce et al. 1993). Proper reprocessing and disinfection practices reduces the potential for cross-contamination of samples and eliminates unnecessary diagnostic and therapeutic interventions.

Disinfectants are also routinely employed to control the spread of nosocomial infections from inanimate surfaces other than medical devices (Favero and Bond 1991; Groschel 1988). Examples of these types of surfaces are door knobs, handles, instrument carts, trays and machines that do not come into direct contact with patients, but may frequently

become contaminated with infected material. Although not directly implicated in transmission of disease, these surfaces potentially contribute to secondary cross-contamination by hands of healthcare workers repeatedly touching the contaminated surface, or by contact with medical instruments (Favero and Bond 1991). Children may be vulnerable to fomite transmission when mouthing toys and other objects. Many pathogens, e.g. tubercle bacilli and hepatitis B virus, are known to be able to survive dried onto surfaces in an infectious state for weeks and even years, with a potential for environmentally mediated disease transmission (Kunz and Gunderman 1982; Mbithi et al. 1992; Mitscherlich and Marth 1984). Transfer of viruses between hands and inanimate surfaces has been documented and inanimate surfaces are thought to play a role in the transmission of viral infections (Mbithi et al. 1992; Sattar et al. 1989; Sattar 1992; Springthorpe and Sattar 1990).

Finally, infections originating directly from disinfectant solutions contaminated with Gram-negative bacilli have also been reported. Intrinsic contamination of povidone-iodine solutions with *Pseudomonas* spp. has led to clusters of pseudobacteremias and peritonitis cases (Berkelman et al. 1981; CDC 1980; CDC 1989; Parrott et al. 1982). Subsequent laboratory studies revealed that pseudomonads colonizing water distribution pipes in plants

manufacturing iodine solutions were protected from the bactericidal effect of the iodophor solution by their glycocalyx film (Anderson et al. 1984). These organisms were recovered from povidone-iodine solutions up to 68 weeks from the time of manufacture (Anderson et al. 1984; Anderson et al. 1990).

The health hazard posed by ineffective disinfection raises concerns regarding the safety and efficacy of disinfectants. These concerns have led to the re-examination of the efficacy of disinfectants and the tests used to obtain efficacy data.

1.2 Disinfectant products and efficacy claims

The choice of an appropriate, effective disinfectant has become an increasingly difficult task. The disinfectant market is extremely competitive with over 4000 disinfectant products available representing about \$1 billion per year at the retail level in the United States (GAO 1990). These products made about 8,000 different efficacy claims against a variety of target microorganisms (e.g., tubercle bacillus, human immunodeficiency virus) with different levels of intended activity (e.g., a specified reduction in level of the microorganism or a complete kill) (GAO 1990). About 18,000 use patterns were recommended on product labels, ranging from use on counter tops to soaking medical instruments (GAO 1990). Products on the Canadian

market, although somewhat fewer in numbers (approximately 700 products listed), present equally diverse and complex efficacy claims. Fierce competition results in preparations that carry similar active ingredients often carry different label claims. Confusing wording on the label for appropriate dilutions, exposure times and temperatures has contributed to the bewildering amount of information presented to disinfectant users. Infection control practitioners and biosafety officers, responsible for implementing safe disinfection policies and practices in hospitals, laboratories and other health-care institutions, rely heavily on purchased products to work as claimed on the label or in promotional literature. Label claims may not always be true if efficacy data have been generated using an array of diverse, unreliable tests.

There is considerable concern on the efficacy of numerous disinfectant products available on the market (Fox 1986; Groschel 1983; Groschel 1988; Gurevich, Yannelli and Cunha 1990; Rhodes 1983). Studies evaluating randomly selected disinfectants were often unable to reproduce manufacturers' efficacy claims (Rutala and Cole 1987). In one report, disinfectant claims for 22% of hospital disinfectants could not be reproduced (Rhodes 1983). The effectiveness of disinfectants with tuberculocidal claims has also been questioned (Best et al. 1990B; Rutala et al. 1991). Given the seriousness of acquiring such noscomial

illnesses as multidrug-resistant tuberculosis, these findings are alarming.

In response to these concerns, the US General Accounting Office (GAO), an agency responsible for monitoring government activities, evaluated the regulation of disinfectant products in the US. Their recent report concluded that up to 20% of disinfectant products on the market may be ineffective (GAO 1990). This was attributed to, in part, the test methodology currently used to evaluate the efficacy of disinfectants and substantiate product label claims. Unreliable test methods permit ineffective products to gain access to the disinfectant market.

1.3 Limitations of currently available efficacy tests

The scientific validity, reproducibility and accuracy of the currently accepted Association of Official Analytical Chemists (AOAC) protocols for evaluating bactericidal, tuberculocidal and sporicidal efficacy claims have been criticized by the scientific community (Ascenzi 1991; Beloian 1990; Groschel 1991; Myers 1988; Rhodes 1983). The fundamental design of the AOAC methods, which are at best semi-quantitative, contain many uncontrollable variables. Specifically, several deficiencies were identified: 1) the inconsistent surfaces of the stainless steel carriers, 2) the lack of standardization of the

inoculum on the carrier surface, 3) the loss of test organisms into the disinfectant solution, 4) the poor recovery medium that does not support the growth of healthy or sublethally damaged cells, and 5) a difficult-to-follow, vague, and ambiguous methodology (Alfano, Cole and Rutala 1988; Ascenzi et al. 1986; Ascenzi et al. 1987; Ascenzi 1991; Cole et al. 1987A; Cole et al. 1987B; Cole et al. 1988A; Cole and Rutala 1988B; Cole and Rutala 1988C; Cole et al. 1990; Rubino et al. 1993).

Extreme variability of test results was detected among laboratories conducting several collaborative studies testing the bactericidal activity of identical disinfectant products using the AOAC use-dilution protocol (Cole and Rutala 1988C; Rubino et al. 1993). It was decided by the AOAC that the development of a new reproducible method was necessary. Research on the tuberculocidal and sporicidal test methods was also initiated. Regulatory agencies in Canada and the US agreed and supported research into the development of new methodologies (CGSB 1991; GAO 1990).

In addition to poor inherent test design, the currently accepted protocols have been criticized for generating data that cannot be extrapolated to a disinfectant's performance under in-use conditions. The time a disinfectant under test is exposed to the contaminated carrier surface (contact time) is fixed at 10 minutes in the AOAC protocol. This time differs

significantly from actual use conditions where the contact time can vary from a few seconds to several hours. The tests are not conducted in the presence of organic matter (e.g. blood, serum) and therefore do not adequately simulate in-use conditions where such materials are likely to be encountered. Furthermore, testing carried out using distilled water as diluent for the disinfectant does not simulate the routine use of tap water in the field to prepare in-use solutions of disinfectants

There are no approved AOAC methods for assessing virucidal activity of germicides. The present virucidal methods set forth by the American Society for Testing and Materials (ASTM) are also under scrutiny by the scientific community (ASTM 1989; Groschel 1991). Many disinfectant products are tested for their bactericidal activity only and such products can carry general label claims, including "hospital disinfectant" and "medical environmental disinfectant" for use in areas where viruses are most likely to be present. The corresponding virucidal testing is rarely done and their proven efficacy against bacteria is not necessarily indicative of their effectiveness against viruses. Supplementary claims against specific viruses are supported by data generated from tests that may be technically sound, but do not simulate the conditions under which the product is intended for use, or, have been carried out using relatively susceptible viruses that are

not representative of all viruses.

The limited information that is available on the virucidal activity of disinfectants has been generated using a wide variety of test methods and viruses, the results of which cannot be absolutely compared (Chen 1991; Springthorpe and Sattar 1990). A general survey of virucide testing laboratories reported the use of 39 types of viruses representing eight major virus groups as target viruses in their testing (Chen 1991). It is not practical to test disinfectants against a wide spectrum of viruses and then use different products for different viruses. The viral targets are often not known and, ideally, a disinfectant chosen for a particular use should be one that will potentially inactivate all types of viruses of concern. Selection of a model virus is a reasonable approach to virucidal testing providing that it's relative resistance to disinfection is comparable to the virus group.

Research aimed at improving the currently accepted test protocols has focused on the modification of existing AOAC protocols (Cole et al. 1987B; Cole and Rutala 1988C; Cole et al. 1990; Rubino et al. 1993). The hard surface carrier test method for testing the bactericidal activity of disinfectants has recently replaced the highly criticized use-dilution method and modifications to the tuberculocidal and sporicidal test are under investigation.

AOAC methods designed for testing bactericides have also been modified for virucidal testing (Chen 1991). Such approaches are of limited value as the inherent design of the AOAC protocols is flawed in a way that severely limit their usefulness. A new test incorporating a unique design that eliminates the inherent variability of the AOAC protocols is preferable.

1.4 Modifications required for a suitable protocol

Disinfectant test methods are generally of two types: (1) "suspension" tests in which the test organism is mixed with the disinfectant for a specified contact time, and (2) "carrier" tests in which the test organism is dried onto a surface and subsequently exposed to the disinfectant for a specified contact time. Carrier tests represent a more realistic and stringent challenge to the disinfectant under evaluation. In many studies comparing the efficacy of disinfectants in suspension and carrier tests, test organisms dried onto surfaces were much more difficult to inactivate (Best et al. 1988; Best et al. 1990A; Best et al. 1990B; Lloyd-Evans et al. 1986; Springthorpe et al. 1986). Most studies on developing new methods use suspension tests, the results of which have little practical value for assessing disinfectants intended for general use on contaminated surfaces or medical instruments (Ascenzi et al. 1987; Collins 1987; Robison 1988; Tanner

1989; Van Klingeren 1987). Several proposed carrier methods are also problematic due to poor carrier design, loss of test organisms that are not accounted for, inadequate recovery systems, and lack of an organic load (Kleiner and Trenner 1988; Lind et al. 1986; Tyler et al. 1990; Walder et al. 1989).

In many carrier tests, carriers are dipped into the test suspension and allowed to drain, resulting in an unknown initial titre on the carrier surface (Beloian 1990). This can be overcome by depositing known amounts of standardized test inocula directly onto the carrier surface. Placing the disinfectant solution onto the contaminated area of the carrier and subsequent immersion of the entire carrier into an eluent allows for complete recovery of the test inocula and eliminates the problems associated with wash-off and loss of test organisms from dipping contaminated carriers into the disinfectant. This method has been used successfully for mycobacteria, *Listeria spp.* and viruses and represents a superior design over other published methods (Best et al. 1988; Best et al. 1990A; Best et al. 1990B; Lloyd-Evans et al. 1986; Mbithi et al. 1990; Sattar et al. 1989).

Official test methods recommend the use of distilled water as a diluent for the disinfectant under evaluation. This could over-estimate the efficacy of certain products because their activity has been known to be neutralized by

usual impurities contained in tap water, the diluent most often used in the field to prepare in-use solutions (Cremieux and Fleurette 1991). Recognizing that the chemical composition of tap water, including its degree of hardness, varies geographically and temporally, the use of water of a defined hardness should be a recommendation in standard test methods.

The inadequacy of standard protocols that do not incorporate an organic load has been mentioned previously. Simulating organic materials in carrier tests greatly increases their practical relevance. Organic soil occludes microorganisms, hinders disinfectant penetration and may even contain varied microbial populations. An ideal organic load is well defined, reproducible and representative of body fluids that are likely to be encountered. Serum has been recommended in many germicidal test protocols (ASTM 1989; CGSB 1991). It is however, inhibitory for certain types of microorganisms and subject to batch-to-batch variations. Other proposed organic materials have included feces, sputum, mucin, milk powder, tryptose phosphate broth and yeast extract (Best et al. 1990A; Best et al. 1990B; Cremieux and Fleurette 1991; Gelinas and Goulet 1983; Kleiner and Trenner 1988; Mbithi et al. 1990; Sattar et al. 1989; Springthorpe et al. 1986). Further work is required to determine if these or other products would be suitable as a universally applicable organic load.

The use of surrogate organisms for standardized testing is advantageous because it is neither practical to test large numbers of strains nor those that are highly pathogenic. Model strains should be carefully selected to ensure that they represent the group; several strains of mycobacteria have been suggested in response to concerns that *M. bovis* BCG may be more sensitive to many germicides than *M. tuberculosis* (Collins 1987; Croshaw 1971; Gunderman 1987; Manowska et al. 1979; Russell 1992; Van Klingerren and Pullen 1987). Although there are no currently accepted AOAC virucidal tests, the use of poliovirus as a surrogate organism for evaluating virucidal claims has been recommended in the protocols of both the American Society for Testing and Materials and the Canadian General Standards Board (ASTM 1989; CGSB 1991). It is generally recognized that lipid containing viruses are more readily inactivated by most chemical disinfectants than are nonenveloped viruses such as poliovirus (Chen 1991; Sattar et al. 1989; Springthorpe and Sattar 1990). Nonenveloped viruses differ from one to another in their sensitivity to many disinfectants, suggesting the need to investigate the use of surrogates in place of, or, in addition to poliovirus; the use of hepatitis A may serve this purpose (Mbithi et al. 1990; Sattar et al. 1989; Springthorpe and Sattar 1990).

Use of a proper recovery system in disinfectant

testing is essential for determining "actual" vs "apparent" reductions in the numbers of test organisms. The recovery procedure should facilitate the growth of stressed or injured organisms that may have been sublethally damaged by the disinfectant. The AOAC tuberculocidal test has been criticized because of the inability of the recommended recovery media to detect injured mycobacteria; alternative media were able to recover higher numbers (Beloian 1990; Cole 1990). The recovery system for virucidal testing is somewhat more complex. It should similarly promote growth of the organism but must also render the disinfectant nontoxic for the cell culture assay. This second requirement can be achieved by dilution of the disinfectant to a level which does not kill the host cells. Virus challenge of the target cells (previously exposed to one dilution of the disinfectant below cytotoxic level) should be with a small number of infectious viruses to ensure that small numbers of virus can be accurately quantitated.

To make specific efficacy claims, disinfectants under evaluation must meet a criterion of efficacy set for the test protocol. The criteria of efficacy of the AOAC protocols vary depending on the claim intended and the test method employed but are all based on simple qualitative pass/fail criteria (Beloian 1990). For example, a disinfectant is considered to have failed the test if it fails to kill a microorganism in a specified number of

replicates. Quantitative protocols establish efficacy criteria based on $\log_{10}$ reductions in the starting titre of microorganisms. The criteria usually set vary from a 99.9% to 99.9999% reduction in titre depending on the nature of the claim i.e. bactericidal, tuberculocidal, virucidal (Best et al. 1990A; Best et al. 1990B; Springthorpe and Sattar 1990). This approach provides valuable information on the level of inactivation produced by the disinfectant and is preferred over qualitative methods.

1.5 Testing multiple classes of microorganisms

There is a lack of harmonization between efficacy tests for different classes of microorganisms i.e. bactericidal, mycobactericidal, virucidal, fungicidal and sporicidal tests. For example, the AOAC test for sporicidal activity and AOAC hard surface carrier test for evaluating bactericidal claims are both carrier tests but use different carriers (silk suture loops and porcelain penicylinders, and, glass penicylinders, respectively) (Beloian 1990). The AOAC test for fungicidal activity is a suspension test and does not require the use of carriers (Beloian 1990). In addition to the original AOAC tuberculocidal test which utilizes porcelain penicylinders, a suspension method based on membrane filtration has been approved for evaluating tuberculocidal claims (Beloian 1990; Groschel 1991). Virucidal claims can be supported by data

obtained from any scientifically sound carrier test (Springthorpe and Sattar 1990).

This lack of uniformity in disinfectant testing techniques yields different results that cannot be absolutely compared (Ayliffe 1989; Groschel 1991; Reybrouck 1991). Incorporating a mixed challenge of multiple classes of microorganisms would be advantageous. This mixed challenge would allow for concurrent evaluation of efficacy claims and provide more stringent and realistic test conditions than could be obtained using a single organism. One usually does not know what the microbial targets are and, secondly, microorganisms rarely occur as a single species on materials to be disinfected.

The incorporation of different classes of microorganisms into a single test also permits a more reliable means of classifying germicides based on their spectrum of activity. Historically, the Spaulding classification of germicidal action has been widely accepted and is included in recommendations for disinfection and sterilization (Favero and Bond 1991; Groschel 1988; Rutala 1990). The three levels ("high", "intermediate" and "low") of disinfectant activity described are based on the categorization of microorganisms according to their innate resistance to a spectrum of disinfectants. High-level disinfectants can be expected to destroy all microorganisms and are capable of sporicidal

activity after prolonged exposure; intermediate-level disinfection inactivates mycobacteria, vegetative bacteria, most viruses and most fungi; low-level disinfection can kill most bacteria, some viruses and some fungi.

Spaulding divided instruments and items for patient care into categories based on the degree of risk of infection and assigned to each a level of appropriate disinfectant activity. Critical items enter sterile tissue or the vascular system and present a high risk of infection (e.g., scalpels, needles, other surgical instruments). Most of these objects can be purchased as sterile or be steam sterilized. If heat labile, the object may be treated with a chemical sterilant. Semicritical items such as laryngoscopes and endoscopes which come into contact with mucous membranes and non-intact skin must receive high-level disinfection. Noncritical items such as stethoscopes which come into contact with intact skin require intermediate or low-level disinfection.

This disinfectant classification scheme was based on the general belief of the descending order of resistance of various classes of microorganisms to chemical germicides as follows: bacterial spores, mycobacteria and nonlipid viruses, fungal conidia, vegetative bacteria and lipid-containing viruses (Crowshaw 1971; Favero and Bond 1991; Russell et al. 1986). The ordering was established from the results of studies with individual types of

organisms, limited types of disinfectants and wide variations in test protocols; data have not been derived from tests on mixtures of organisms. Given that the mechanisms of inactivation of microorganisms by disinfectants is not fully understood, there is no reason to assume that this hierarchy of sensitivity/resistance would remain the same for all classes of disinfectants. For example, the relative resistance of nonlipid viruses compared to mycobacteria remains unclear, as does that of lipid-containing viruses to vegetative bacteria. Indeed, these relative sensitivities may depend to a large extent on the disinfectant formulation and the individual preparations of microorganisms that were tested.

2. STATEMENT OF OBJECTIVES

There is mounting concern regarding the efficacy of many germicides on the market because officially recognized germicidal tests for various classes of microorganisms vary widely and often lack reproducibility and proper quantitation. The objective of this thesis project is to examine the feasibility of a combined efficacy test for the simultaneous disinfection of microorganisms of varying degrees of resistance. The microorganisms are prepared as a mixture and used to contaminate carrier surfaces.

Microorganisms to be included in the test represent the major classes of microorganisms (vegetative bacteria, mycobacteria, fungal spores, bacterial spores and viruses). There are no previously published test protocols for the simultaneous examination of disinfectant efficacy against a variety of microorganisms from different classes. The test design will also address the deficiencies identified in existing test methods. Furthermore, it will permit a better assessment of disinfectant potency and a comparison between or ranking of specific formulations.

Specific objectives are listed as follows:

1. To develop a combined test protocol for simultaneously and quantitatively assessing disinfectant efficacy against a mixture of microorganisms.

2. To determine the best recovery methods for specific quantitation of the individual microorganisms from the mixture after disinfectant treatment.
3. To examine the possibility and extent of physical protection among components of the mixture.
4. To test the influence of organic materials on disinfectant activity.
5. To determine the influence of standard hard water, when used as a diluent for disinfectants, on germicidal activity.
6. To compare the resistance of currently accepted test strains with possible alternate surrogates within a class of microorganisms i.e. bacteria, mycobacteria, viruses and bacterial spores.

3. MATERIALS AND METHODS

3.1 Cell cultures

FRhK-4 cells, an established line derived from fetal rhesus monkey kidney, were obtained from Dr. M. Sobsey of the University of North Carolina, Chapel Hill. They were grown in 75 cm² plastic cell culture bottles (Costar, Cambridge, Mass.) using Eagle minimum essential medium (EMEM; GIBCO, Grand Island, N.Y.) with 50 µg of gentamicin sulfate (Cidomycin; Hoechst-Roussel Canada Inc., Montreal, Quebec, Canada.) per mL, 50 µg of kanamycin (GIBCO) per mL, 146 µg of L-glutamine (GIBCO) per mL, 2 µg of amphotericin B per mL, 0.015 M HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid; GIBCO), 50 uL of non-essential amino acids (GIBCO), 0.113% sodium bicarbonate (BDH Chemicals, Toronto, Ontario, Canada) and 10% fetal bovine serum (FBS). For virus plaque assays, cell monolayers were prepared in 12-well cell culture plates (Costar).

3.2 Microorganisms and cultivation of stock cultures

Sabin poliovirus type 1 (LSc2ab), was obtained from Dr. P. Payment, Institut Armand-Frappier, Laval, Quebec, Canada. Virus stock was prepared by infecting FRhK-4 monolayers in 75 cm² flasks. The virus was allowed to adsorb to the cells for 60 min at 37°C and the infected monolayers kept in EMEM,

without any antibiotics and FBS but containing 0.015 M HEPES, 146 µg per mL of glutamine, 50 µg of nonessential amino acids, and 0.113% sodium bicarbonate until about 75% of the monolayer was affected by virus cytopathology. The cultures were then frozen (-20°C) and thawed three times and the suspension centrifuged at 1,000 Xg for 10 min to remove cellular debris.

The HM-175 strain of hepatitis A virus (HAV) was obtained from Dr. M.D. Sobsey, University of North Carolina, Chapel Hill. Stock suspensions were prepared as described for poliovirus with an adsorption time of 90 minutes.

Staphylococcus aureus (ATCC-6538) was obtained from the American Type Culture Collection, Rockville, Md. Stock suspensions were prepared by culturing the bacteria in tryptic soy broth (Quelab Laboratories, Montreal, Quebec, Canada.) for 24 hours at 37°C.

Pseudomonas aeruginosa (ATCC-15442) and *Salmonella choleraesuis* (ATCC-10708) were obtained from the American Type Culture Collection. Stock cultures were prepared and stored as described for *S. aureus*.

A clinical isolate of *Trichophyton mentagrophytes* was obtained from the Microbiology Laboratory, Ottawa General Hospital, Ottawa, Canada. Stock suspensions of conidia were obtained by inoculating the center of a Mycobiologic Agar (Quelab) plate and incubating it at 28°C for 9 days. Mycelial mats were harvested from the agar surface,

homogenized with sterile glass beads in normal saline and filtered through sterile cotton to remove hyphae.

Bacillus stearothermophilus (ATCC-12980) was obtained from a biological indicator ampule (Chemspore 2; American Sterilizer Co., Erie, PA) for monitoring steam sterilization. Stock suspensions of the spores were prepared by inoculating plates of tryptic soy agar (TSA; Quelab) and incubating them at 56°C for 7 days. Growth was harvested with saline using a bent glass rod and heat treated for 10 min at 85°C.

B. subtilis (ATCC-19659) was obtained from the American Type Culture Collection. Stock suspensions of *B. subtilis* spores were prepared as described for *B. stearothermophilus* spores but with 11 days of incubation at 37°C.

The following mycobacteria cultures were obtained from the Mycobacteriology Section of the Laboratory Centre for Disease Control, Health Canada: *Mycobacterium bovis* BCG (ATCC-35743), *Mycobacterium tuberculosis* (NT 91-160) resistant to four first-line drugs (rifampicin, isoniazid, streptomycin and ethambutol), *Mycobacterium avium-intracellulare* (NT 92-598), *Mycobacterium terrae* (ATCC-15755) and *Mycobacterium gordonae* (ATCC-14470). Stock cultures were maintained on Lowenstein-Jensen agar. Liquid stock cultures of *M. bovis* BCG were prepared by growing the mycobacterium in 7H9 broth containing ADC enrichment (Difco

Laboratories, Detroit, Mich.) for 21 days at 37°C. The cell suspension was then homogenized with sterile glass beads.

Glycerol was added to the suspensions of all bacteria and mycobacteria to a final concentration of 10% and the suspensions of all microorganisms were separately aliquoted in 2 mL quantities and stored at - 80°C.

Preliminary studies on development of the combined test method, including the determination of elution efficiencies, were carried out using stock cultures prepared as described above. All disinfectant studies were carried out using a second set of stock cultures prepared in the same manner but to a higher titre to permit the demonstration of higher $\log_{10}$ reductions in CFU.

3.3 Disinfectants

Table 1 lists the disinfectants, their common applications and in-use concentrations tested in this study; the pH of the in-use solutions is also given. Except for 70% ethanol, all were commercial formulations and were purchased on the open market. Other similar formulations may be available but have not been tested. Furthermore, many disinfectants are marketed on a national rather than international basis, and the same formulation may be sold under a variety of trade names in different countries.

Table 1. Relevant information on the disinfectants tested.

Disinfectant	Dilution tested	Uses (pH)
1) 70% ethanol EtOH (BDH Chemicals, Toronto, ON)	undiluted	Soak and wipe (5.2)
2) 3% hydrogen peroxide H₂O₂ (Valmo Labs, Chambly, Quebec)	undiluted	Contact lenses and skin (6.8)
3) 1.5% chlorhexidine gluconate Chlorhex (Savlon, Ayerst Labs, Montreal, Quebec)	1:200	Hospital equipment & antiseptic (6.7)
4) 10% povidone-iodine P-I₂ (Proviiodine, Rougier Inc., Chambly, Quebec)	undiluted (1% iodine)	Antiseptic (4.0)
5) 7.05% quaternary ammonium compounds ^a Quat (Unicide, Brulin & Co., Indianapolis, IN)	1:128 (550 ppm quat)	Cleaning & disin- fection of environmental surfaces (3.0)

Table 1. (cont'd)

6)	7.3% <i>o</i> -phenylphenol and 7.4% <i>p</i> -tertiary amylphenol Phenol (LpH, Calgon Vestal Labs, St. Louis, MO)	1:256 (575 ppm phenolic)	Cleaning & disin- fection of environmental surfaces (2.5)
7)	0.1% <i>o</i> -phenylphenol and 79% ethanol Phenol-EtOH (Lysol Spray, L&F Products, NJ)	undiluted	Spray for hard surfaces (8.2)
8)	0.4% quaternary ammonium compound ^b and 23% HCl Quat-HCl (Bo-Lav, Avmor, Montreal, Quebec)	undiluted	Toilet bowl cleaner (0.4)
9)	6% sodium hypochlorite NaOCl (Javex, Colgate Palmolive, Toronto)	1:11 (5000 ppm free chlorine)	Domestic bleach and general disinfectant (11.2)
10)	2% alkaline glutaraldehyde Glutarald (Cidex, Surgikos, Peterborough, ON)	undiluted	Instrument soak (7.5)

Table 1. (cont'd)

11) peroxygen ^c	1:33	Environmental
Peroxygen		
(Virkon, Antec Int., Suffolk, UK)		surfaces (2.3)
12) mixed phenolic ^d	1:80	Tuberculocide for
M-Phenolic		
(DRX Germicide)		environmental surfaces (9.3)

^aquaternary ammonium compound - alkyl dimethyl benzyl ammonium chloride 2.680%, octyl decyl dimethyl ammonium chloride 2.010%, didecyl dimethyl ammonium chloride 1.355% and dioctyl dimethyl ammonium chloride 1.005%

^bquaternary ammonium compound - alkyl dimethyl benzyl ammonium chloride

^c50% triple salt of potassium monopersulphate, potassium hydrogen sulphate and potassium sulphate + 18% sodium hexametaphate, 15% sodium dodecyl benzene and 10% malic acid, 5% sulfanic acid

^d3.4% o-phenylphenol, 1.7% o-benzyl p-chlorophenol, tetra sodium ethylene diamine, 2.0% tetra acetate, 4.5% ethyl alcohol, and 0.5% methyl alcohol

The types and amounts of the active ingredients in the formulations are listed. Disinfectants requiring dilution were diluted in tap water according to the instructions of their respective manufacturers. Free chlorine in sodium hypochlorite solutions was measured by the DPD (*N,N*-diethyl-*p*-phenylenediamine) method using a commercial kit (Hach Chemical Co., Ames, Iowa). A minimal contact time of 1 min was selected for initial testing; contact times were extended to 10 minutes, 30 minutes, and 3 hours in accordance with product label claims.

3.4 Combined test suspensions

Efficacy tests were carried out on both a mixture of five microorganisms (*S. aureus*, *M. bovis* BCG, *T. mentagrophytes* spores, *B. stearothermophilus* spores and poliovirus) and each individual microorganism to determine any influence the mixture may have on the disinfectants' efficacy. A mixture of the five organisms was prepared by separately centrifuging for 5 min (Eppendorf 5412; Brinkmann Instruments Inc., Westbury, N.Y.) 1 mL each of stock suspensions of the bacteria and the two types of spores, and the resuspending the pellets together in 1 mL of the poliovirus stock containing 5% FBS but no antibiotics. Each type of microorganism was assayed, as described below, before and after resuspension in the virus stock to determine any influence the mixture may have on

their viability and enumeration. When individual organisms were tested, the suspending medium was the supernatant (with 5% FBS and no antibiotics) from uninfected monolayers of FRhK-4 cells that had been frozen and thawed and clarified by centrifugation as described above.

Each individual organism and the mixed suspension was examined by scanning electron microscopy to assess the possibility of physical protection from disinfection that the components of the mixture may afford each other. Glass cover slips (10 mm; Chance Propper Ltd., Warley, England) were inoculated and dried as described for the carriers in the test protocol below. Samples were fixed in 2% glutaraldehyde and dehydrated in ethanol as described elsewhere (Schroeter et al. 1984). Critical point drying, gold coating and examination in the scanning electron microscope (Zeiss DSM940A) was carried out by Mr. Ann-Fook Yang, Electron Microscopy and Spectroscopy, Research Branch, Agriculture Canada.

3.5 Combined test method

Earle's balanced salt solution (EBSS) was used as both diluent and eluent for the virus, and as eluent in all other tests. Phosphate buffered saline (pH 7.2) was used as diluent for the remaining types of microorganisms.

Glass cups (catalog no. 8-733; Fisher Scientific, Nepean, Ontario), 10 mm wide x 14 mm long, were used as the

carriers to represent non-porous inanimate surfaces. In the test, 10 μ L of the test suspension was placed at the center of the inside flat surface of each carrier and the inoculum was allowed to air dry for 60 min in a Class II biological safety cabinet (with the blower on); during these experiments, the room temperature was $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and the relative humidity ranged from 30% to 40%. The dried inoculum was then covered with 60 μ L of the disinfectant under test; for the controls, 60 μ L of EBSS was used instead. After the required contact time at room temperature, each carrier was picked up with a pair of sterile forceps and immediately immersed in a 10 mL screw-cap glass vial (Wheaton 651907; Wheaton, Millville, N.J.) containing 2,940 μ L of EBSS and vortexed for 3 seconds to arrest the action of the disinfectant by a 1:50 dilution and to elute the microorganisms from the carrier surface.

In preliminary tests, the 50-fold dilution of the microorganism-disinfectant mixture was found to be effective in reducing the germicidal activity to an undetectable level with all the formulations tested. Previous testing found cell cultures exposed to 50-fold dilutions of glutaraldehyde and sodium hypochlorite capable of supporting the growth of the virus (cells were not fixed). Although cytotoxicity testing was not carried out with all products no degenerative changes in cell

cultures were observed with 50-fold dilutions. The glass cups could be used repeatedly after thorough washing and autoclave sterilization.

Each type of microorganism was assayed before and after drying the inoculum to determine any loss in its viability titer during the drying period as well as to determine the efficiency of its recovery from the carrier surface.

Separation and quantitation of the specific types of microorganisms in the mixture were achieved using membrane filtration, selective media and specific growth conditions (Figures 1, 2a, 2b, 2c). For microorganisms other than the viruses, the eluates were serially diluted in 10-fold steps and 0.1 mL of each dilution to be tested was spread separately on at least two plates of various recovery media as follows: *M. bovis* BCG was recovered on 7H11 Agar (Quelab) containing Middlebrook OADC enrichment (Quelab) and 10 µg/mL of griseofulvin (Sigma Chemical Co., St. Louis, Mo.) to suppress fungal growth; the plates were incubated at 37°C for 4 weeks. *T. mentagrophytes* spores were grown on Mycobiotic Agar, a selective medium containing 0.05 g/L of cyclohexidimide and 0.05 g/L of chloramphenicol, inhibitory for saprophytic fungal and bacterial growth, incubated at 28°C for 5 days. *B. stearoothermophilus* spores were grown on TSA plates, and incubated at 56°C for 48 hours. Columbia Sheep Blood Agar

(CSBA; Quelab) was used as the recovery medium for *S. aureus* and the plates were incubated at 37°C for 48 hours. The growth on the plates was counted as colonies and the results expressed as colony forming units (CFU)/mL.

Testing of two additional recovery media for the mycobacterium and fungus was carried out in order to determine that the use of a selective medium containing antibiotics was not interfering with the growth of sublethally damaged organisms. *M. bovis* was recovered from a mixed challenge containing all but the fungus on both 7H11 agar with and without griseofulvin (10 µg/mL). *T. mentagrophytes* spores were recovered from a mixed challenge containing all but *S. aureus* on Mycobiotic agar and Sabouraud Dextrose Agar (Quelab), an enrichment medium for the isolation of dermatophytes.

For recovering the virus, 1.0 mL of the eluate was passed through a 0.22 µm syringe filter unit (Millex-GV; Millipore, Bedford MA.). The filtrates were diluted in 10-fold steps in EBSS and plaque assayed in monolayers of FRhK-4 cells in 12-well plates. Each well was inoculated with 0.1 mL of the test sample, using at least 3 wells for each eluate dilution tested; cell culture control wells received 0.1 mL of EBSS instead. After 60 minutes of virus adsorption at 37°C in a 5% CO₂ atmosphere, each monolayer was overlaid with EMEM containing 2% FBS and 0.75% agarose (type II; Sigma). The plates were sealed in clear plastic

Fig. 1. Flow chart showing the main steps in the combined carrier test.

Mixture of *S. aureus*, *M. bovis*, poliovirus 1 (Sabin), *T. mentagrophytes* and *B. stearothermophilus* spores in 5% FBS

↓

10 μ L inoculated onto 3 control and 3 test carriers

↓

Inoculum allowed to dry for 1 hour at room temperature

↓

60 μ L disinfectant (EBSS for controls) placed on dried inoculum for desired contact time

↓

Carrier placed in glass vial containing 2,940 μ L of EBSS to arrest disinfectant action and elute the microorganisms

↓

1 mL eluate passed
through 0.22 μ m filter

↓

Filtrate plaque assayed
for virus

↓

1 mL eluate serially diluted
and media inoculated

↓

- 1) *M. bovis* - 7H11 agar with griseofulvin (4 wks, 37°C)
- 2) *S. aureus* - Blood agar (48 hrs, 37°C)
- 3) *B. stearo.* - Tryptic soy agar (48 hrs, 56°C)
- 4) The fungus - Mycobiotic agar (5 days, 28°C)

Test and control CFU/mL or PFU/mL ($\log_{10}$ reduction) compared

Fig. 2a. Combined carrier test showing application of disinfectant on contaminated carriers.

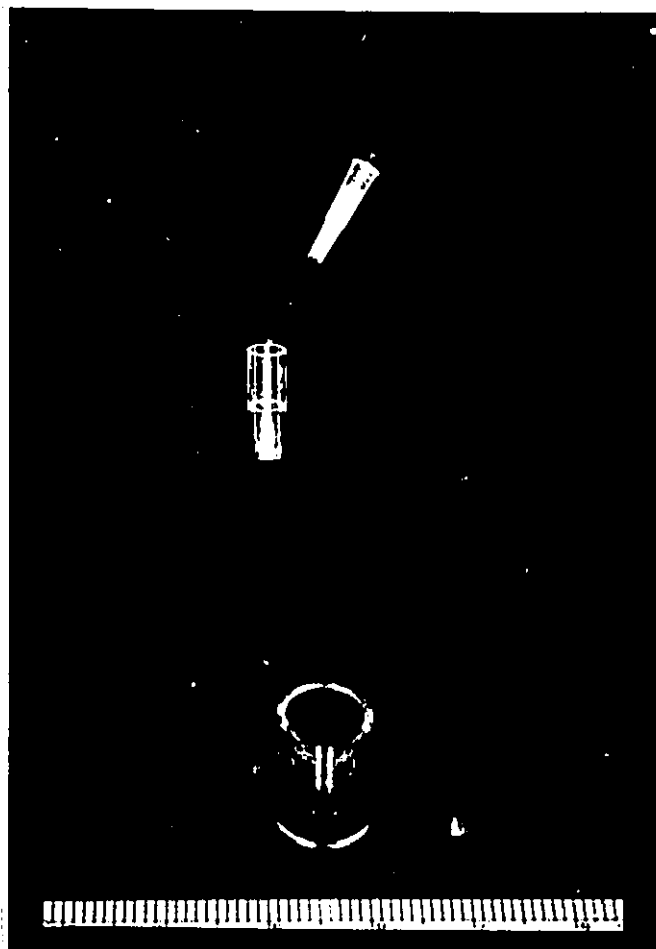


Fig. 2b. Combined carrier test showing disinfectant neutralization by dilution and elution of microorganisms from carrier surface.

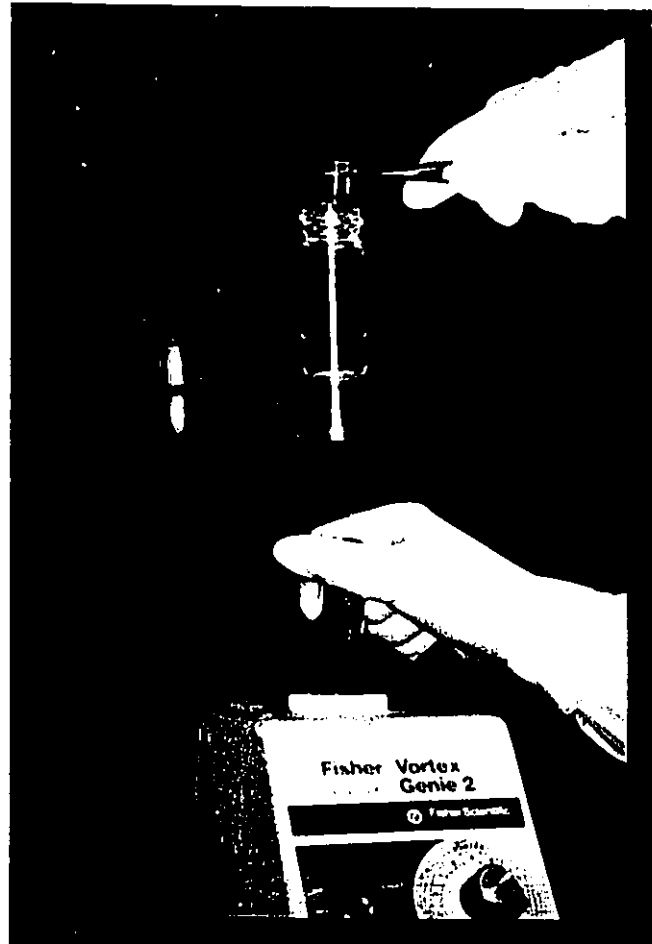
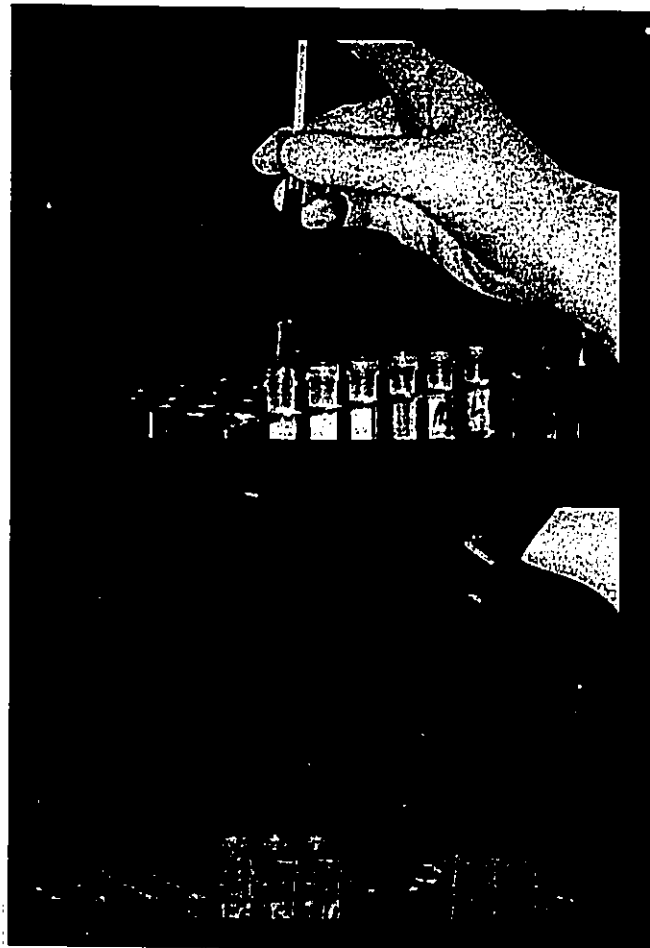


Fig. 2c. Combined carrier test showing filtration of eluate for viral separation and quantitation, and serial dilution of eluant for inoculation onto various recovery media.



bags (Dazey Corp., Industrial Airport, Kansas) and held for 40 hours at 37°C in a walk-in incubator. The monolayers were then fixed overnight at room temperature by adding 2.0 mL of 10% Formalin (BDH) in normal saline to each well. The overlay was then removed and the cells stained for one minute in a 1% aqueous solution of crystal violet. The stain was washed off in running tap water and virus plaques were counted and the results expressed as plaque forming units (PFU)/mL.

Tests were carried out at least three times with each of two batches of all formulations except for the quaternary ammonium-based formulation (Product #5) and the mixed phenolic (Product #6) where two samples from one batch were tested. Disinfectant activity was determined by comparing the growth on the control and test plates and is reported as the mean reduction in CFU or PFU for each disinfectant. The maximum levels of detection allowed for testing of up to a 6- $\log_{10}$ reduction in CFU of *S. aureus*, up to a 5- $\log_{10}$ reduction in CFU of *T. mentagrophytes* spores, *B. stearothermophilus* spores and *M. bovis*, and up to a 4- $\log_{10}$ reduction in PFU of the poliovirus.

Sample calculation of maximum levels of detection (*M. bovis*):

Control carrier

- 0.1 mL sample of eluate from the carrier was serially diluted, inoculated onto 7H11 agar and

yielded a concentration of 1.8×10^5 CFU/mL

- this represents a concentration of 1.8×10^6 CFU/mL in 3 mL of eluant or 5.5×10^6 CFU/mL in the carrier
- since the carrier was inoculated with 10 uL, this represents a concentration in the initial suspension of 5.5×10^8 CFU/mL

Test carrier:

- 0.1 mL sample of eluate from the carrier serially diluted and inoculated onto 7H11 agar yielded no growth
- this result was compared to the concentration obtained in the control carrier (1.8×10^5 CFU/mL)
- the $\log_{10}$ reduction in CFU is therefore > 5

In this study, the criterion of disinfectant efficacy was a demonstrated reduction in CFU or PFU of at least 3 log₁₀.

3.6 Effect of organic load

The organic load used in this study was 5% FBS as recommended in germicidal test protocols (ASTM 1989; CGSB 1991). It is however, expensive, and there is a need to find an alternative organic load which is reproducible and representative of the protein load of body fluids. Tryptose phosphate broth (TPB) (Difco) at 7.6 g/L was studied as a possible substitute for FBS. This concentration of TPB was found to contain 1.9 g/L total protein, as does 5% FBS.

Protein determinations of the two substances were carried out by the Clinical Biochemistry Dept., Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada using the Coomassie Brilliant Blue G-250 method (Bradford 1976). Efficacy tests were carried out using the mixed challenge suspended in TPB instead of FBS using the previously described test protocol.

3.7 Effect of diluent for disinfectant

Tap water (50 ppm CaCO_3 , tested by the Regional Municipality of Ottawa-Carleton) was used as the diluent for disinfectants under test that required dilution. The chemical composition of tap water varies geographically and it would be necessary to use water of a specific hardness for standard test protocols. Some tests are now requiring that the disinfectant's use-dilution be prepared in water to a minimum specified hardness (CGSB 1991). In order to determine the effect of water hardness on disinfectant efficacy, testing was carried out using disinfectants diluted in water prepared according to AOAC 960.09 at a hardness of 200 ppm CaCO_3 (Beloian 1990).

3.8 Comparative resistance of bacterial spores

Since *B. stearothermophilus* spores were selected for this study their resistance was first compared with that of the spores of *Bacillus subtilis*, the organism generally

recommended for disinfectant testing (Beloian 1990). Test suspensions were prepared by centrifuging the stock suspension and resuspending the pellet in 5% FBS. Resistance tests to 2% alkaline glutaraldehyde were carried out using the protocol described above with contact times of 1, 10 and 30 minutes, and 3 hours. *B. subtilis* spores were recovered on TSA plates incubated at 37°C for 48 hours. The resistance of both spores was also tested against 2.5N HCl for 2 minutes as recommended in the AOAC sporicidal test (Beloian 1990).

3.9 Comparative resistance of vegetative bacteria

In addition to *S. aureus*, two other bacteria, *Pseudomonas aeruginosa* (ATCC-15442) and *Salmonella choleraesuis* (ATCC-10708), are recommended for evaluating bactericidal disinfectant efficacy claims (Beloian 1990). Their resistance to disinfectants was compared with *S. aureus*, the bacteria used in this study. Each test suspension was prepared by centrifuging the stock suspension and resuspending the pellet in 5% FBS. Efficacy testing was carried out using the protocol described above.

3.10 Comparative resistance of mycobacteria

The disinfectant resistance pattern of *M. bovis* BCG, the currently accepted surrogate for mycobactericidal testing, was compared to that of two clinically important

species of mycobacteria: 1) a clinical isolate of *Mycobacterium tuberculosis* (NT 91-160) resistant to four first-line drugs (rifampicin, isoniazid, streptomycin and ethambutol; drug susceptibility testing using Canetti Proportion Method on Lowenstein-Jensen media was carried out by Mycobacteriology Laboratory, Laboratory Centre for Disease Control, Health Canada), and 2) *Mycobacterium avium-intracellulare* (NT 92-598) isolated from an AIDS patient. The disinfectant sensitivity of two potential surrogates (*Mycobacterium terrae* [ATCC-15755] and *Mycobacterium gordonae* [ATCC-14470]) was also investigated. All testing on *M. tuberculosis* and *M. avium-intracellulare* was carried out in the Mycobacteriology laboratory of the Laboratory Centre for Disease Control. Test suspensions were prepared by suspending harvested bacterial cells in 5% FBS and homogenizing them for 30 seconds with sterile glass beads.

Efficacy testing was carried out using the protocol described above. All species of mycobacteria were recovered on 7H11 agar; plates were incubated at 37°C for 28 days for cultivation of *M. tuberculosis* and *M. avium-intracellulare*, and for 21 days for cultivation of all other *Mycobacteria* spp..

3.11 Efficacy of disinfectants against hepatitis A virus

Efficacy tests were carried out using the method

described above with a mixed suspension of hepatitis A virus, the poliovirus, *S. aureus*, *M. bovis* BCG and the bacterial and fungal spores. The mixed test suspension was prepared by resuspending the bacteria and spores into a 1:1 mixture of poliovirus:hepatitis A virus stock containing 5% FBS but no antibiotics.

Recovery of the viruses from the mixture was achieved by passing the eluate through a syringe filter as described previously. Further separation and quantitation of hepatitis A virus in the filtrate was achieved using a neutralizing monoclonal antibody (Mab) (PP12 1A42A1) specific for the poliovirus serotype used in this study, which was obtained from Dr. P. Payment, Institut Armand Frappier, Laval, Quebec. Neutralization assays were performed to determine the optimal neutralizing dilution of the Mab for the poliovirus and any crossreactivity with hepatitis A virus. The Mab was diluted 1:1000 in EBSS and incubated with the filtrate for 30 min at 37°C to neutralize the poliovirus. Plaque assays for quantitation of HAV were carried out as described previously for the poliovirus with an adsorption time of 90 min. Plates were overlaid with EMEM containing 2% FBS, 0.75% agarose and 26 mM magnesium chloride (BDH) and incubated for 8 days at 37°C.

4. RESULTS

4.1 Combined efficacy test

Table 2 outlines the results of preliminary studies on the development of the combined test to determine any influence the mixture may have on the titer of the five organisms, to determine any loss of titer during drying, and to determine the efficiency of recovery from the carrier. All microorganisms in the mixture retained a titer suitable for demonstrating $\log_{10}$ reductions. The reductions observed between the titer of the individual organisms and the titer of each in the mixture may be due to physical loss during the preparation of the mixed suspension or physical interference during enumeration. The five organisms did not show any significant loss during the drying of the mixed inoculum. High titers were recovered from the carrier indicating that the elution procedure was efficient in recovering the microorganisms.

Sabouraud Dextrose agar and 7H11 agar without griseofulvin did not recover higher numbers of the fungus and mycobacteria, respectively, in comparison to Mycobiotic agar and 7H11 agar with griseofulvin. The use of selective recovery media containing antibiotics does not interfere with the growth of stressed or injured organisms that may have been sublethally damaged by the disinfectant.

Scanning electron micrographs of each individual

Table 2. Viability titer of the organisms before and after preparation of the mixture and efficiency of inoculum recovery from the carriers.

Organism	Titre of organism/1.0 mL suspension		
	Mean (SD)		
	Individual	Mixed	Mixed & dried
<i>S. aureus</i>	1.4 (0.12) X10 ⁹	1.1 (0.11) X10 ⁹	1.0 (0.10) X10 ⁹
<i>T. mentag.</i>	2.3 (0.09) X10 ⁷	1.1 (0.17) X10 ⁷	1.2 (0.20) X10 ⁷
spores			
<i>M. bovis</i> BCG	6.5 (1.60) X10 ⁷	6.0 (1.36) X10 ⁷	4.5 (1.52) X10 ⁷
<i>B. stear.</i>	4.9 (0.33) X10 ⁷	4.7 (0.50) X10 ⁷	4.4 (0.75) X10 ⁷
spores			
Poliovirus	3.3 (0.21) X10 ⁷	3.3 (0.18) X10 ⁷	2.6 (0.46) X10 ⁷

Note: The titres of organisms indicated in this table were derived from preliminary stock cultures. Stock cultures of higher titre were used for all disinfectant efficacy testing to permit the demonstration of higher log₁₀ reductions in CFU.

organism and the mixed inoculum dried onto glass surfaces are shown in Figures 3a to 3e. The physical interactions between the different components in the mixed inoculum are shown in Fig. 3e. Each microorganism is randomly distributed in the mixture and there is a limited degree of shielding of the smaller bacteria (*S. aureus*) by the larger organisms (bacterial rods, fungal conidia). The fibrous material surrounding the microorganisms may be attributed to the presence of serum in the mixed inoculum.

Fig. 3a. Scanning electron micrograph of *S. aureus* suspended in 5% FBS and dried onto a glass surface at 5000 magnification.

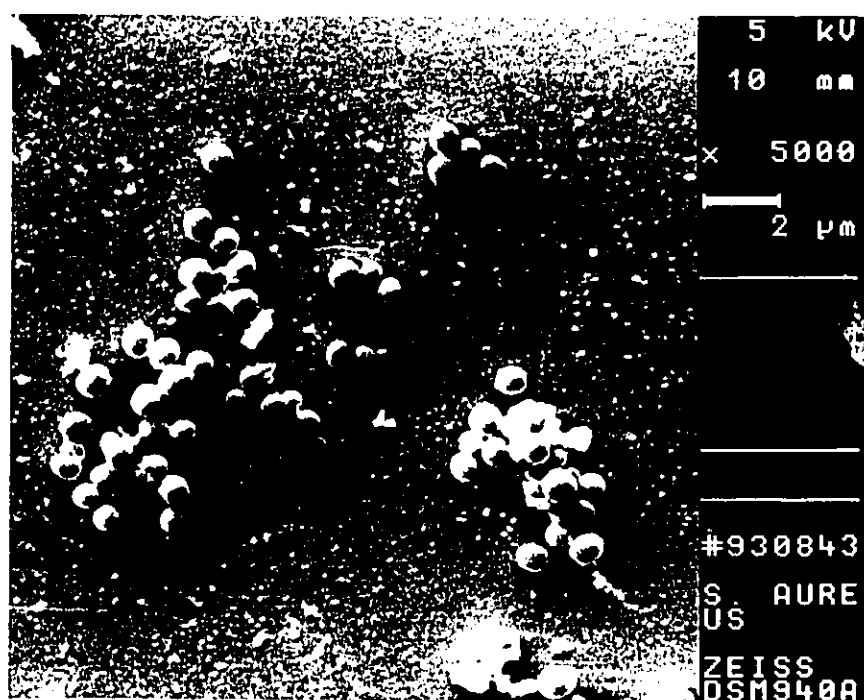


Fig. 3b. Scanning electron micrograph of *M. bovis* BCG
suspended in 5% FBS and dried onto a glass surface at 5000
magnification.

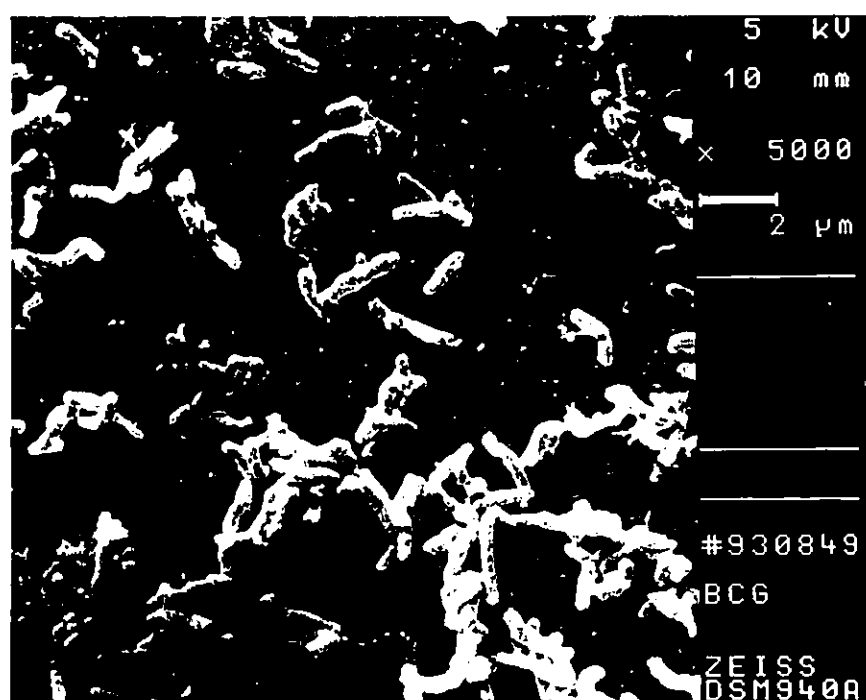


Fig. 3c. Scanning electron micrograph of *T. mentagrophytes* spores suspended in 5% FBS and dried onto a glass surface at 5000 magnification.

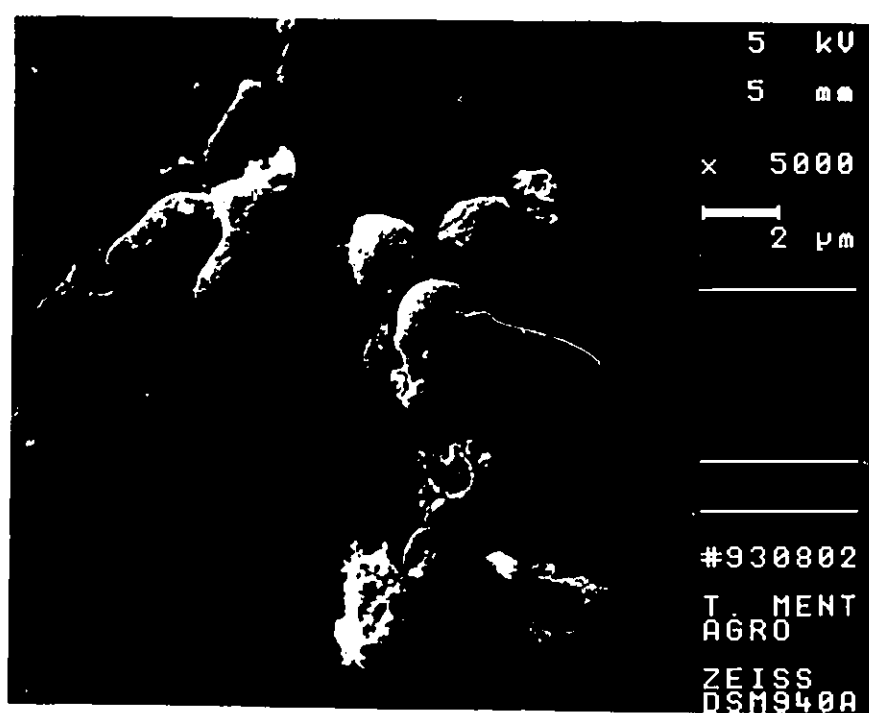


Fig. 3d. Scanning electron micrograph of *B. stearothermophilus* spores suspended in 5% FBS and dried onto a glass surface at 5000 magnification.

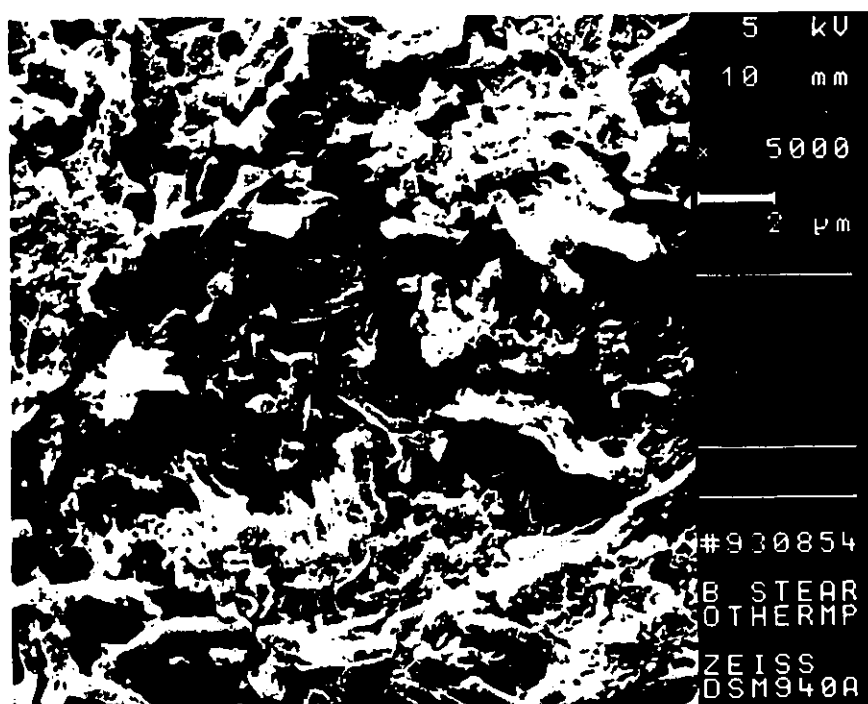
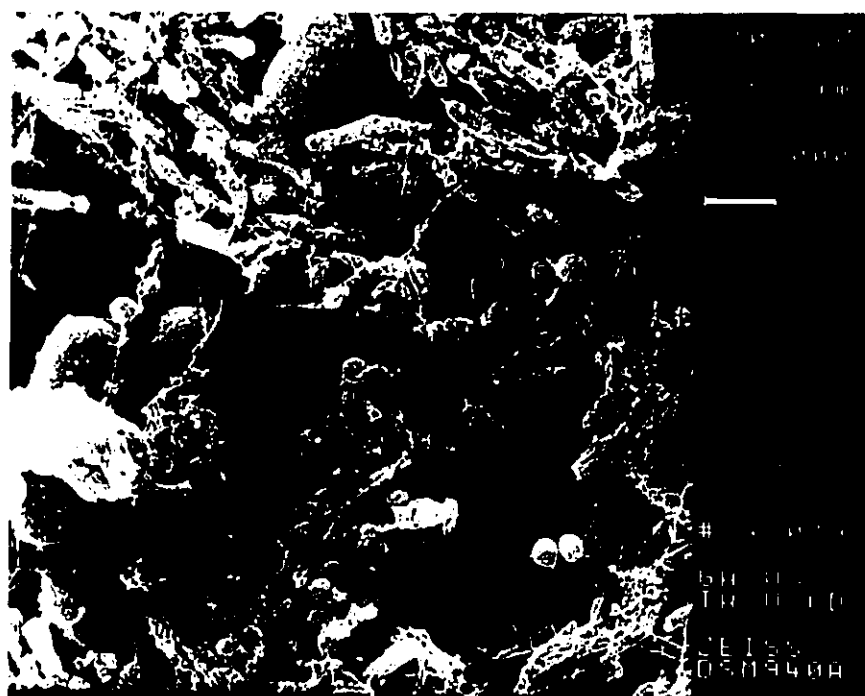


Fig. 3e. Scanning electron micrograph of a mixture of *S. aureus*, *M. bovis* BCG, *T. mentagrophytes* spores and *B. stearothermophilus* spores suspended in 5% FBS and dried onto a glass surface at 5000 magnification.



10000

50000

100000

200000

300000

The results of the efficacy tests with the 11 disinfectants against the five microorganisms alone and in the mixture are presented in Tables 3 and 4, respectively. Three products (0.4% quaternary ammonium and 23% HCl, 0.6% sodium hypochlorite, and 2% alkaline glutaraldehyde) were effective in causing a greater than 3 $\log_{10}$ reduction against all 5 challenge organisms, although longer contact times were required to effectively reduce the number of bacterial spores. Product #7 (0.1% *o*-phenylphenol with 79% ethanol) was effective against all but bacterial spores. Povidone-iodine (1% iodine) and 70% ethanol were similarly ineffective against bacterial spores but also exhibited no activity against the poliovirus. Product #11 (a mixture of peroxygen compounds) exhibited virucidal and bactericidal activity only. Products #3 (1.5% chlorhexidine gluconate), #5 (0.06% quaternary ammonium), #6 (0.03% *o*-phenylphenol + 0.03% *p*-tertiary amylphenol) were effective against *S. aureus* only; $\log_{10}$ reductions obtained against *S. aureus* tested alone were higher than obtained against *S. aureus* in the mixture. Three percent hydrogen peroxide (Product #2) was ineffective against all five of the test organisms even after a contact time of 10 minutes. Figure 4 is a composite of the data for the spectrum of germicidal activity of the 11 disinfectants in relation to contact time.

Table 3. Efficacy of disinfectants tested against each individual microorganism.

<u>Disinfectant Log₁₀ reduction in viability units - Mean (SD)</u>					
(contact time)	<i>S.aureus</i>	<i>M.bovis</i> BCG	<i>T.mentag.</i> spores	<i>B.stear.</i> spores	<i>Polio-</i> virus
1. EtOH					
(1 min)	4.3(0.23)	2.7(0.34)	< 1	< 1	< 1
(10 min)	> 6	> 5	> 5	< 1	1.4(0.30)
2. H ₂ O ₂					
(1 min)	< 1	< 1	< 1	< 1	< 1
(10 min)	1.2(0.40)	< 1	< 1	< 1	1.0
3. Chlorhex					
(1 min)	3.7(0.32)	< 1	< 1	< 1	< 1
(10 min)	> 6	< 1	< 1	< 1	< 1
4. P-I ₂					
(1 min)	> 6	> 5	> 5	< 1	< 1
(10 min)	ND	ND	ND	< 1	1.1(0.20)
5. Quat					
(1 min)	4.2(0.36)	< 1	< 1	< 1	< 1
(10 min)	> 6	< 1	< 1	< 1	< 1
6. Phenol					
(1 min)	3.2(0.13)	< 1	< 1	< 1	< 1
(10 min)	> 6	< 1	< 1	< 1	< 1

Table 3. cont'd

7. Phenol-EtOH					
(1 min)	> 6	> 5	> 5	< 1	3.5(0.21)
(10 min)	ND	ND	ND	< 1	> 4
8. Quat-HCl					
(1 min)	> 6	> 5	> 5	2.8(0.23)	> 4
(10 min)	ND	ND	ND	> 4.8	ND
9. NaOCl					
(1 min)	> 6	> 5	> 5	< 1	> 4
(30 min)	ND	ND	ND	2.5(0.31)	ND
(3 hrs)	ND	ND	ND	> 4.5	ND
10. Glutarald					
(1 min)	> 6	1.6(0.13)	> 5	< 1	> 4
(10 min)	ND	> 5	ND	< 1	ND
(30 min)	ND	ND	ND	3.5(0.41)	ND
(3 hrs)	ND	ND	ND	> 4.5	ND
11. Peroxygen					
(1 min)	> 6	< 1	< 1	< 1	> 4
(10 min)	ND	< 1	< 1	< 1	ND

ND=not done

Table 4. Efficacy of disinfectants tested against a mixture of the five microorganisms.

<u>Disinfectant Log₁₀ reduction in viability units - Mean (SD)</u>					
(contact time)	<i>S.aureus</i>	<i>M.bovis</i> BCG	<i>T.mentag.</i> spores	<i>B.stear.</i> spores	<i>Polio-</i> virus
1. EtOH					
(1 min)	3.9(0.45)	ND	ND	ND	ND
(10 min)	> 6	> 5	> 5	ND	ND
3. Chlorhex					
(1 min)	3.4(0.23)	ND	ND	ND	ND
(10 min)	3.6(0.37)	ND	ND	ND	ND
4. P-I ₂					
(1 min)	> 6	> 5	> 5	ND	ND
5. Quat					
(1 min)	2.8(0.32)	ND	ND	ND	ND
(10 min)	3.4(0.12)	ND	ND	ND	ND
6. Phenol					
(1 min)	2.4(0.56)	ND	ND	ND	ND
(10 min)	3.9(0.47)	ND	ND	ND	ND
7. Phenol-EtOH					
(1 min)	5.0(0.35)	3.0(0.21)	3.4(0.50)	ND	2.2(0.14)
(10 min)	ND	> 5	> 5	ND	> 4

Table 4. cont'd

8. Quat-HCl

(1 min)	> 6	> 5	> 5	ND	> 4
(10 min)	ND	ND	ND	> 4.5	ND

9. NaOCl

(1 min)	> 6	> 5	> 5	ND	> 4
(30 min)	ND	ND	ND	2.6 (0.35)	ND
(3 hrs)	ND	ND	ND	> 4.5	ND

10. Glutarald

(1 min)	> 6	ND	> 5	ND	> 4
(10 min)	ND	> 5	ND	ND	ND
(30 min)	ND	ND	ND	3.9 (0.42)	ND
(3 hrs)	ND	ND	ND	> 4.5	ND

11. Peroxygen

(1 min)	> 6	ND	ND	ND	> 4
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ND=not done

Fig. 4. A composite of the results from germicidal tests showing the spectrum of activity of the disinfectants in relation to contact time. The criterion of efficacy in this study was a $> 3 \log_{10}$ reduction in the viability of the test organism. If a disinfectant did not meet this criterion with an organism tested individually it was not tested against the mixture. The contact time was extended, e.g., from 1 minute to 10 minutes, only when a disinfectant failed to meet this criterion.

4.2 Effect of organic load

Table 5 gives the results of testing disinfectants against a mixed challenge suspended in tryptose phosphate broth. All products exhibited a similar efficacy pattern against each organism in the mixture as was observed when 5% FBS was used as the organic load (Table 3).

4.3 Effect of diluent for disinfectant

The use of standard hard water (200 ppm CaCO_3) as a diluent for disinfectants adversely affected the efficacy of two out of the six products tested (Table 6). Four disinfectants, namely, 70% ethanol (#1), 1.5% chlorhexidine gluconate (#3), and 0.6% sodium hypochlorite (#9) produced similar log reductions when diluted in either standard hard water or tap water. A mixture of peroxygens (#11) diluted in hard water, although still efficacious, produced a lower reduction in CFU of *S. aureus* than was obtained when tap water was used as the diluent. Product #6, a phenolic, was ineffective against *S. aureus* when diluted in hard water. This is in contrast to its observed effectiveness against this organism when tap water was used as the diluent.

Table 5. Efficacy of disinfectants tested against a mixture of the five microorganisms suspended in 7.6 g/L Tryptose Phosphate Broth.

<u>Disinfectant Log₁₀ reduction in viability units - Mean (SD)</u>					
(contact time)	<i>S.aureus</i>	<i>M.bovis</i> BCG	<i>T.mentag.</i> spores	<i>B.stear.</i> spores	<i>Polio-</i> virus
1. EtOH					
(10 min)	> 6	> 5	> 5	ND	< 1
3. Chlorhex					
(1 min)	> 6	> 5	> 5	ND	< 1
5. Quat					
(10 min)	3.5 (0.21)	< 1	< 1	ND	< 1
6. Phenol					
(10 min)	3.8 (0.35)	< 1	< 1	ND	< 1
7. Phenol-EtOH					
(1 min)	5.6 (0.60)	3.2 (0.21)	2.1 (0.16)	ND	1.1 (0.23)
9. NaOCl					
(1 min)	> 6	> 5	> 5	ND	> 4
(30 min)	ND	ND	ND	3.9 (0.57)	ND
10. Glutarald					
(1 min)	> 6	ND	> 5	ND	> 4
(10 min)	ND	> 5	ND	ND	ND
(30 min)	ND	ND	ND	> 4	ND
11. Peroxygen					
(1 min)	> 6	< 1	< 1	ND	> 4
(10 min)	ND	< 1	< 1	< 1	ND

ND=Not done

Table 6. Efficacy of disinfectants diluted in hard water (200 ppm CaCO₃) against a mixed microbial challenge in 5% FBS.

<u>Disinfectant Log₁₀ reduction in viability units - Mean (SD)</u>					
(contact time)	<i>S.aureus</i>	<i>M.bovis</i> BCG	<i>T.mentag.</i> spores	<i>B.stear.</i> spores	Polio- virus
1. EtOH					
(10 min)	> 6	> 5	> 5	ND	< 1
3. Chlorhex					
(10 min)	3.8 (0.21)	ND	ND	ND	ND
5. Quat					
(1 min)	2.9 (0.10)	ND	ND	ND	ND
(10 min)	3.6 (0.38)	ND	ND	ND	ND
6. Phenol					
(1 min)	< 1	ND	ND	ND	ND
(10 min)	1.2 (0.34)	ND	ND	ND	ND
9. NaOCl					
(1 min)	> 6	> 5	> 5	ND	> 4
(30 min)	ND	ND	ND	2.7 (0.61)	ND
(3 hrs)	ND	ND	ND	> 4	ND
11. Peroxygen					
(1 min)	4.0 (0.03)	ND	ND	ND	ND

ND=Not done

4.4 Comparative resistance of bacterial spores

The comparative resistance of *B. stearothermophilus* and *B. subtilis* spores to 2% alkaline glutaraldehyde is shown in Figure 5. Both spore suspensions exhibited a similar resistance pattern with no significant loss after 10 minutes of contact, and a 3 to 4 $\log_{10}$ reduction in numbers of viable spores occurring after 30 minutes of contact with the glutaraldehyde. Both spore suspensions resisted 2.5N HCl after 2 minutes of contact.

4.5 Comparative resistance of vegetative bacteria

The results of tests comparing the efficacy of the three bacteria species recommended for bactericidal testing are presented in Table 7. All three bacteria were similarly sensitive to six of the eleven disinfectants tested. *P. aeruginosa* was slightly more resistant than the other two bacteria to Product #3 (1.5% chlorhexidine gluconate), #5 (0.06% quaternary ammonium), #6 (0.03% *o*-phenylphenol + 0.03% *p*-tertiary amylphenol) and #11 (a mixture of peroxygen compounds). Conversely, *S. choleraesuis* was found to be the most sensitive to Product #2, #3, #5 and #6.

Fig. 5. Comparative resistance of *Bacillus*
stearothermophilus and *Bacillus subtilis* spores to 2%
alkaline glutaraldehyde.

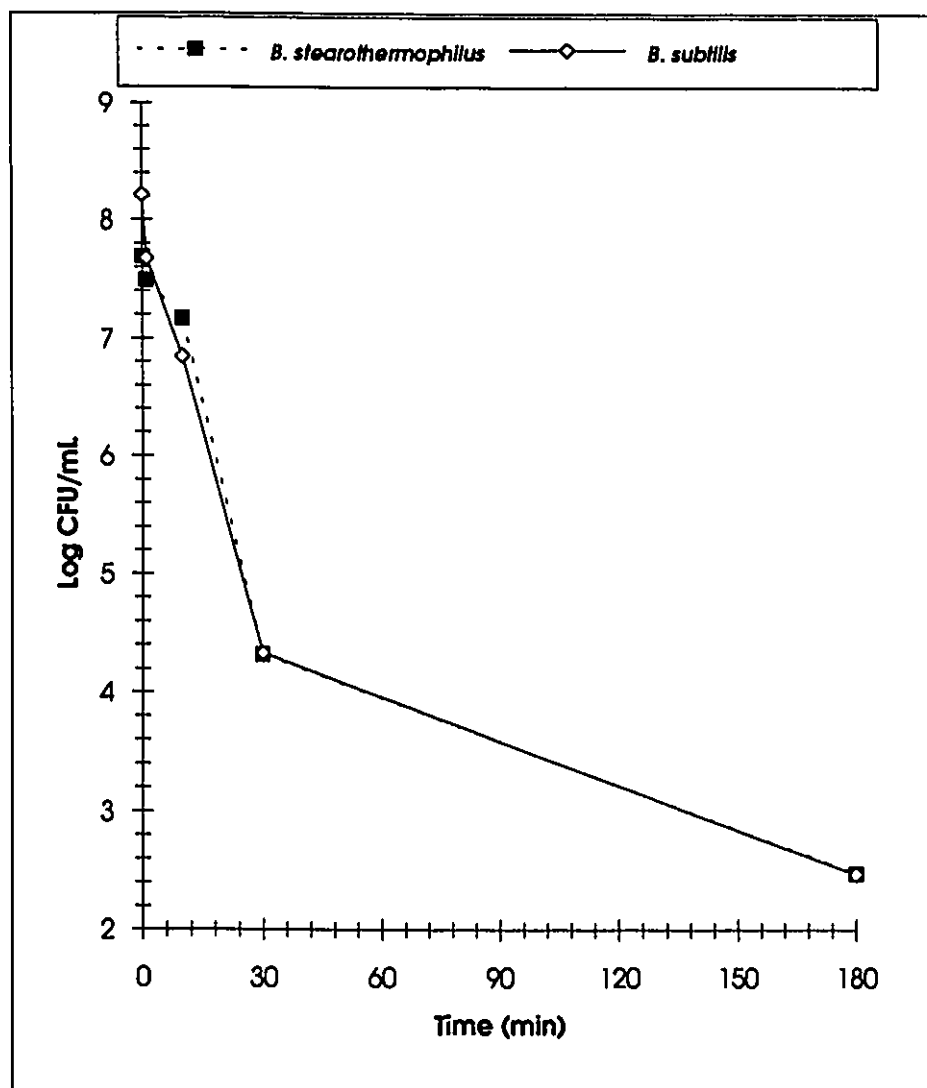


Table 7. Comparative disinfectant resistance patterns of vegetative bacteria recommended by the AOAC for evaluating bactericidal efficacy claims.

Disinfectant (contact time)	<u>Log₁₀ reduction in CFU</u>		
	<i>S.aureus</i>	<i>P. aeruginosa</i>	<i>S. choleraesuis</i>
1. EtOH			
(1 min)	4.3 (0.24)	4.7 (0.35)	4.5 (0.47)
2. H ₂ O ₂			
(1 min)	< 1	<1	> 6
(10 min)	< 1	1.2 (0.32)	> 6
3. Chlorhex			
(1 min)	3.2 (0.13)	2.7 (0.57)	3.8 (0.39)
4. P-I ₂			
(1 min)	> 6	> 6	> 6
5. Quat			
(1 min)	3.9 (0.81)	2.3 (0.35)	> 6
6. Phenol			
(1 min)	3.1 (0.42)	2.1 (0.22)	3.4 (0.41)
7. Phenol-EtOH			
(1 min)	> 6	> 6	> 6
8. Quat-HCl			
(1 min)	> 6	> 6	> 6
9. NaOCl			
(1 min)	> 6	> 6	> 6
10. Glutarald			
(1 min)	> 6	> 6	> 6
11. Peroxygen			
(1 min)	> 6	3.8 (0.76)	> 6

4.6 Comparative resistance of mycobacteria

As outlined in Table 8, all *Mycobacteria* spp. tested were sensitive to 1% iodine (#4) after 1 minute and 70% ethanol (#1) after 10 minutes contact. MDR-TB was more resistant than *M. bovis* to 0.1% o-phenylphenol with 79% ethanol (#7) and 0.6% sodium hypochlorite (#9). *M. avium-intracellulare* was similarly more resistant than *M. bovis* to these two disinfectants and to 2% alkaline glutaraldehyde (#10). *M. terrae* exhibited a resistance pattern similar to MDR-TB for most formulations tested while *M. gordonae* was slightly more susceptible to disinfection than the two clinical isolates of mycobacteria tested. A mixture of peroxygens (#11) and a mixed phenolic compound (#12) were ineffective in all tests.

4.7 Efficacy of disinfectants against hepatitis A virus

The results of the efficacy of chemical disinfectants against hepatitis A virus in the mixed inoculum containing 5% FBS are presented in Table 9. In comparison to the poliovirus, HAV was similarly resistant to five of the products tested (#1, #2, #3, #5 and #6) and similarly sensitive to three products (#8, #9 and #10). A mixture of peroxygens (#11) required a longer contact time to effectively inactivate HAV, while 0.1% o-phenylphenol with 79% ethanol (#7) was ineffective against this virus even after 10 minutes of contact. Unlike the resistance of the

Table 8. Comparative efficacy of chemical disinfectants against *Mycobacteria* spp. in 5% FBS.

<u>Disinfectant Log₁₀ reduction in viability units - Mean (SD)</u>					
(contact time)	<i>M. bovis</i>	MDR-TB	<i>M. avium-</i> <i>intrac.</i>	<i>M. terrae</i>	<i>M. gord.</i>
1. EtOH					
(10 min)	> 5	> 5	> 5	> 5	> 5
4. P-I ₂					
(1 min)	> 5	> 5	> 5	> 5	> 5
7. Phenol-EtOH					
(1 min)	> 5	3.7 (0.18)	4.3 (0.48)	4.4 (0.18)	4.0 (0.25)
(10 min)	ND	4.3 (0.17)	4.2 (0.31)	> 5	> 5
9. NaOCl					
(1 min)	> 5	< 1	< 1	4.3 (0.14)	> 5
(10 min)	ND	> 5	> 5	> 5	ND
10. Glutarald					
(1 min)	1.64	< 1	< 1	2.8 (0.67)	2.8 (0.26)
(10 min)	> 5	> 5	3.9 (0.15)	> 5	> 5
(20 min)	ND	ND	> 5	ND	ND
11. Peroxygen					
(20 min)	< 1	< 1	< 1	< 1	1.9 (0.05)
12. M-Phenolic					
(10 min)	2.4 (0.39)	< 1	< 1	2.3 (0.89)	1.5 (0.81)

ND=Not done

Table 9. Comparative efficacy of chemical disinfectants tested against hepatitis A virus and poliovirus in a mixed inoculum containing 5% FBS.

Disinfectant (contact time)	<u>Log₁₀ reduction in PFU</u>	
	hepatitis A virus	poliovirus
1. EtOH (10 min)	< 1	< 1
2. H ₂ O ₂ (10 min)	< 1	< 1
3. Chlorhex (10 min)	< 1	< 1
4. P-I ₂ (1 min)	> 4	< 1
5. Quat (10 min)	< 1	< 1
6. Phenol (10 min)	< 1	< 1
7. Phenol-EtOH (1 min)	< 1	2.2 (0.14)
	(10 min)	1.9 (0.30)
8. Quat-HCl (1 min)	> 4	> 4
9. NaOCl (1 min)	> 4	> 4
10. Glutarald (1 min)	> 4	> 4
11. Peroxygen (1 min)	< 1	> 4
	(10 min)	> 4
		ND

ND=Not done

poliovirus to 1% iodine (#4), HAV was rapidly inactivated by this product.

5. DISCUSSION

The rationale for a combined disinfectant efficacy test becomes clear when one examines the currently used methods for evaluating germicides and the efficacy claims based on them. For the disinfectant user, product selection becomes extremely difficult when faced with a bewildering array of data obtained under an equally diverse set of conditions, many of which are non-quantitative. Moreover, in the field, two specific conditions make a combined test a more realistic challenge. Firstly, one often does not know what the microbial targets are and, secondly, microorganisms rarely occur as a single species on materials to be disinfected. Each of these situations suggests the need for product(s) with a certain spectrum of efficacy. In view of this, the development of a combined test for disinfectants and the results presented above show that it is both feasible and practical. As can be seen from Fig. 1, the test protocol itself is relatively simple to perform and fully quantitative.

In the test, the recovery methods allowed for both separation and quantitation of the reduction in numbers of the test organisms. All microorganisms in the mixture retained a titre suitable for the test. Although there was no interference between the organisms in the mixture, various components of the mixture may afford each other

physical protection shielding them from the action of disinfectants. This may present a more stringent challenge to the disinfectant than would exist when using a single test organism. As observed by scanning electron microscopy (Figures 3a to 3e), the individual components of the mixture were randomly distributed and there was a limited degree of protection of the smaller bacteria, particularly *S. aureus*, by the larger organisms in the mixture (e.g. bacterial rods, fungal conidia). Further, the efficacy testing results do not indicate that the mixed inoculum presents a more stringent challenge as the combined inoculum affected the efficacy of only those products that were also marginal in dealing with a single organism.

The fundamental design of this test eliminates the inherent variability of currently accepted AOAC test protocols, and, as such, is superior to them. By placing the disinfectant solution directly onto the contaminated area of the carrier and subsequently immersing the entire carrier into an eluant, the complete inoculum can be recovered. In the AOAC tests, the carrier is dipped into the test suspension and allowed to drain, resulting in an unknown initial titre (Beloian 1990). Furthermore, carriers that are subsequently dipped into the disinfectant prevent a distinction between inactivation of the test organism and that which has been washed from the carrier into the disinfectant. The reductions in numbers obtained with such

tests may not be a true reflection of disinfectant activity.

Disinfectant activity was arrested by a 1:50 dilution in EBSS. The 50-fold dilution was effective in rendering the disinfectant ineffective. The EBSS used as the diluent may have had an additional neutralizing effect on the disinfectants. The buffering capacity of EBSS may also have been responsible for rendering the disinfectants non-cytotoxic for cell cultures and neutralizing acid containing disinfectants.

The use of glass cups as carriers has several advantages over certain other carrier designs. In addition to its nonporosity (nonporous surfaces are better models as it is difficult to elute test organisms from porous surfaces [Sattar et al. 1986]), glass surfaces have been found to be very smooth when examined by scanning electron microscopy (Cole et al. 1987A). By contrast, stainless steel and porcelain surfaces, which are often used as carrier materials, showed severe grooves and pitting (Cole et al. 1987A; Springthorpe and Sattar 1990). Microorganisms residing in these grooves may be protected from disinfectant exposure or physical removal from the carrier surface. Glass penicylinders have recently replaced the stainless steel carriers traditionally used in the AOAC standard use-dilution method (Rubino et al. 1992).

The use of a cup in place of a ring, penicylinder or a

flat surface allows for an increase in the volume ratio of disinfectant to inoculum. While the importance of this ratio has not been fully studied, the inoculum volume is generally much smaller than that of the disinfectant. Finally, glass cups are of a simple design allowing for consistent, reproducible results in a standard test. Disinfectants which pass under such conditions could then be tested using more intricate carriers which may reflect more closely the objects to be disinfected in the field e.g., endoscopes and bronchoscopes, .

The incorporation of an organic load in a standardized test is important in order to simulate natural challenges that a disinfectant is presented with under in-use conditions. Organic material can occlude organisms from or neutralize the action of disinfectants. As observed under scanning electron microscopy, the inclusion of serum results in the presence a "fibrous" proteinaceous coating of the microorganisms in the test mixture. Serum was included in this study because it is recommended as an organic load in many germicidal test protocols (ASTM 1989; CGSB 1991). It is however, expensive, inhibitory for certain types of microorganisms and subject to batch-to-batch variations. Tryptose phosphate broth is free of such limitations and was found to present a comparable challenge to disinfectants when used at approximately the same protein concentrations. This protein digest is harmless to

microorganisms and has a better defined and more reproducible composition. The results are promising and further work is required to determine the suitability of this product as a universally applicable organic load.

Tap water is routinely used in the field to prepare in-use solutions of disinfectants. For this reason, it was used in our study for all those products which required dilution before testing. Recognizing that the chemical composition of tap water varies geographically and temporally the use of water of a defined hardness was also tested. Water of a standard hardness of 200 ppm calcium carbonate was selected for use as this level of hardness is now being recommended in several standard test methods (CGSB 1991). Studies evaluating the effect of water hardness on the activity of disinfectants are limited. In one such study, raising the hardness from 0 to 400 ppm did not have an inhibitory action on the antibacterial activity of hypochlorite solutions, a finding in agreement with the results of this study (Dychdala 1991). Other investigators did however observe a negative influence of hard water on the activity of phenolic disinfectants against a variety of test bacteria (Cremieux 1986; Economou-Stamatelopoulou and Papavssiliou 1988). One of the phenolic products evaluated in this study was similarly sensitive to the presence of hard water.

Although five major classes of microorganisms were

included in the mixed inoculum in this study, the basic test can be adapted for use with any combination of two or more microorganisms including species other than those recommended for disinfectant testing. However, with the exception of bacterial spores, this study used currently accepted strains (Beloian 1990). *B. stearothermophilus* spores are used for evaluating heat sterilization processes and their requirement for an elevated growth temperature provided a simple means of separating and quantitating the spores from the mixture. The comparative resistance studies have shown that these spores are at least as resistant as those of *B. subtilis*, the currently recommended species for evaluating sporicidal claims.

S. aureus was included in the mixture as it is one of three bacterial species recommended for evaluating bactericidal efficacy claims (Beloian 1990). In comparative testing, this organism exhibited an intermediate resistance pattern between that of the more susceptible *S. choleraesuis* and more resistant *P. aeruginosa*. *Pseudomonas* spp. are well known for their ability to contaminate quaternary ammonium compounds, povidone-iodine solutions, liquid soaps and detergent creams, contributing to their role as an important source of nosocomial infections (Russell 1986). These special problems of resistance posed by pseudomonads appear to be associated with the chemical composition of the outer cell membrane and glycocalyx-like

structure which could shield the bacteria from disinfectants (Gelinas et al. 1984; Russell et al. 1986). Bactericidal disinfectants intended for general use are required to demonstrate efficacy against *S. choleraesuis* and *S. aureus*; products intended for use in hospitals must also demonstrate effectiveness against *P. aeruginosa* (GAO 1990). The combined test protocol is flexible and substituting *P. aeruginosa* for *S. aureus* to evaluate hospital bactericidal claims can be achieved with slight modifications of the recovery media.

The use of surrogate organisms for standardized testing is advantageous because it is neither practical to test large numbers of strains nor those that are highly pathogenic. Model strains should be carefully selected to ensure that they represent the group. In response to concerns that *M. bovis* BCG may be more sensitive to many germicides than clinically relevant species of mycobacteria, a concern supported by the findings of this study, several alternate surrogates have been suggested. *Mycobacterium smegmatis*, although recommended for presumptive testing by the AOAC because it is a relatively fast growing and non-pathogenic organism, is not a good model because of its proven sensitivity to disinfectants (Best et al. 1988; Collins 1986; Van Klingeren and Pullen 1987). *M. goodii* was found to be more resistant to disinfection with 2% glutaraldehyde than BCG and the

tubercle bacilli (Collins 1986). In a second study, *M. gordonae* and *M. tuberculosis* were similarly eliminated from contaminated bronchoscopes. However, the investigators utilized the attenuated H37RA strain of *M. tuberculosis* whose comparative resistance to the more virulent wild-type strains is not known (Davis et al. 1984). The results presented here do not support the use of *M. gordonae* as a suitable model as it was found to be more sensitive than both *M. tuberculosis* and *M. avium-intracellulare*.

M. terrae has previously been shown to be similar to *M. tuberculosis* in disinfectant resistance and exhibited a resistance pattern similar to MDR-TB for most formulations tested in this study (Gundermann 1987; Sipus 1986; Sonntag and Hingst 1985; Van Klingeren 1987). Sonntag and Hingst based their selection of *M. terrae* for comparative disinfectant testing on the results of serological differentiation methods in which *M. terrae* displayed high rates of common antigens with *M. tuberculosis* (Sonntag and Hingst 1985). *M. terrae* is a relatively fast growing organism, is non-pathogenic and may be sufficiently reliable to assess the tuberculocidal activity of disinfectants. The use of *M. terrae* has been incorporated into the latest revision of testing required by the regulatory agency in Germany (Ascenzi 1991).

The relative resistance to disinfectants of MDR-TB has not been previously tested. A recent clinical isolate of *M.*

tuberculosis resistant to four first-line drugs (rifampin, isoniazid, streptomycin and ethambutol) and *M. avium-intracellulare* recently isolated from an AIDS patient were selected for evaluation because of their potential clinical significance. Both of these organisms were slightly more resistant than the currently used model for tuberculocidal testing for some of the products evaluated. In accordance with other investigators who evaluated the sensitivity of the MAIS group, *M. avium-intracellulare* presented a higher resistance to 2% alkaline glutaraldehyde than did *M. tuberculosis* (Collins 1986; Hanson et al. 1988; Russell et al. 1992; Slosarek et al. 1990). While additional studies are necessary with more mycobacterial species and a wider range of agents, these findings raise concerns about the effectiveness of currently used disinfection practices for controlling the spread of tuberculosis.

Although there are no currently accepted AOAC virucidal tests, the use of a poliovirus as a surrogate organism for evaluating virucidal claims has been recommended in the protocols of both the American Society for Testing and Materials and the Canadian General Standards Board (ASTM 1989; CGSB 1991). Nonenveloped viruses represent a better model than the more sensitive enveloped viruses. However, they are also known to differ from one another in their sensitivity to many disinfectants (Sattar et al. 1989; Springthorpe and Sattar 1990). For

this reason, the comparative resistance of a second nonenveloped virus, hepatitis A virus, chosen for its demonstrated resistance to disinfectants, was tested (Mbithi et al. 1990). Differences in resistance were observed with these two viruses whereby the poliovirus was more readily inactivated by a mixture of peroxygens and a disinfectant containing o-phenylphenol and ethanol, and was more resistant to 1% iodine when compared to HAV. This inconsistency of activity against different nonenveloped viruses presents a problem of selection of an acceptable model virus and demonstrates the need for a further understanding of the mechanisms of viral inactivation by disinfectants.

The data generated in the combined efficacy study clearly showed *Staphylococcus aureus* to be the most sensitive to the disinfectants among the five classes of microorganisms tested (Fig. 4). This is in agreement with the general belief of the descending order of resistance of various classes of microorganisms to be as follows: bacterial spores, mycobacteria and nonlipid viruses, fungal conidia, vegetative bacteria and lipid-containing viruses (Favero and Bond 1991; Russell et al. 1986). However, the relative sensitivity/resistance of the fungus, the mycobacterium and the viruses varied depending on the nature of the disinfectant under test and the contact time. The testing of many other disinfectants will make it

possible to determine whether such differences relate to the class of disinfectants or individual formulations. Extrapolation of the results of this study to products with other formulations would not be possible without further work.

As would be expected, the bacterial spores proved to be the most resistant and, depending on the product, contact times of 10 minutes to 3 hours were needed to show a 3.53 to $>4.8 \log_{10}$ reduction in the viability count of the spores. The scanning electron micrograph of the spore preparation (Fig. 3d) reveals the presence of numerous vegetative bacteria. Although heat inactivated, their presence provides for an increased organic load than would be present using a "clean" spore suspension. However, a spore suspension prepared by removing vegetative cells may not simulate natural conditions where spores and vegetative cells undergoing various stages of sporulation are likely to be encountered (Russell 1990). The AOAC sporicidal test does not require the removal of dead vegetative cells from the spore preparation used for contaminating carriers (Beloin 1990). The practicality and value in using a spore suspension that has been separated from the vegetative bacteria requires further investigation.

An increase in the input titer of the bacterial spores will be required to show a 6 $\log_{10}$ or greater reduction in the spore count to declare a product as sporicidal. Whereas

bacterial spores were included in the mixture to demonstrate that the basic protocol was suitable for working with them as well, the longer contact times and the generally higher levels of $\log_{10}$ reductions required for sporicidal activity may make it preferable to test them separately.

Two percent glutaraldehyde is highly effective even in the presence of organic material and is widely used as a high-level disinfectant for medical equipment such as flexible fiberoptic endoscopes (Gelinas and Goulet 1983; Gelinas et al. 1984). Its activity demonstrated here supports previous efficacy data and confirms its classification as a high-level disinfectant (Best et al. 1990B; Cole et al. 1990; Hanson et al. 1991; Hanson et al. 1992; Mbithi et al. 1990; Power and Russell 1990; Russell 1990; Rutala and Cole 1987; Sattar et al. 1989; Tyler et al. 1990; Weller et al. 1988). Despite this broad range of activity, reports of adverse health effects, including allergic dermatitis, mucous membrane irritation and occupational asthma preclude its use as a general disinfectant in many areas (Burge 1989; Norback 1988).

Another problem encountered in practice is a reduction of the in-use concentration of glutaraldehyde from aging and dilution during reprocessing of medical instruments. Glutaraldehyde is usually supplied as a 2% solution along with a buffer that must be added to the solution to

"activate" it and once activated, solutions are only stable for a limited time. Recent studies have demonstrated dramatic falls in glutaraldehyde levels, from a starting concentration of over 2% to less than 1%, during the manufacturers' post-activation life of 14 or 28 days (Babb et al. 1992; Leong et al. 1991; Mbithi et al. 1993; Whyman et al. 1991). Further investigations determining the minimum effective concentration of glutaraldehyde will be valuable for setting guidelines and implementing a policy on the re-use of glutaraldehyde solutions.

Sodium hypochlorite (0.6%) and a quaternary ammonium-based formulation containing 23% HCl could also be classified as having high-level activity based on these investigations. However, both of these products are corrosive. The highly acidic quaternary ammonium formulation (Product #8), a toilet bowl cleaner, could only be used on resistant surfaces and its use is further limited for safety reasons. Its germicidal activity may be primarily due to its low pH. Sodium hypochlorite solution is stabilized with sodium hydroxide and is highly alkaline (pH 11.2); its activity is extremely dependent on the level of available chlorine and presence of organic material and varying spectrums of activity are reported in the literature (Best et al. 1990B; Coates et al. 1992; Mbithi et al. 1990; Russell 1990; Rutala et al. 1991; Sattar et al. 1991; Tanner 1989; Tyler et al. 1990).

Seventy percent ethanol was only effective against the vegetative bacteria, the fungus and the mycobacteria after a contact of 10 minutes; no activity was seen against either of the viruses tested even after 10 minutes. This is in agreement with several virucidal studies where the activity of ethanol was demonstrated against enveloped viruses but it failed to inactivate non-enveloped viruses. Previous evaluations of ethanol showed variable results against the poliovirus (Mbithi et al. 1990; Sattar et al. 1989; Tyler et al. 1990). The tuberculocidal activity of ethanol appears to be time dependent; earlier observations demonstrated effectiveness after 15 min but not after 1 min (Best et al. 1990B; Lind et al. 1986). However, extended contact times with environmental surfaces are difficult to achieve unless the items are immersed. Ethanol has little or no sporocidal activity (Russell 1990).

Povidone-iodine solutions are highly stable, provide a sustained release of the effective iodine, and are more active in the presence of organic material than equivalent chlorine-based products. The bactericidal, tuberculocidal and fungicidal activity demonstrated here (1% available iodine) is in accordance with previous efficacy data (Best et al. 1990B; McLure and Gordon 1992; Schubert 1985; Tanner 1989). The sporicidal activity of iodine-releasing agents has been demonstrated, however they are much less active against bacterial spores than are chlorine-containing

compounds (Russell 1990; Williams and Russell 1991). The virucidal activity of povidone-iodine solutions is limited; it failed to inactivate the poliovirus in this test as well as in earlier studies with polio- and other non-enveloped viruses (Sattar et al. 1989; Tyler 1990). Surprisingly, hepatitis A virus was rapidly inactivated. This is in contrast to the ineffectiveness of three iodine-based compounds when tested against fecally suspended HAV (Mbithi et al. 1990).

Although there is a lack of published efficacy data for many of the numerous phenolic products on the market, the spectrum of activity of phenolics has been reported to be quite varied, depending on the specific product formulation tested (Best et al. 1990B; Hegna 1977; Mbithi et al. 1991; Rutala and Cole 1987; Rutala et al. 1991; Sattar et al. 1989; Tanner 1989). As discussed earlier, some phenolics are sensitive to interfering substances present in hard water; this sensitivity may have contributed to the array of efficacy data available, depending on the diluent used in the test methodology. One of the phenolic products tested here was unsatisfactory as an intermediate level disinfectant, exhibiting marginal activity against the vegetative bacteria only. Of particular concern is the lack of activity of a phenolic product (Product #12), specifically marketed for tuberculosis laboratories, when tested against five

different species of mycobacteria. O-phenylphenol in combination with ethanol demonstrated good bactericidal, tuberculocidal, fungicidal and some virucidal activity. Even at high concentrations, phenolic disinfectants are reported to be poorly sporicidal (Russell 1990).

A mixture of peroxygens, of which potassium monopersulphate is the primary microbiocidal ingredient, also exhibited limited intermediate-level activity; it was ineffective against the bacterial spores, all *Mycobacteria* spp. tested and the fungal spores. This formulation is marketed as a possible alternative for surface disinfection but efficacy data is limited to a few studies. When tested as a possible alternative disinfectant for disinfecting spills and for use in laboratory discard jars, it was found to be effective against vegetative bacteria in the presence of defibrinated horse blood (Coates and Wilson 1992; Coates et al. 1992); marginal effectiveness was demonstrated against polioviruses (Tyler et al. 1990). However, mycobactericidal activity of this peroxygen-based disinfectant was not demonstrated either in this test or in a study with *M. tuberculosis* and *M. avium-intracellulare*, even after 120 min (Broadley et al. 1993).

The poor germicidal activity of the quaternary ammonium compound (Product #5) and chlorhexidine gluconate was not unexpected, and is consistent with their rating as low-level disinfectants (Favero and Bond 1991; Rutala

1990). Despite the widespread use of quaternary ammonium compounds as environmental surface disinfectants, they generally do not possess sporicidal, tuberculocidal, virucidal or fungicidal activity and show variable bactericidal activity (Best et al. 1990B; Mbithi et al. 1990; Russell 1990; Rutala and Cole 1987; Rutala et al. 1991; Sattar et al. 1989; Tyler et al. 1990).

The disinfectant properties of hydrogen peroxide have long been accepted and 3% solutions are widely used for general surface disinfection, the disinfection of contact lenses and general antisepsis. Studies evaluating its efficacy against various classes of microorganisms are however limited and the efficacy data that have been reported in the literature are conflicting. In the present study, 3% hydrogen peroxide was ineffective against all microorganisms tested. Greater activity against gram-negative than gram-positive bacteria such as *S. aureus* has been reported (Block 1991; Baldry 1983). Other investigations have failed to show activity of solutions containing 6% hydrogen peroxide against hepatitis A virus and *M. bovis* (Mbithi et al. 1990; Rutala et al. 1991). In contrast, marginal activity has been demonstrated against poliovirus and *M. tuberculosis* (Rutala et al. 1991; Tyler et al. 1990). The sporicidal activity of hydrogen peroxide solutions has received increased attention due to pressures to find sterilants that could be used in place of toxic

aldehydes to decontaminate equipment that is not amenable to heat treatment. Higher concentrations (6 to 25%) have exhibited sporicidal activity (Baldry 1982; Block 1991; Eskenazi et al. 1982; Russell 1990).

The combined test described here should be of interest to disinfectant manufacturers and regulators as well as users. The application of a suitable mixture of microorganisms could also be very helpful in screening new disinfectant formulations for their spectrum of germicidal activity. Just how easy is the combined test to perform? It is certainly more complex than testing against a single organism, but it is a great deal more realistic and simpler than conducting several tests against an equivalent number of test organisms. More work is required before a combined test could be adopted as a standard test.

6. CONCLUSIONS

1. The feasibility of a novel combined test protocol for simultaneously and quantitatively assessing the disinfectant efficacy against a mixture of microorganisms has been demonstrated.
2. The fundamental test design eliminates the inherent variability of currently accepted protocols. The combined test is quantitative as the recovery methods used allowed for both separation of the microorganisms in the mixture and the quantitation of the reduction in their infectivity titre.
3. Scanning electron microscopy revealed a limited degree of physical protection among individual microbial components of the mixed inoculum. The mixed inoculum does not generally present a more stringent challenge than a single organism as the combined inoculum affected the efficacy of only those products that were also marginal in dealing with a single organism.
4. Tryptose phosphate broth (7.6 g/L) presented a comparable challenge to disinfectants when used at approximately the same protein concentration as 5 % fetal bovine serum.
5. The use of standard hard water as a diluent for disinfectants negatively influenced the efficacy of a mixture of peroxygens and a phenolic disinfectant.

6. *B. stearothermophilus* and *B. subtilis* spores exhibited a similar disinfectant resistance pattern.

S. aureus exhibited an intermediate resistance pattern between that of the more susceptible *S. choleraesuis* and more resistant *P. aeruginosa*.

M. bovis BCG and *M. gordonae* were more sensitive to disinfectants than multi-drug resistant *M. tuberculosis* and *M. avium-intracellulare*. *M. terrae* exhibited a resistance pattern similar to MDR-TB for most formulations tested in this study.

Differences in resistance were observed with hepatitis A virus and poliovirus depending on the nature of the disinfectant under test and the contact time.

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8. PUBLICATIONS

1. Best, M., V.S. Springthorpe and S.A. Sattar. 1994. Feasibility of a combined carrier test for disinfectants: studies with a mixture of five types of microorganisms. Amer. J. Infect. Control, accepted for publication.

PRESENTATIONS

1. Sattar, S.A., M. Best, V.S. Springthorpe and M.E. Kennedy. 1993. Simultaneous evaluation bactericidal, mycobactericidal, virucidal, fungicidal and sporicidal activity of chemical disinfectants. To be presented at the 61st Conjoint Meeting on Infectious Diseases, Nov. 21-25, Vancouver, B.C.
2. Best, M., M.E. Kennedy, V.S. Springthorpe and S.A. Sattar. 1993. Efficacy of disinfectants against *Mycobacteria spp.*, including drug-resistant *M. tuberculosis* and *M. avium-intracellulare* isolated from an AIDS patient. To be presented at the 36th Biological Safety Conference, Oct. 11-13, Albuquerque, NM.
3. Best, M., V.S. Springthorpe and S.A. Sattar. 1993. A new carrier method for assessing germicidal efficacy of disinfectants. Presented at the 20th Meeting of the Association for Practitioners in Infection Control, May 23-28, Orlando, FLA.
4. Best, M., V.S. Springthorpe and S.A. Sattar. 1992. Development of a combined efficacy test method for disinfectants. Presented at the 35th Biological Safety Conference, Oct. 25-28, San Francisco, CA.