RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS* AND OTHER MYCOBACTERIA OF INCREASING CLINICAL IMPORTANCE TO CHEMICAL AGENTS

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The resistance of *Mycobacterium tuberculosis* and other mycobacteria of increasing clinical importance to chemical agents

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Summary

Tuberculosis is a major public health problem which has been compounded by the emergence of multi-drug-resistant strains of *Myco. tuberculosis* (MDR-TB), an increased use of immunosuppressive therapy and increasing numbers of HIV infection. To further complicate the infection control issues, many of the environmentally associated mycobacteria, commonly referred to as opportunistic pathogens, are being incriminated in human infection with increasing frequency. Information is required on the mycobactericidal effectiveness of disinfectants, especially those associated with heat sensitive equipment such as bronchoscopes, which may be contaminated with mycobacteria. The activity of disinfectants against *Myco. tuberculosis* is well documented. However, there is much variation in test methodology resulting in conflicting efficacy data. Therefore a standard, reproducible and practical method must be developed which will give useful and reliable data on the resistance of *Myco. tuberculosis* and other mycobacteria of increasing clinical importance to current disinfection procedures.

A standard test method was developed for use in this study. Suspension and carrier tests were carried out in the presence and absence of 10% serum as the organic load. The test organisms were type strains of *Myco terrae, Myco chelonae, Myco. fortuitum* and *Myco. tuberculosis*. Two endoscope washer disinfector isolates of *Myco. chelonae* and a clinical isolate of *Myco. avium-intracellulare* were also used. The type strains of *Myco. chelonae* and *Myco. fortuitum* were very sensitive to all disinfectants tested. *Myco. terrae* was slightly more resistant than *Myco. tuberculosis*. This is in agreement with published data. *Myco. avium-intracellulare* was without doubt the most resistant of all the test organisms. The two machine isolates of *Myco. chelonae* were extremely resistant to 2% glutaraldehyde. This prompted further work to assess if these two strains differed from the type strain in other ways.

Key words: *Mycobacterium tuberculosis*, Disinfectant test methods, Resistance to disinfectants

To my mother and father

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Abbreviations

ACDP	Advisory Committee on Dangerous Pathogens
AFNOR	Association Francaise de Normalization (French Association of Normalization)
AIDS	Acquired Immunodeficiency Disease
AOAC	Association of Official Analytical Chemists
APIC	Association of Practitioners in Infection Control
BATH	Bacterial adherence to hydrocarbons
BAUS	British Association of Urological Surgeons
BSG	British Society for Gastroenterology
BSI	British Standards Institute
BTS	British Thoracic Society
CDC	Center for Disease Control
CDR	Communicable Disease Report
CEN	Centralisation European de Normalisation
CIP	Cleaning-In-Place
COSHH	Control of Substances Hazardous to Health
CsCDC	Consultants for Communicable Disease Control
CSF	Cerebro-spinal fluid
DGHM	Deutsche Gesellschaft fur Hygiene und Mikrobiologie (German Society for Hygiene and Microbiology)
DoH	Department of Health
ETM	Ethambutol
FAMEs	Fatty Acid Methyl Esters
FDA	Food and Drugs Administration
GC	Gas Chromatography
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Lipid Chromatography
INH	Isoniazid
IWGMT	International Working Group for Mycobacterial Taxonomy
KOH	Potassium hydroxide
MAC	*Mycobacterium avium* complex

mAGP	mycolylarabinogalactan-peptidoglycan
MAI	*Mycobacterium avium-intracellulare*
MBC	Minimum Bactericidal Concentration
MBTH	3-Methyl-2-benzothiazoline hydrazone hydrochloride
MDA	Medical Devices Agency
MDR-TB	Multi-drug resistant tuberculosis
MIC	Minimum Inhibitory Concentration
Myco	*Mycobacterium*
NaDCC	Sodium dichloroisocyanurate
NaOH	Sodium hydroxide
NCTC	National Collection of Type Cultures
PAA	Peracetic acid
ppm	parts per million
RMP	Rifampicin
SAB	Safety Action Bulletin
STM	Streptomycin
TB	Tuberculosis
TVC	Total Viable Counts
UK	United Kingdom
USA	United States of America
WP	Working Party
ZN	Ziehl-Neelsen stain for acid fast bacilli

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1.0 INTRODUCTION

1.1 GENERAL INFORMATION

Tuberculosis, the most important life-threatening bacterial disease (Collins, 1993), remains a major public health problem. The World Health Organisation estimates 6 - 8 million new cases per year with approximately 2 - 3 million deaths (Dolin *et al.*, 1993). An age old disease, it had been in steady decline in most countries for many years. However, this trend has now been reversed. Historically, tuberculosis has been a disease of the population, irrespective of race or class, but by 1980, it had become a disease of the underprivileged (McGowan & Blumberg, 1995). The re-emergence of tuberculosis as a major public health concern can now be seen primarily in the developing countries and the inner cities of the US and Europe.

Kochi (1991) estimated that about one third of the world's population (i.e. 1.7 US billion) was infected with *Mycobacterium* (*Myco.*) *tuberculosis*. As the great majority of the world's population reside in developing countries, so too do the majority of infected persons. Tuberculosis has a devastating effect in these countries, where approximately 95% of the worlds cases occur, 80% of which are persons in their productive years, i.e. 15 - 59 years old (Snider *et al.*, 1994). Murray *et al.* (1990) remind us that the mortality rate of tuberculosis in the pre-chemotherapy era was 50-60%, however even with widespread availability and use of appropriate chemotherapy regimes, tuberculosis still causes 25% of the avoidable adult deaths in the developing world.

In the UK, notifications of tuberculosis have declined since statutory notifications were introduced in 1913, with a 10-fold decrease reported

between 1948 and 1987. The decline then stopped. Between 1987 and 1993, notifications of tuberculosis increased by 34% in London and 15% in the East and West of the UK, despite apparent under-notification in some parts of London (McEvoy & McGuire, 1995). In response to this worrying increase in London, the London Consultants for Communicable Disease Control (CsCDC) Working Party on TB was established in 1992 to review current surveillance data. They were also concerned with the emergence of multi-drug resistant tuberculosis which had already been reported in New York. The working party made recommendations to improve the surveillance of tuberculosis in London and established a city wide surveillance function in 1994 to collate and monitor data on TB. McEvoy & McGuire (1995) draw attention to the similarities between the concerns of the London CsCDC WP on TB in 1994 and the Minister for Health for the London County Council in 1893. In the USA, the declining rates of tuberculosis reversed in the mid-1980s. Between 1985 and 1992, the annual number of reported tuberculosis cases increased by 20%. Of note is the increase from 28% to 35% of reported cases in large cities, while rural cases decreased from 54% to 46% (McGowan & Blumberg, 1995).

In the United States and in the developing countries of the African subcontinent, the re-emergence of tuberculosis as a major public health problem coincided with the advent of the HIV epidemic. Human Immunodeficiency Virus reactivates latent *Myco. tuberculosis* infection (Watt *et al.*, 1993). Therefore HIV-infected patients who are also infected with *Myco. tuberculosis* are at risk of developing clinical tuberculosis which can then be transmitted to others, including health care workers. This is causing great concern in those countries with a

large overlap of individuals infected with both the Human Immunodeficiency Virus and *Myco. tuberculosis*. To further compound these problems, multiple-drug-resistant strains of *Myco. tuberculosis* (MDR-TB) have emerged and have been the cause of numerous outbreaks in the USA, particularly in hospitals, prisons and institutions (Tapper, 1995). Homelessness and immigration are also factors responsible for the rising numbers of reported cases of tuberculosis in the USA.

HIV-associated tuberculosis is not yet a major concern in the UK and the rest of Europe as the overlap of HIV-infected and TB infected individuals is not large enough. Reasons for the increase of tuberculosis in the UK are mainly linked with the increase in the numbers of immigrants from the Indian-subcontinent. Poverty, poor hygiene, institutionalization and reactivation of latent infection in the elderly, have also been implicated (Blair, 1993; Pearson, 1995). Multi-drug resistant tuberculosis has not yet become a major problem in the UK with only a few reported outbreaks to date. The first reported outbreak of hospital acquired MDR-TB occurred on an HIV unit in London in 1995 (CDR, 1995).

The increase of tuberculosis has been accompanied by an increase in infections due to the atypical mycobacteria, many of which are naturally resistant to antibiotics (Sanders *et al.*, 1977; Wolinsky, 1992; Sattar *et al.*, 1995) including *Myco. avium-intracellulare* (MAI), *Myco. chelonae* and *Myco. fortuitum*. These opportunistic pathogens are ubiquitous in nature and until the last decade had only sporadically been reported as aetiological agents in human infection (Carson *et al.*, 1978).

MAI is reported to infect over 50% of AIDS patients (Hellyer *et al.*, 1993; Hanson, 1988). *Myco. chelonae* and *Myco. fortuitum* have been shown not only to survive, but to multiply in water samples, particularly those used for rinsing diagnostic and therapeutic instruments, e.g. flexible bronchoscopes. Problems have been ascribed to their presence as contaminants in haemodialysis fluids, pharmaceutical and disinfectant preparations (Carson *et al.*, 1978). They are being isolated from endoscope washer disinfectors and medical devices with alarming frequency. This leads to increased infection risk through cross-contamination and also a risk of misdiagnosis from false positive results due to the presence of acid fast bacilli introduced during equipment processing (Pappas *et al.*, 1983; Nye *et al.*, 1990; Duckworth, 1988; Griffiths *et al.*, 1997).

The increasing infection risk of nosocomial and iatrogenic spread of mycobacteria has forced a review of infection control procedures (Sattar *et al.*, 1995). Tuberculosis is most likely to be transmitted by the airborne route from patient to patient and from patient to healthcare worker. However, because of its resistance to drying, environmental surfaces may also act as potential vehicles of transmission of the infection. Improperly decontaminated medical devices including flexible endoscopes, resuscitation and lung function equipment and ventilators have also been implicated in the transmission of *Myco. tuberculosis* and atypical mycobacteria (Sattar *et al.*, 1995). Reducing airborne transmission is usually accomplished by isolating the patient in a negative pressure ventilated room. Ultraviolet light has also been shown to be highly effective in reducing the number of tubercle bacilli in the air and as a result, is used in some countries, e.g. USA, as an additional precaution (Rubin, 1991).

To reduce the increasing mycobacterial infection rates appropriate and effective decontamination procedures are necessary for the skin, instruments and environmental surfaces (Sattar *et al.*, 1995).

Microbial decontamination is a process which reduces the number of organisms to a level which will be insufficient to initiate an infection in a susceptible site. It is achieved by cleaning, disinfection or sterilization. Each of these processes tends to be progressively more effective, costly and more likely to damage the treated item. Cleaning is an essential pre-requisite to disinfection and sterilization. It removes contaminants including dust, soil, large numbers of micro-organisms and the organic matter that protects them (Ayliffe, 1991). Sterilization is a process which destroys or removes all living micro-organisms including bacterial spores. It is an absolute process, which renders objects free from viable organisms and is recommended for all items penetrating, or in contact with, broken skin and mucous membranes, or entering otherwise sterile body areas, e.g. surgical instruments, implants, dressings etc. (Babb, 1992). Disinfection as defined by Ayliffe *et al.* (1993) is a process used to reduce the number of micro-organisms but not usually bacterial spores. The process does not necessarily kill all micro-organisms but reduces their numbers to a level which is not harmful to health. The term is applicable to the treatment of inanimate objects and materials and may also be applied to the treatment of skin, mucous membranes and other body tissues. Disinfection may also be used for invasive items or those in contact with a breach in the skin or mucous membranes if no practical means of sterilization is available (Babb, 1992).

The Association of Practitioners in Infection Control (APIC) in the USA, have subdivided the term "disinfection" into high-level, intermediate-level and low-level disinfection based on the Spaulding classification of 1968 (Rutala, 1990). High-level disinfection can be expected to destroy all micro-organisms including *Myco. tuberculosis* with the exception of high numbers of bacterial spores. Intermediate-level disinfection inactivates *Myco. tuberculosis*, vegetative bacteria, most viruses and fungi but does not necessarily kill bacterial spores. Low-level disinfection can kill most bacteria, some viruses and fungi, but cannot be relied on to kill resistant micro-organisms such as tubercle bacilli and bacterial spores.

The choice of method of disinfection or sterilization will depend on a number of factors, including the type of material to be treated, the organisms involved, the time available for decontamination and the risks to staff and patients. Based on the risks to patients from equipment and the environment Ayliffe *et al.* (1993) have categorized high, intermediate and low level risk and proposed suitable methods of decontamination based on these categories (Table 1.1.1). Similarly, Spaulding has categorized items into critical, semi-critical and non-critical based on their risk to patients (Spaulding, 1968).

Table 1.1.1: Classification of equipment and environment based on risks to patient with proposed suitable methods of decontamination

Taken from Ayliffe, Coates and Hoffman (1993), The Public health Laboratory Service

Heat-tolerant medical devices can be thermally disinfected by exposure to hot water or steam. This is the preferred method of disinfection. It is the most effective and least expensive method. Boiling water, sub atmospheric steam at 73^{0}C or processing in a washer disinfector at time/temperature ranges of 65^{0}C for 10 minutes, 71^{0}C for 3 minutes, 80^{0}C for 1 minute or 90^{0}C for 1 second are highly effective in destroying non-sporing bacteria including *Myco. tuberculosis* and most viruses (Collins BJ *et al.*, 1986). However, little is known about the susceptibility of other species of mycobacteria to heat.

Heat-sensitive items that do not tolerate thermal disinfection or sterilization require chemical disinfection i.e. the use of disinfectants. Microorganisms vary in their resistance to these disinfectants and mycobacteria are generally more resistant to disinfectants than enveloped viruses and other types of vegetative bacteria (Ayliffe *et al.*, 1993). Mycobacteria are therefore often considered suitable as efficacy indicators for high or intermediate level disinfection.

1.2 MYCOBACTERIA

1.2.1 History

The name "*Mycobacterium*" means "fungus bacterium" and arose from the characteristic fungus/mould-like pellicle produced by the tubercle bacilli when grown in liquid media (Collins *et al.*, 1985; Wayne & Kubica, 1986). The genus was first named in an "Atlas of Bacteriology" by Lehmann and Neumann in 1896. It then contained the leprosy bacillus of Hansen 1874 and the tubercle bacillus of Koch 1882, named *Mycobacterium leprae* and *Mycobacterium tuberculosis* respectively. This genus was then, and remains today, the only genus in the family Mycobacteriaceae, which belongs to the Actinomycetes order, Actinomycetales class (Schinnick & Good, 1994).

Originally, the defining characteristics were morphology and acid-alcohol fastness (Schinnick & Good, 1994). The acid-alcohol fastness is a characteristic staining reaction, discovered by Ehrlich in 1882 (Draper, 1982). It is due to the ability of the mycobacteria to resist decolourization by acidified alcohol after staining with hot carbol fuschin or other arylmethane dye. Both the cultural hydrophobic property and characteristic acid-alcohol fastness of mycobacteria are due to the possession of a thick, complex, lipid-rich cell wall (Russell, 1992a). The leprosy bacillus was included in the genus on the basis of its acid-alcohol fastness alone, as it had not been cultured. In fact, it has not to this day been grown convincingly *in vitro* (Collins *et al.*, 1985).

As time went on, more acid-alcohol fast bacilli, with cultural properties similar to Koch's bacillus were being isolated from both diseased animals and environmental sources, all suitable for inclusion in the genus (Collins *et al.*, 1985). Koch's original bacillus and these other acid-alcohol fast bacilli isolated from diseased animals became known as the "tubercle bacilli" and were divided into groups, e.g. mammalian, avian and cold-blooded tubercle bacilli, reflecting their source of isolation (Grange, 1983)

The "mammalian tubercle bacilli" were further divided by Theobald Smith in 1898 into *Myco. tuberculosis hominis* and *Myco. tuberculosis bovis*, the human and bovine types. This division was based on small but constant cultural differences (Collins *et al.*, 1985). Two other species were added to the "mammalian tubercle bacilli": the vole bacillus of Wells (1946), and *Mycobacterium africanum*, the African variant of tuberculosis. The vole bacillus was named *Mycobacterium microti* (Reed, 1957). All are obligate parasites. *Myco. tuberculosis* is the causative agent of most human cases of tuberculosis, although some cases are due to the bovine type, which is the main cause of tuberculosis in cattle and other mammals. It was not until 1970, that this bovine type was officially given the name *Mycobacterium bovis* (Karlson & Lessel, 1970).

The "avian tubercle bacilli" belong to the *Myco. avium* species and the "cold-blooded tubercle bacilli", the turtle, frog and fish bacilli to the species currently known as *Myco. chelonae*, *Myco. fortuitum* and *Myco. marinum* respectively (Collins *et al.*, 1985). *Myco. avium* was first found to cause disease in chickens with rabbits being reported to be highly susceptible, but humans relatively resistant to infection (Wolinsky,

1979). The first case of human infection was reported in 1949 and the causative organism was called *Nocardia intracellulare* (Cuttino & McCabe, 1949). Runyon later demonstrated this organism to be a mycobacterium and renamed it *Mycobacterium intracellulare* (Runyon, 1965). It is currently the second species in what is commonly referred to as the *Mycobacterium avium* complex (MAC) (Schinnick & Good, 1994), where a complex is defined as 2 or more species whose distinction is of little or no medical importance.

The species described as the "cold-blooded tubercle bacilli" may be pathogenic to man. In 1972, the species name *Mycobacterium chelonae* was given to the mycobacteria previously known as *Myco. friedmanii, Myco. abscessus, Myco. runyonii* and *Myco. borstelense. Mycobacterium fortuitum* was used to replace *Myco. minetti* and *Myco. ranae. Myco. chelonae* and *Myco. fortuitum* are both rapid growers and tend to be referred to as opportunistic pathogens (Wolinsky, 1979). *Mycobacterium marinum* meaning "of the sea" which reflects its original source of isolation by Aronson in 1926 is a slow grower. It is known to cause granulomatous skin lesions in humans (Roberts *et al.*, 1991).

Runyon (1975) proposed that the term "tubercle bacilli" should incorporate the three separate species of *Myco. tuberculosis, Myco. bovis* and *Myco. africanum*, all pathogenic to man and all obligate parasites. This proposal was accepted by the International Committee on Systematic Bacteriology in 1980, although many disagree that they should have specific status. They prefer that these bacilli be listed as subspecies of *Mycobacterium tuberculosis*, to avoid possible serious misunderstandings. Collins *et al.* (1985) refer to a story told by

Symons of a surgeon whose son had enlarged cervical lymph nodes. When told that *Mycobacterium bovis* was isolated, he expressed delight that it was not tuberculosis. For this reason, Collins *et al.* (1985) grouped the three species as variants of *Myco. tuberculosis*. Nowadays they are commonly referred to as the *Mycobacterium tuberculosis* complex. Some authors also include *Myco. microti* in the complex, which, although non pathogenic to man, is pathogenic to animals and is very closely related to *Myco. tuberculosis*.

All species other than the "tubercle bacilli" based on Runyon's proposal (1975), normally exist as saprophytes of soil and water. Although many were first described at the turn of the 19th century, little interest was aroused in these until the 1950s, when their numbers increased and it was suspected that at least some of them were associated with human disease (Buhler & Pollak, 1953; Timpe & Runyon, 1954; Collins *et al.*, 1985). In 1959 Runyon established a grouping for these mycobacteria based on their rate of growth, morphology and production/non-production of a pigment. Groups 1-3 are slow growers and are distinguished by production of a pigment. Group 1, the Photochromogens, produce a pigment on exposure to light, Group 2, the Scotochromogens, produce a pigment in the absence of light and Group 3, the Nonchromogens do not produce a pigment. Group 4 contains the rapid growers, those that require less than 7 days to produce visible colonies on solid medium (Runyon, 1959).

The Index Bergeyana listed 128 legitimate names for the various mycobacterial species in 1966 (Draper, 1982) and in 1967 an International Working Group on Mycobacterial Taxonomy (IWGMT)

was established to reorganize the classification of these mycobacteria. The group employed numerical taxonomy (Schinnick & Good, 1994). In 1980 the International Committee on Systematic Bacteriology published the "Approved List of Names" with just 41 species of mycobacteria to replace the original 128 (Draper, 1982).

Apart from numerical taxonomy, other techniques have been used to assist in the classification, i.e. antigenic studies, DNA relatedness, the chemical composition of whole cells as determined by pyrolysis mass spectrometry and, more recently, semantide studies. The result of all these studies was the development, with time, of additional criteria to clearly distinguish the many different species of mycobacteria and also to differentiate this genus from other closely related genera (Draper, 1982). Some species in the *Rhodococcus*, *Nocardia* and *Corynebacterium* genera all exhibit varying degrees of acid fastness and cultural properties not dissimilar to those of the mycobacteria. Because of all this work on the genus, *Mycobacterium* is perhaps, currently, the best classified of all the bacterial genera. It is now far more clearly defined than simply acid-alcohol fastness and morphology. The current minimal standards for inclusion in the genus *Mycobacterium* are:

1. Acid-alcohol fastness
2. Presence of mycolic acids containing 60-90 carbons which are cleaved to C22 to C26 fatty acid methyl esters by pyrolysis.
3. A guanine and cytosine content of the DNA of 61 to 71 mol %

(Levy-Febrault & Portaels, 1992)

There are currently 71 recognized or proposed species in the genus *Mycobacterium* based on conformity with these standards (Schinnick & Good, 1994). For convenience, these species fall into 2 main groups; the slow growers and the rapid growers (Table 1.2.1.1). Unofficially, the species have been divided into various groups which are believed to be useful from a clinical point of view (Woods & Washington, 1987). However, Runyon's groupings of 1959 are still perhaps the most useful (albeit unofficial from a taxonomical standpoint) both clinically and in identification schemes.

A summary of some of the notable dates in the history of mycobacteria is shown in Table 1.2.1.2

The mycobacterial species employed in this study are described in more detail in the following sections.

Table 1.2.1.1: Species of the genus *Mycobacterium*

Taken from Schinnick & Good (1994)

Table 1.2.1.2: Summary of some notable dates in the history of the mycobacteria

1874	Armauer Hansen observes the leprosy bacillus, the causative agent of leprosy. It is the first bacterium to be associated with human disease.
1882	Robert Koch observes and cultures the causative agent of tuberculosis, the tubercle bacillus
	Paul Ehrlich discovers the acid alcohol fastness property of the bacillus
1883	Zopf proposes the specific name *Bacterium tuberculosis* for the tubercle bacillus
1896	Ziehl and Neelsen propose a procedure for staining the acid alcohol fast bacilli, really a modification of Ehrlich's
	Lehmann and Neumann first name the genus *Mycobacterium* in "An Atlas of Bacteriology"
1898	Theobald Smith separates the mammalian tubercle bacilli into the human and bovine types based on small but constant cultural differences - *Mycobacterium tuberculosis hominis* and *Mycobacterium tuberculosis bovis*
1899	Lehmann and Neumann describe *Mycobacterium smegmatis*
1903	Friedman isolates the turtle bacillus, now officially known as *Mycobacterium chelonae*
1905	Küster describes the "cold-blooded" or frog tubercle bacillus, now officially known as *Mycobacterium fortuitum*
1946	Demonstration of the efficacy of Streptomycin
1950	Richard & Cummings isolate *Myco. terrae* from washings of radish
1952	Isoniazid becomes available
1959	A botanist, Ernest Runyon, establishes a grouping for mycobacteria other than the tubercle bacilli- Runyon Groups 1 - 4, based on morphology, and production/nonproduction of pigment
1961	Ethambutol is discovered among synthetic compounds screened for antituberculous activity.
1966	Index Bergeyana lists 128 validly published legitimate names for the various mycobacterial species
1967	An International Working group on Mycobacterial Taxonomy (IWGMT) is set up, to bring some order to the chaos of classification of mycobacteria. They achieve this using numerical taxonomy

1967	Rifampicin is introduced for clinical trials
1970	The bovine tubercle bacillus is officially given the name *Mycobacterium bovis* by Karlson and Lessel
	Rifampicin introduced as a first line anti tuberculous agent
1975	Runyon proposes that the term "tubercle bacilli" should include *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium africanum*
1980	The International Committee on Systematic Bacteriology publish the "Approved Lists of Bacterial Names" with just 41 valid species names in the genus *Mycobacterium*, to replace those listed in the Index Bergeyana in 1966
	Runyon's proposal of 1975 is accepted and approved by the International Committee on Systematic bacteriology, with the names being given specific status
	Myco. chelonei is changed to *Myco. chelonae*
1985	Tuberculosis case rates increase in the US for the first time in 20 years
1986	71 recognised or proposed species meet the current minimal standards for inclusion in the genus *Mycobacterium*
1987	CDC and Prevention Advisory Committee for the Elimination of Tuberculosis is established with the aim of total eradication of tuberculosis from the US by the year 2010
	CDC include extrapulmonary tuberculosis in HIV positive persons in the AIDS surveillance case definition
	Tuberculosis notifications increase in England and Wales for the first time since 1913
1992	New minimal standards for inclusion in the genus Mycobacterium
	The London CsCDC Working Party on tuberculosis is established
1995	First reported outbreak of MDR-TB in the UK

1.2.2 General Characteristics

Mycobacteria are aerobic, acid-alcohol fast, non-spore forming, non-motile bacteria. They are straight or slightly curved bacilli, in the range of 0.2-0.6 x 1.0-10μm in size. Most strains occur as unicellular rods, but some develop as mycelia. However, early fragmentation of the mycelium occurs to produce either rods or branched rods.

The mycobacterial cell wall differs from other bacterial cell walls in several respects. These differences are believed to convey an above-average resistance to drying, alkali and many antibiotics and disinfectants. This general resistance is thought to be related to the unusual structure and resultant low permeability of the mycobacterial cell wall. It is universally agreed that knowledge of the cell wall composition is central to an understanding of these organisms including their acid-alcohol fastness and above-average resistance. Many investigations have been carried out and much information is available regarding the structure and ultrastructure of the wall; however, further work is still required (McNeil & Brennan, 1991; Brennan & Nikaido, 1995).

The mycobacterial cell wall, classified as a chemotype IV (Brennan & Nikaido, 1995), is rich in lipid content, including free lipids, Wax D (believed to be an autolysis product of the cell wall) and acetylated trehaloses (Russell, 1992a). The cell wall skeleton (also known as the insoluble matrix of the cell wall and the cell wall core) is made up of peptidoglycan, arabinogalactan and mycolic acids covalently linked to give mycolylarabinogalactan-peptidoglycan (mAGP) (Jarlier & Nikaido, 1994; Brennan & Nikaido, 1995). The peptidoglycan itself differs

slightly from that of other bacteria in that it contains N-glycolmuramic acid as opposed to N-acetylmuramic acid (Fig. 1.2.2.1). Mycolic acids are high molecular weight, α-branched-β-hydroxy fatty acids with the main chain approximately 50-60 carbons in length and the branch typically about 24 carbons in length (Fig 1.2.2.2). The branch is a simple alkyl chain but the main chain contains (in *Myco. tuberculosis*) cyclopropyl, methoxyl or keto and methyl groups. They are present mostly as bound esters of arabinogalactan but also in extractable lipids, mainly as cord factor (α_1-α^2-D- trehaloses). The arabinogalactan is a branched chain polysaccharide containing D-arabinose and D-galactose in the ratio 5:2. The peptidoglycan is linked to the arabinogalactan via phosphodiester bonds. In turn, the distal ends of the arabinogalactan are esterified to 1 molecule of mycolic acid (Fig 1.2.2.3).

Fig.1.2.2.1 Peptidoglycan

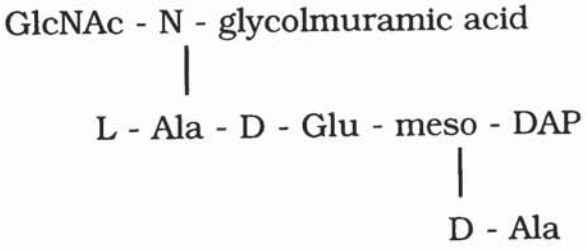
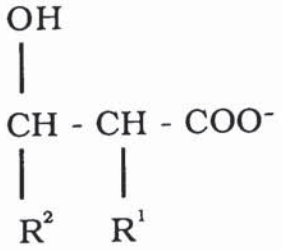
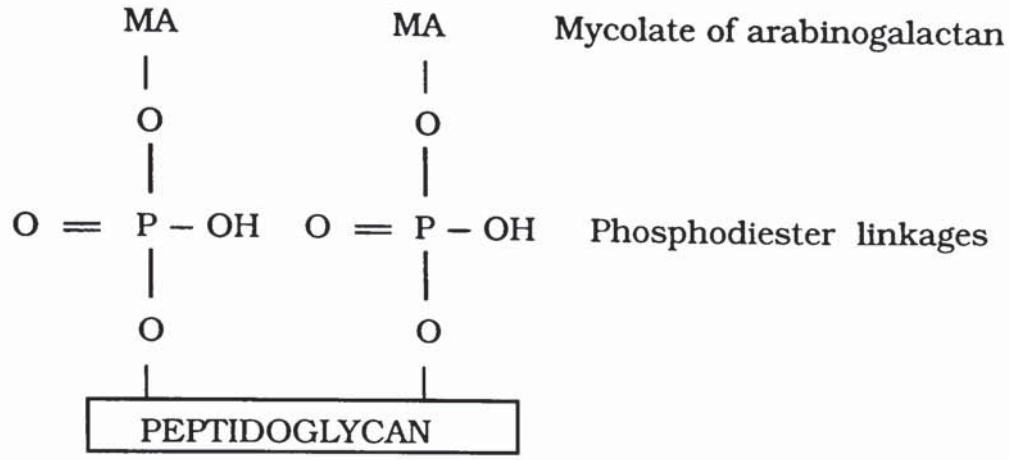


Fig. 1.2.2.2 Mycolic Acid



R^1 and R^2 are alkyl groups that may be saturated or unsaturated

Fig. 1.2.2.3 Mycobacterial Cell Wall Skeleton



The cell wall skeleton is constant among mycobacterial species and is located inside 2 outer layers. These outer layers are believed to comprise glycolipids and proteins and, unlike the mAGP, are considered to vary between species. McNeil & Brennan (1991) among others, have studied the structure of the mycobacterium cell wall in great detail. As a result of their investigations, they have produced, in their own words "our most daring, but still reasonable, model of the cell wall of mycobacteria (Fig.1.2.2.4).

Note: Figs. 1.2.2.1, 1.2.2.2, and 1.2.2.3 taken from Russell (1992a)

Fig.1.2.2.4 Schematic Representation of the mycobacterial cell wall as presented by McNeil and Brennan (1991).

1.2.3 *Mycobacterium tuberculosis*

This organism was first cultured and observed by Robert Koch in 1882. The specific name *Bacterium tuberculosis* was proposed by Zopf in 1883. Then in 1896, Lehmann and Neumann assigned the species to the genus *Mycobacterium* (Schinnick & Good, 1994). *Myco. tuberculosis* is a very slow grower and under optimal conditions, it requires 16-18 hours to undergo one cycle of replication (Wayne, 1976; Wayne, 1994). Therefore in theory, with such a generation time, a single bacillus can yield a visible colony on solid medium in 14 days at 35-37°C. In practice, this tends to be nearer 3 weeks and bacilli isolated from clinical specimens, or cultured after exposure to antibiotics or disinfectants, may require 4-8 or even 10 weeks to produce visible colonies on solid medium, due perhaps to the need to repair injury/damage to the cells. Colonies are rough and dry with a characteristic buff colour (Roberts *et al.*, 1991). Although classed as aerobic, *Myco. tuberculosis* has in fact the ability to grow and/or survive under a wide range of partial oxygen pressures (Wayne, 1994). Bacilli are typically thin and slightly curved, approximately 0.3-0.6 x 1-4 μm in size with a distinctive beaded appearance. They are strongly acid fast.

Myco. tuberculosis is an obligate parasite and is the primary causative agent of tuberculosis in humans (Roberts *et al.*, 1991). There is palaeopathologic evidence of spinal tuberculosis in neolithic, pre-Columbian and early Egyptian remains. It is believed to have occurred sporadically but not in epidemic form. Tuberculosis probably occurred as an endemic disease among animals long before it affected humans. Daniel *et al.* (1994) suggest that *Myco. bovis* was most likely the

infecting organism in human disease but, as *Myco. tuberculosis* infects all primate species, it is also possible that this species existed in subhuman primates before it became established in humans. Haas & Des Prez (1995) report that tuberculosis did not become a major problem until the crowded living conditions of the Industrial Revolution created circumstances favourable to the spread of the disease.

In the 17th and 18th centuries, tuberculosis was responsible for 1/4 of the adult deaths in Europe. In the pre-chemotherapy era, treatment of the disease centred around prolonged rest in the open air, which led to the emergence of the specialized sanitoria. Then, in 1946 streptomycin (STM) was introduced as the first effective anti-tuberculous drug and, although it was rapidly replaced by a far more effective agent, isoniazid (INH) in 1952, 1946 was the beginning of the modern era of tuberculosis therapy. Treatment with these agents was such that patients rapidly became non-infectious, there was no longer the need for prolonged rest, and more importantly tuberculosis was curable in the great majority of cases. This led to the gradual disappearance of the specialized sanitoria and the emergence of tuberculosis control programmes. INH remains today the first choice anti-tuberculous agent. Other first line agents are rifampicin (RMP), ethambutol and pyrazinamide. RMP was introduced in 1970 and was shown to be at least as effective as INH. Treatment may involve one or more of the agents in combination over a prolonged period (INH alone, 18-24 months; INH in combination with RMP, 9 month regimens; multiple drug therapy, including INH and RMP, 4-6 months). Of the newer antibiotics, *Myco. tuberculosis* is susceptible to some of the quinolones (ciprofloxacin and oxofloxacin), some cephalosporins (ceftizoxime and

cephapirin) and also clavulanic acid if combined with amoxicillin or ticarcillin (Roberts *et al.*, 1991; Haas & Des Prez, 1995).

The effectiveness of these agents in the treatment of tuberculosis, combined with low cost and ease of administration made it practical to treat not only active cases of tuberculosis, but also people who might, on the basis of a positive tuberculin skin test, harbour the tubercle bacilli. However, poor supervision of treatments, led to non-compliance with many of the regimens and this is believed to be one of the factors which led to the emergence of multi-drug resistant tuberculosis (MDR TB).

1.2.4 *Mycobacterium chelonae* (formerly *Myco. chelonei*)

(Wayne & Kubica, 1986)

The turtle bacillus of Friedman, 1903, named *Myco. chelonae* by Bergey *et al.* in 1923, is a rapid grower, producing colonies in 2-3 days when subcultured on nutrient agar or egg medium. It does not produce a pigment and colonies are white, moist, soft and domed, approximately 2-3 mm in diameter. The bacilli tend to be fat and stain solidly. They range from 2-3 x 0.5 μm in size.

In 1969, Stanford & Beck proposed that *Myco. abscessus*, *Myco. borstelense* and *Myco. runyonii* be reduced to synonyms of *Myco. chelonae*. Stanford *et al.* (1972) then found that this species could be divided into 2 groups based on geographical variation. One subgroup contained the strains previously classified as *Myco. abscessus* and *Myco. runyonii* and the second subgroup contained those strains previously classified as *Myco. chelonae* and *Myco. borstelense*. Kubica *et*

al. (1972) also recognized the same subgroups and reclassified the species as *Myco. chelonae subsp. abscessus* and *Myco. chelonae subsp. chelonae.* A third subspecies has also been included termed *Myco. chelonae-* like organisms. *Mycobacterium chelonae var. abscessus* was given specific status in 1994 and is now called *Mycobacterium abscessus* (Schinnick & Good, 1994).

Mycobacterium chelonae is an environmental organism and has been found not only to survive but to multiply in both natural and treated waters, including tap water (Goslee & Wolinsky, 1976; Collins *et al.*, 1984). It was obtained occasionally from human resources as early as 1904 and generally regarded as a commensal in man. Friedman was so convinced of its non-pathogenicity, that he introduced the turtle vaccine to prevent and treat tuberculosis (Brown, 1985). The first report of human infection with this organism was in 1953, when it was discovered to be the causative agent in a septic knee and gluteal abscess in a patient. *Myco. chelonae* is classed as a weak pathogen and most human disease associated with it consists mainly of soft tissue abscesses or wound infections. Numerous nosocomial infections and pseudo-infections have been observed since 1953, derived from contaminated medical devices and equipment, and implants such as porcine heart valves (Bolan *et al.*, 1985; Safranek *et al.*, 1987; Cooper *et al.*, 1989; Wenger *et al.*, 1990). Recent literature also suggests that *Myco. chelonae* is the causative agent of disseminated infection in several immunocompetent individuals contrary to the belief that some form of immunosuppression or tissue damage is necessary for infection to occur (Pappas *et al.*, 1983; Spach *et al.*, 1993; Ingram *et al.*, 1993). *Myco. chelonae* is very resistant to antibiotics although some isolates have been shown to be sensitive to amikacin and a sulphonamide.

Imipenem, tobramycin and erythromycin may have some effect on a few isolates.

1.2.5 *Mycobacterium fortuitum*

This species was first introduced by daCosta Cruz in 1938 to describe an apparently new strain isolated from an abscess at an injection site (Roberts *et al.*, 1991). However, Stanford & Gunthorpe (1969) showed that this *Myco. fortuitum* was in fact the same organism as Küster's frog tubercle bacillus of 1905 which was given specific status as *Myco. ranae* by Bergey *et al.* in 1923. In 1972, Runyon proposed that the species *Myco. fortuitum* should replace that of *Myco. ranae.*

This species shares many properties with *Myco. chelonae.* It is a rapid grower giving colonies in 2-3 days on nutrient agar or egg medium. They are white/buff, approximately 2-3mm in diameter. Bacilli stain solidly and range from 2-3 to 0.5μm in size. Because of these similarities, and others, *Myco. chelonae* and *Myco. fortuitum* have been referred to as the *Myco. fortuitum-chelonae* complex. As defined by Silcox *et al.* (1981), any organism must be acid-fast, non-pigmented, grow within 7 days and grow on McConkey agar without crystal violet to be placed in this complex. However, the species can be separated biochemically and, since many strains of *Myco. fortuitum* are more susceptible to antituberculous drugs, they should be considered separate. *Myco. fortuitum* has three biovariants, *Myco. fortuitum var. fortuitum, Myco. fortuitum var. peregrinum* and an unnamed subspecies simply called the third group (Roberts *et al.*, 1991). Similar to *Myco. chelonae, Myco. fortuitum* can be found in many natural sources including soil, water and dust. Wallace *et al.* (1983) recently reviewed

125 human infections caused by rapidly growing mycobacteria and found that *Myco. fortuitum* and *Myco. chelonae* were equally encountered. *Myco. fortuitum* has been responsible for numerous infections including skin and wound infections, pulmonary infection and disseminated infection similar to *Myco. chelonae*. *Myco. fortuitum* is invariably resistant to the first line anti-tuberculous drugs, but seems to be susceptible to the quinolones, sulphonamides, doxycycline and amikacin. Some isolates have also been shown to be sensitive to vancomycin, erythromycin and imipenem (Roberts *et al.*, 1991).

1.2.6 *Mycobacterium avium-intracellulare*

According to Grange *et al.* (1990) mycobacterial disease in birds was first described by Malfucci in 1890 and the causative organism was found by Straus & Gamalcia in 1891. Chester named the organism *Mycobacterium avium* in 1901. It was recognised as being distinct from the human tubercle bacillus. Classic *Myco. avium* is highly pathogenic to chickens and rabbits but not guinea pigs. Swine and cattle are also susceptible but man was reported to be relatively resistant. Branch *et al.* (1933) reported non-pathogenic strains of *Myco. avium* originating from humans. Then, Cuttino & McCabe (1949) isolated and named *Nocardia intracellulare*, an organism similar to *Myco. avium* which caused disseminated disease in a patient. This was later renamed *Mycobacterium intracellulare* (Runyon, 1965). Runyon separated the virulent and the avirulent strains of *Myco. avium* in an attempt to reduce the widespread confusion. He proposed that the strains virulent for chickens and rabbits be called *Myco. avium* and those virulent for humans *Myco. intracellulare* (Wolinsky, 1979). *Myco. intracellulare* became known as the "Battey Bacillus" because one of the

earliest reports recognizing this organism as being distinct from tuberculosis was drawn from patient data at the Battey State hospital in Georgia USA (Roberts *et al.*, 1991). Runyon also recommended that *Myco. avium* and *Myco. intracellulare* be considered separately due to differences in the host range, optimum growth temperatures and sources of infection. However, in 1979, Wolinsky decided it was reasonable to consider them together as *Mycobacterium avium-intracellulare* (MAI) or the *Mycobacterium avium* complex (MAC) as they resembled each other strongly and a routine diagnostic laboratory would be unable to distinguish the one from the other. At that time the IWGMT had reported that numerical taxonomy would not distinguish one from the other and lipid analysis showed both to be very similar. In recent years, however, DNA homology studies have proven successful in distinguishing between the two.

MAI is found in soil, dust, water and other environmental sources. It is of low pathogenicity and was described as a colonizer that rarely caused disease. However in the past two decades, reports of infections due to MAI have steadily increased and by 1980 only *Mycobacterium tuberculosis* was recovered with a higher frequency than MAI. This huge increase and steady rise in MAI infections is closely associated with the HIV epidemic, with an isolation rate of over 50% from AIDS patients. (Hanson, 1988; Hellyer *et al.*, 1993)

MAI grows slowly, requiring 14-21 days incubation on defined medium at 37°C. Colonies are thin, transparent or opaque, homogenous and very smooth, of approximately 1mm in diameter. Many of the young colonies have very distinctive "asteroid" margins and after subculture more eugonic colonies may occur with domed centres. Eventually all

colonies become hemispherical and may become yellow with age. The bacilli are short, 1.0 by 0.5μm, and usually stain solidly. They appear cocco-bacillary although under certain conditions long thin bacilli can be seen. MAI is generally resistant to anti-tuberculous drugs; however vancomycin, amikacin and some cephalosporins may be effective (Wolinsky, 1979; Grange *et al.*, 1990; Roberts *et al.*, 1994; Haas & Des Prez, 1995).

1.2.7 *Mycobacterium terrae*

Richmond & Cummings first recovered this organism from washings of a radish in 1950, hence it is known as the radish bacillus. It grows slowly, 14-21 days at 25°C and 30°C. Colonies tend to be white, smooth and 1-2mm in diameter. The bacilli are 2-3 by 0.4μm in size and stain solidly. It is resistant to INH and most strains are also resistant to STM and RMP, but, sensitive to other anti-tuberculous drugs. According to Woods & Washington (1987), this organism is a saprophytic mycobacterial species rarely causing human disease, i.e. usually of no clinical significance. However, there have been a number of cases reported. *Myco. triviale* is now included with *Myco. terrae* and known as the *Mycobacterium terrae-triviale* complex.

1.3 CHEMICAL AGENTS (DISINFECTANTS)

A chemical disinfectant is a compound that has disinfecting properties i.e. it can destroy vegetative bacteria and most viruses. Some disinfectants can also destroy bacterial and fungal spores, and these may be called sporicidal disinfectants/sterilants. Non-toxic disinfectants which are safe to apply to the skin are termed antiseptics. The three main uses for disinfectants are the environment, skin and heat sensitive instruments. Chemical disinfection is inherently complicated because of the number and variety of factors that influence the antimicrobial activity of disinfectants. Heat sterilization and disinfection methods are therefore preferred (Ayliffe *et al.*, 1993).

1.3.1 History

(The following section has been compiled from the reviews of Block, 1987, 1991a; Hugo, 1991)

Disinfection of sorts has been used since biblical times. Block (1987) reminds us of the requirement of soldiers returning from battle to disinfect their clothing and equipment: "Everything that may abide fire" had to be put into the fire and the rest immersed in boiling water. He also recalls the reference by Homer in his tale "The Odyssey" to the use of sulphur to fumigate his house. Sulphur was also used for this purpose during the great plagues of the Middle Ages. Hugo (1991) reports the use of wine, vinegar and honey as dressings and cleansing agents for wounds, and notes with interest the recent recommendation of acetic acid for the topical treatment of wounds and surgical lesions infected with *Pseudomonas aeruginosa*.

The common property of all disinfectants used during this period was their ability to overcome odours. Bad odours were associated with disease and death and any agent which could nullify these odours was deemed effective. This was one of those misconceived ideas that sometimes resulted in a change in practice, e.g. the use of chlorine, iodine, phenol etc. to mask odours. Many of these agents are still in use today.

Chlorine was discovered in 1774 by Scheele, a Swedish chemist. Then in 1789, Bertholet discovered the hypochlorites. In 1825, in France, Labarraque reported the use of calcium hypochlorite in many areas including prisons, ships, hospital wards and mortuaries, for "general sanitation". Also, surgeons were using a 1:8 solution for ulcers, burns and other wounds. In England, although Alcock reported these favourable results in 1827, the British Board of Health in 1832, refused to accept that chlorine or hypochlorites could be of any medical value. Oliver Wendell Holmes, in1843, was the first person to show that puerperal fever was transmitted from patient to patient on the hands and clothes of doctors and nurses. Four years later, without any knowledge of Holmes' work, Ignaz Semmelweiss, reached the same conclusion at his hospital, in Vienna. In each case, washing hands in a solution of calcium hypochlorite solution was shown to produce a spectacular decrease in the death rates due to puerperal fever. Traube, in 1894, established the disinfection properties of hypochlorites in water treatment, an area in which they are still widely used today and in 1915, during World War 1, Dakin used a solution of sodium hypochlorite (0.5%) with alkali for disinfecting open wounds.

Phenol ie. carbolic acid, although synonymous with Lister, was known since 1842. Kuchenmeister (Germany) was using pure phenol and Lemaire (France) an extract of coal tar as wound dressings. In 1862, Lund (England) reported on the use of carbolic acid in wounds. Unaware of all this work, Lister, in the 1860's, introduced his "antiseptic surgery". He poured phenol on walls and floors and in all parts of the wound. Although phenol at full strength is toxic to tissue, it was far less harmful than the infection, and in his later surgery he showed that a 1:20 and even 1:40 dilution of phenol was still effective. He published his work and reported a spectacular decrease in infection rates. Others who tried his "antiseptic surgery" also reported wonderful success rates. The interest in phenols aroused by Lister's work led to a search for other phenols with a greater range of antimicrobial activity.

Alcohol, mainly in the form of wine, was used through the ages as an antiseptic both internally and externally for all sorts of complaints. However, the concentration of alcohol in wine is so low, it has little or no value as an antiseptic (Block, 1987). Then in the 16th and 17th centuries distilled spirits were introduced into Europe and they too were used as skin disinfectants. The spirits would have been better antiseptics than wine due to their higher concentrations of alcohol. However, it was not until this century that the true value of alcohol as a disinfectant was appreciated

Hydrogen peroxide is known since 1818. Richardson proposed its use as a disinfectant in 1858 based on its ability to eradicate bad odours. It was marketed from 1920-50's as a 3% solution and was very popular.

However, it was shown to be quickly destroyed by the catalase in tissues.

Other disinfectants still in use today include quaternary ammonium compounds whose antimicrobial activity have been known since 1916 and iodine which was first used for the treatment of goitre in 1816 and in wounds from 1839. Mercury and sulphur share the longest history of continued use of disinfectant.

The efficacy and suitability of disinfectants has improved with advances in microbiology, chemistry research and industrial technology. The well established disinfectants have been improved and in many cases their mechanisms of action elucidated and newer formulations have been introduced.

Block (1987) summarizes the evolution of the use of various inorganic and organic chemicals for disinfection, antisepsis and sterilization as "interesting, varied and side-tracked but has resulted in the development of very useful germicides (disinfectants)"

1.3.2 General Characteristics

The primary criterion for any disinfectant is its antimicrobial activity, which can be influenced by a number of factors including: the concentration and exposure time of the agent; the number, type and location of the microorganisms; the presence of organic matter or other interfering substances; and environmental factors such as temperature and pH.

Extremes of temperature and pH can themselves provide an efficient means of controlling or killing microorganisms by presenting them with such adverse conditions as to prevent their growth or survival. Much smaller changes however which might exert a negligible influence on the microorganisms, can greatly affect the efficacy of a disinfectant. Generally the effectiveness of a disinfectant increases with increasing temperature and the efficacy of some agents is particularly temperature dependant. Alkaline glutaraldehyde (2%), for example, is a much more effective sporicidal agent at 20^{0}C than its acid form. However, by increasing the temperature to 40^{0}C and above, the acid form becomes more effective and the alkaline form tends to lose its activity, to a point where the difference between the activity of the 2 forms is negligible, although the alkaline form is less stable (Russell, 1994). The effect of temperature can be measured using the formulae

1. $\theta^{(T_2 - T_1)} = k_2/k_1$

or

2. $\theta^{(T_2 - T_1)} = t_1/t_2$

where k_2 and k_1 are the rate (velocity) constants at temperatures T_2 and T_1 respectively or t_2 and t_1 are the respective times to bring about a complete kill at T_2 and T_1. The temperature coefficient, θ, refers to the effect of temperature per 1^{0}C rise and is usually between 1.0 and 1.5 (Bean, 1967). It is sometimes more useful to specify the θ^{10} value which is the change in activity per10^{0}C rise in temperature.

pH can influence the activity of a disinfectant in a number of ways. Extremes of pH can effectively limit the growth of microorganisms, pH

4.5 - 9 being a limiting range for many organisms (Kostenbaunder, 1991). Changes in pH can alter the ionization of the molecule or the cell surface. A rise in pH leads to an increase in the dissociation of acidic substances such as phenols and benzoic acid, which in turn increases their effectiveness as disinfectants, as they are effective only or mainly in their unionized forms (Russell, 1992b). The activity of glutaraldehyde is very pH dependent, with greatest activity at alkaline pH (pH 7.5 - 8.5) at 20°C (Gorman *et al.*, 1980). This is due to differing chemical states of the glutaraldehyde (see section 1.3.6) molecule which are altered with changing pH. In contrast, the chlorine-based disinfectants are most active in the pH range of 6 - 8. The active form of a chlorine based disinfectant is unionized hypochlorous acid. Ionic forces are not involved in the reaction of the disinfectant with the microorganisms (Russell, 1992b). At alkaline pH, hypochlorite anions predominate in the solution and exhibit negligible inhibitory effect on microorganisms. The number of negatively charged groups on proteins on the bacterial cell surface increase with increasing pH. This makes available more binding sites for those agents which are active as cations, i.e. positively charged ions e.g. quaternary ammonium compounds (Hugo, 1965; Russell, 1992b).

The concentration of the disinfectant is another important factor which can influence antimicrobial activity. It might be expected that an increase in the concentration of an agent would lead to a shorter exposure time necessary to destroy the microorganisms. This is not always true, however, alcohol being a prime example. 70% alcohol is far more effective than 100% alcohol which exhibits negligible activity. The concentration effect of an agent can be calculated mathematically

using the "concentration exponent" (η) This is also known as the "dilution coefficient".

Russell (1992b) recommended measuring the time needed to produce a comparable level of death of a bacterial suspension at 2 different concentrations then applying one of the 2 equations

1. $c_1^{\eta} t_1^{\eta} = c_2^{\eta} t_2^{\eta}$

or

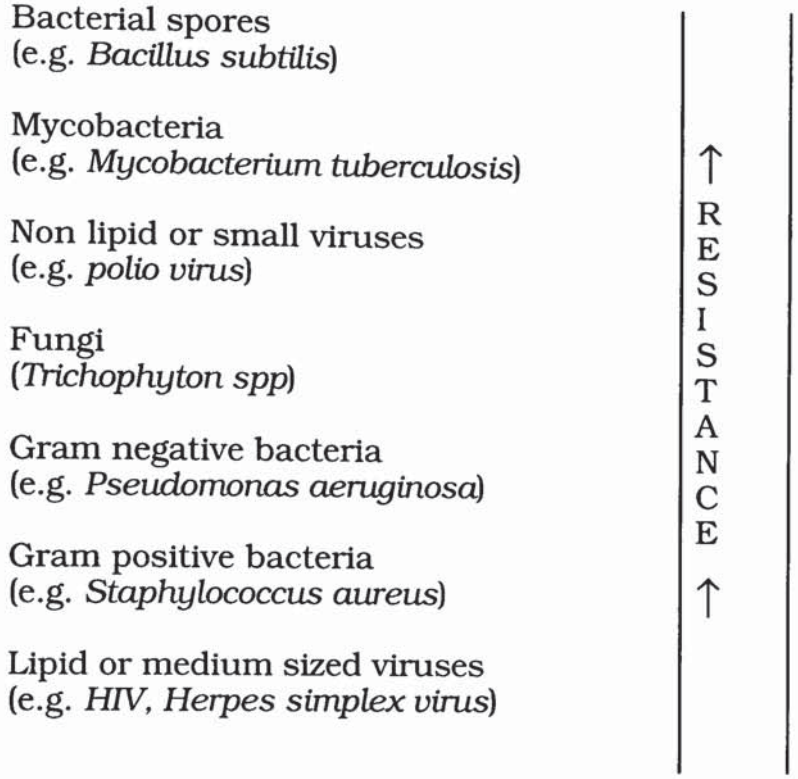
2. $$\eta = \frac{\log t_2 - t_1}{\log c_2 - c_1}$$

where c_1 and c_2 are the 2 concentrations and t_1 and t_2 are the times necessary to obtain a similar reduction in the numbers of viable microorganisms. For many disinfectants the concentration exponent is 1, therefore halving the concentration means the exposure time must be doubled. Phenols have a very high coefficient which means that a small change in concentration can greatly increase the exposure time.

The number, locations and type of microorganisms will obviously influence the activity of a disinfectant. Reduction of initial contamination levels is very important to any decontamination process. By reducing the challenge to the disinfectant by cleaning, the effectiveness of the chemical disinfection procedure is increased. Similarly, microorganisms on a smooth, flat surface are easier to destroy than those deposited in narrow lumen, crevices and hinges as they are more accessible to the agent. Microorganisms themselves vary in their sensitivity to disinfectants as shown in Fig. 1 (Favero, 1994).

Most disinfectants are effective against a number of microorganisms but very few are sporicidal in any realistic exposure time.

Fig 1.3.2.1 Order of resistance of microorganisms to disinfectants



Many disinfectants are inactivated by organic matter e.g. blood, faeces, sputum, serum etc (Gelinas & Goulet, 1983). This inactivation may be due to the disinfectant reacting with the organic load, thereby reducing the level of agent available to react with the microorganisms. An example of this is the inactivation of chlorine-releasing agents, particularly solutions with low levels of chlorine in the presence of organic matter. For those disinfectants that do not react with the soil, their efficacy can also be reduced, as the organic matter may act as a protective barrier, shielding the microorganisms from the action of the chemical agent. This emphasizes again the necessity for cleaning prior to disinfection, which will remove all the organic soil. In

situations where prior cleaning is not possible, e.g. blood spillages, higher concentrations of disinfectant should be used or disinfectants which are not affected by organic mater. Inactivation of disinfectants may also result from hard water, cork, rubber, plastics and detergents with which they are incompatible (Ayliffe *et al.*, 1993).

Although the antimicrobial activity of a disinfectant must be the primary consideration when choosing a disinfectant, it is only one of many. Other factors which influence the choice of a disinfectant include corrosiveness, toxicity, irritancy and cost. In 1957, Spaulding listed "arbitrary criteria" for the ideal disinfectant and concluded that it was unlikely the perfect disinfectant would ever be found or created based on those criteria. Over three decades later the basic properties of an ideal disinfectant as described by Rutala & Weber (1995) have changed little from those of Spaulding although a number of additional criteria have been added, and to date no one disinfectant has been found or created which meets all the requirements listed in Fig. 1.3.2.2

Fig. 1.3.2.2 Properties of an Ideal Disinfectant

1. A broad spectrum of activity
2. Rapid in action
3. Retains most if not all of its activity in the presence of organic matter and other interfering substances.
4. Non irritating and non-toxic to the user with no irritating fumes
5. Exerts a residual effect on surfaces
6. Any residues remaining on instruments are non irritating to tissues
7. Non corrosive and non damaging
8. Economical
9. Easy to use
10. Stable in concentrate or at use dilutions
11. Soluble in water and non-flammable
12. Pleasant smelling or odourless
13. Environmentally friendly and biodegradable

1.3.3 Alcohols

Harrington & Walker (1903) showed that solutions of 60-70% alcohol were effective bactericides but no concentration was sporicidal. Then in 1904, Wirgin reported that the bactericidal activity of the alcohols (i.e. methyl, ethyl, propyl, butyl and amyl) increased with the increase in their molecular weights, with the exception of the tertiary alcohols. Tilley & Schaeffer (1926), using the Rideal-Walker method, demonstrated that activity did indeed increase from the methyl to amyl alcohols. This work was expanded by Tanner & Wilson (1943) who

concluded that the activity of alcohols containing a similar number of carbon atoms varied in the following order;
primary-normal > primary-iso > secondary-normal > tertiary-normal.

The alcohols possess many of the features desirable for a disinfectant or antiseptic (Ayliffe *et al.*, 1993). They are inexpensive, readily obtainable and relatively non-toxic for topical use. They are colourless (may be coloured if desired) and evaporate easily. They have rapid activity against bacteria including acid-fast bacilli, although they are not sporicidal and exhibit poor activity against many viruses. Ethanol and isopropanol are the two most widely used for disinfection purposes. The presence of water is essential for activity, the most effective concentration for ethanol is 70% and isopropanol 60-70%. Concentrations below 50% show a marked decrease in activity and those of 95% and above have too little water to be effective (Ayliffe *et al.*, 1993). It is reported that the alcohols act on microorganisms by denaturing proteins (Larson & Morton, 1991). This would explain why alcohols are more active in the presence of water, as proteins are not denatured as readily in its absence and therefore, why absolute ethanol, a dehydrating agent is less effective than mixtures of ethanol and water. Other studies suggest that alcohols may also interfere with the metabolism of the microorganisms and may even cause lysis of the cells (Pulvertaft & Lumb, 1948; Razin & Argaman, 1963; Larson & Morton, 1991)).

Alcohols are also widely used preceding procedures such as venipunctures, hypodermic injections and other procedures that break intact skin. Isopropanol is slightly more effective than ethanol. They are often also used as a base for other bactericides, e.g. solutions of

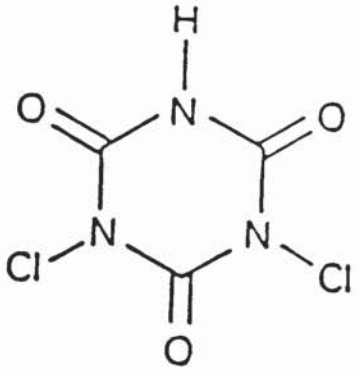
chlorhexidine and iodine in 70% ethanol may be employed for preoperative skin disinfection. Alcohols may be damaging to plastics and rubber and do not penetrate organic matter, especially proteins. Therefore prior cleaning of items to be disinfected is essential if alcohol is the agent of choice (Ayliffe *et al.*, 1993).

1.3.4 Chlorine-releasing Agents

Dakin's solution was the first standard chlorine compound to be used for disinfection in 1915 (Dakin, 1915). Although chlorine was recognised in the first half of the 19th century as having disinfecting properties and was being used for treatment of sewage in London and disinfection in hospital wards as early as 1854, the solutions varied in composition (Block, 1987). Dakin's solution was a sodium hypochlorite solution (NaOCl) of 0.45-0.5% w/v available chlorine (Dychdala, 1991). Hypochlorites are the oldest and most widely used form of active chlorine compounds in the field of chemical disinfection with a wide antibacterial spectrum. They are active against mycobacteria at high concentrations (e.g. 5,000ppm available chlorine) (Dychdala, 1991). According to Lesser (1949) they have many advantages including a proven and powerful broad spectrum of activity, non-toxic residues, ease of handling and are economical. However, the hypochlorites have numerous disadvantages, including inactivation by organic materials. The reactions are pH dependent, and dilute solutions are very unstable and must be prepared fresh daily. Decomposition is accelerated by light, heat and heavy metals. N-chloro compounds are the organic chlorine compounds, e.g. sodium dichloroisocyanurate NaDCC commercially available as Presept or Sanichlor, (Fig. 1.3.4.1) and each contain an $>$N-Cl group. These

have similar properties to the hypochlorites but are less corrosive and more resistant to inactivation by organic materials. They are often more convenient than the hypochlorites as many come in powder or tablet form. Undissolved the powders and tablets are very stable but once in solution suffer similar stability problems as the hypochlorites. Hypochlorites at 10,000ppm available chlorine concentrations will effectively disinfect blood spillages containing HIV and the hepatitis B virus (Coates, 1988; MAC/DoH, 1991). NaDCC can be added directly to the spillage in powder form which gives a larger safety margin because a higher concentration of available chlorine is achieved and it is less susceptible to inactivation by organic matter (Coates, 1988).

Fig. 1.3.4.1: Structure of sodium dichloroisocyanurate (NaDCC)



Chlorine dioxide (1,100ppm av Cl) is another of the chlorine releasing agents which is becoming an increasingly popular disinfectant. It is an extremely reactive compound and consequently cannot be prepared or transported in bulk. It is therefore usually prepared at the point of use. This can be done by mixing a solution of sodium hypochlorite with a solution of chlorine and the reaction follows the order

$$Cl_2 + 2NaClO_2 \rightarrow 2ClO_2 + 2\ NaCl$$

Chlorine dioxide may also be produced by acidification of chlorates with hydrochloric or sulphuric acid, reduction of chlorates in acid medium, reacting acid with chlorites or electrolytically by using sodium chloride, sodium chlorite and water. In the US, it has become popular for disinfection of drinking water, waste water treatment and slime control in cooling towers. It has also been used in the food industry. In the UK, in the last 3-4 years chlorine dioxide at 1,100ppm av. ClO_2 under various commercial names including Tristel and Dexit, is being marketed as an instrument and environmental disinfectant. Although there is no question of its antimicrobial effectiveness, doubts still remain regarding its corrosiveness and user friendliness (Dychdala, 1991).

1.3.5 Peracetic Acid

Peracetic acid (PAA) or more precisely peroxyacetic acid, CH_3CO_3H, is a colourless liquid with a characteristic pungent odour. A peracid, characterized by the presence of the peroxy group (-O-O-), it can be considered as a derivative of hydrogen peroxide (HP) in which one of the hydrogen atoms is replaced by acetic acid (Fig. 1.3.5.1).

Fig. 1.3.5.1: Peracetic acid as a derivative of hydrogen peroxide

Peroxides in general, being high-energy-state compounds are thermodynamically unstable and peracetic acid is considerably more

unstable than hydrogen peroxide. Therefore, when producing peracetic acid, the resultant solution is fortified with acetic acid and hydrogen peroxide to maintain the equilibrium and prevent the decomposition. Additionally, a stabilizer such as a sequestering or a chelating agent may be used.

The antimicrobial activity of PAA was first reported by Freer & Novy (1902) who noted its excellent disinfecting and cold sterilizing actions. However, it was not until 1949 that PAA became generally available. This was the year in which the electrochemical process for producing 90% hydrogen peroxide was developed, a product necessary for the manufacture of peracetic acid (Block, 1986). This development also coincided with a report by Hutchings & Xezones (1949) which showed PAA to be the most effective of 23 agents tested against *Bacillus thermoacidurans*.

Since 1949, numerous investigators have further confirmed the effectiveness of PAA as a disinfectant/sterilant. It can be used either as a liquid or a vapour. As a liquid it is rapidly sporicidal at room temperature even at low concentrations. Gershenfeld & Davis (1952) demonstrated a lethal effect on spores of *B. stearothermophilus* in 15 minutes at 20°C using 500ppm and by increasing the concentration to 3,000ppm, the same result was achieved in 15 seconds. Han *et al.* (1980) using 10,000ppm PAA, also demonstrated its rapid sporicidal activity against *B. subtilis* and *B. stearothermophilus* at 20°C in 15 seconds. Baldry (1983) and Bradley *et al.* (1995) demonstrated a similar rapid sporicidal effect. PAA is also lethal to bacteria, yeast and fungi in 5 minutes or less at concentrations of <100ppm. In the presence of organic matter, the same result can be demonstrated by

increasing the concentration to 200-500ppm. Tests carried out by Holton *et al.* (1995), Lynam *et al.* (1995) and Griffiths *et al.* (1997) have shown a 3,500ppm PAA solution to be effective against *Myco. tuberculosis*, MAI and other atypical mycobacteria in 1-5 minutes, in suspension and dried onto carriers in the presence and absence of organic matter. Virucidal activity has been demonstrated using a wide range of concentrations and exposure times (Block, 1991b).

These numerous studies have also shown that PAA solution remains effective in the presence of organic matter. With the lower concentrations, its function may be slightly impaired, but this can be overcome by small increases in concentration. PAA is also relatively unaffected by pH. As a weak acid it prefers slightly acidic conditions, but again any reduction in activity observed at alkaline pH can be overcome by using higher concentrations. The pH effect is not very evident with bacteria or yeasts at pH 5-8 but decreased activity is observed at pH 9. However, in the case of spores greater activity is observed at lower pH.

Little work has been carried out on the mechanism of action of this agent; there are, however, a number of theories. As a strong oxidising agent, it is likely that it functions much the same as the other peroxides. Block (1991b) speculates that PAA oxidizes sensitive sulphydryl (-SH) and disulphide (S-S) bonds in proteins and other metabolites, thereby causing disruption to important components in cells and membranes. Baldry & Fraser (1988) suggest that it damages/ruptures the cell wall, causing disruption to the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport.

Commercial preparations of peracetic acid are produced by the reaction of acetic acid or acetic anhydride with hydrogen peroxide, using sulphuric acid as a catalyst. Therefore the breakdown products of peracetic acid are acetic acid, oxygen, hydrogen peroxide, water and dilute sulphuric acid (Block, 1991b). For this reason it has gained widespread use in the food industry in the USA where it is said to be ideal for cleaning in place (CIP) systems. There is no need to rinse the systems as the breakdown products in high dilution produce no objectionable taste, odour or residues (Block, 1991b). Peracetic acid also found numerous other uses including gnotobiotics (the production of germfree animals), and treatment of sewage, preparation of pharmaceuticals and industrial water cooling systems.

However, the disadvantages of PAA, which include irritancy, corrosiveness and lack of stability mean it is very unpleasant to work with and not compatible with most equipment. This might explain why the medical community were slower to accept PAA as a disinfectant/sterilant for medical equipment. PAA in its natural state is very corrosive, the materials most likely to be affected being rubber and copper alloys. It will also extract the plasticiser from some seals. Newer commercial formulations appear to have solved many of the problems of corrosiveness and stability and the CDC currently list peracetic acid as a high level disinfectant and sterilant. Wewalka & Werner (1973) recommended its use for the disinfection of respirators and believed its spectrum of activity, rapid action and non-toxic residues would make it an ideal disinfectant for medical machines. Then in 1988 the FDA granted clearance for the "Steris System" to be marketed in the US. This is an enclosed system which uses 0.2% peracetic acid as a "sterilant" for endoscopes. The peracetic acid is

supplied at a concentration of 35% which means it has a substantial shelf life but it is diluted within the enclosed system prior to use. This reduces the risk of irritancy to staff. Corrosion inhibitors are also included (Bradley *et al.*, 1995).

In the UK there are still a number of reservations surrounding the use of PAA. Firstly, the commercial preparations currently available are very expensive and, although claims have been made for the stability and non-corrosiveness of the new formulations, further compatibility tests are required. Furthermore, their effects on the environment and long-term user safety are not yet known.

1.3.6 Virkon

This is a relatively new addition to the ever-increasing list of disinfectants for medical purposes. It is described as a "balanced, stabilized blend of peroxygen compounds, surfactant, organic acids and an inorganic buffer system". The active constituent is a triple salt, potassium monopersulphate/potassium hydrogen sulphate/potassium sulphate, which provides 10% oxygen, and an activity equivalent to 9.75% available chlorine. The inorganic surfactant is 90% biodegradable and is included in the formulation to allow for cleaning and disinfection in combination thus avoiding the problems with prior cleaning. The product is marketed as the "ultimate medical disinfectant" with an unparalleled spectrum of activity (Antec International Ltd., Windham Road, Sudbury, Suffolk, England). Coates *et al.* (1992) demonstrated its bactericidal activity in tests carried out to determine its suitability for use in laboratory discard jars and as a powder for blood and body fluid spillages. A 1%

w/v aqueous solution of Virkon achieved a 5 log reduction of *E. coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris* in the presence of up to 5% defibrinated horse blood. They also demonstrated a 5 log reduction of *B. subtilis* spores in 3 hours under clean conditions. However sporicidal activity was greatly reduced in the presence of organic matter. Based on these and other tests, Coates *et al.* (1992), suggested that Virkon be used as an alternative to clear soluble phenolics in laboratory discard jars, with the exception of those used in mycobacterial work as results on mycobacterial efficacy were unavailable. They also suggested that Virkon powder would be a suitable alternative to NaDCC granules for blood and body fluid spillages as it was rapidly bactericidal, contained a detergent which perhaps compensated its poor absorbency and did not give off fumes when mixed with urine.

Tests carried out on the virucidal and mycobactericidal activity of Virkon however, proved more controversial. Tyler *et al.* (1990) using the poliovirus in suspension and dried onto surfaces showed 3% Virkon to be less effective than 2% glutaraldehyde. A 2.6 log reduction was achieved in 1 minute in the suspension tests with little improvement at 5 and 10 minutes exposure. Similarly a 3.8 log reduction was achieved in 1 minute in the surface test with no further reductions at 5 minutes.

Results on the mycobactericidal activity have only recently become available. Cutler *et al.* (1993, unpublished report available from Antec International) tested the efficacy of 1% Virkon for disinfecting endoscopes contaminated with *Myco. tuberculosis* H37 Ra (the avirulent strain). They demonstrated the complete removal of *Myco.*

tuberculosis from the endoscope channel after 20 minutes exposure. It must be noted however that the initial count in the channels after drying was 6.39 $\log_{10}$cfu/ml and the test method involved a brushing technique. Therefore the results are based on a combination of removal and destruction of the organisms. Holton *et al.* (1994) and Broadley *et al.* (1993) used a suspension test in the presence and absence of organic matter, Holton *et al.* demonstrated a 0.5 - 2.5 $\log_{10}$ reduction in cfu/ml of *Myco. tuberculosis* and MAI in 15-60 minutes. These results were slightly better than Broadley *et al.* who achieved only a 0.5 $\log_{10}$ reduction in *Myco. tuberculosis* and MAI in suspension using a 2% Virkon solution.

1.3.7 Aldehydes

The two most commonly encountered aldehydes are glutaraldehyde (e.g. Cidex, Asep, Totacide) and formaldehyde. In theory, formaldehyde is used as a disinfectant in liquid or vapour form. In practice, however, formaldehyde solution (formalin) is too irritant to be used as a general disinfectant. It is used mainly as a gaseous fumigant to disinfect patient isolators, sealed rooms and category 3 safety cabinets. The concentration, humidity and temperature must be carefully controlled if fumigation is to be effective. It has also been combined with low temperature steam to sterilize heat-sensitive medical equipment. In the liquid form it has been used to disinfect membranes in dialysis equipment. Formaldehyde is generally believed to have good mycobactericidal activity, however, there have been conflicting reports about the liquid.

Glutaraldehyde (Fig. 1.3.7.1) is a 5-carbon dialdehyde, $C_5H_8O_2$, with a molecular weight of 100.12. It was first reported by Harries & Frank in 1908 (Gorman *et al.*, 1980). They synthesised the dialdehyde by boiling the ozonide of cyclopentene which gave several products including glutaraldehyde. The modern production of this chemical involves a 2 step synthesis, starting from an interaction of acrolein with vinyl ethyl ether to give an ethoxy dihydropyran, which is then hydrolyzed with water to form glutaraldehyde and ethanol (Fig. 1.3.7.1) (Russell & Hopwood, 1976).

In its simplest form, glutaraldehyde exists as a monomeric dialdehyde, with carbonyl groups. Under proper conditions these, either singly or together, undergo most of the typical aldehyde reactions, to form acetals, oximes etc. Initially, following research by leather chemists, glutaraldehyde found its first useful role in the leather industry as a tanning agent. It also found uses as a fixative in electron microscopy as a cross-linking agent for protein and enzymes (Russell & Hopwood, 1976).

Then in 1962, Pepper & Lieberman reported on the potential anti-microbial activity of glutaraldehyde which led to further studies and in 1963 Pepper & Chandler reported that glutaraldehyde in alkaline isopropanol was a superior sporicidal agent to formaldehyde and glyoxal (Scott & Gorman, 1991). In the same year Stonehill *et al.* published a paper on "Buffered Glutaraldehyde, a new chemical sterilizing agent". They found glutaraldehyde in aqueous solution to be mildly acidic and although very stable at this acidic pH, sporicidal activity was evident only at pH 7.5-8.5. They advocated a 2% wt/vol glutaraldehyde solution activated (made alkaline) with 0.3% sodium

bicarbonate and including a surface tension depressant, an anti-corrosive compound and a non-staining water soluble dye. For ease of use, they recommended that the 2% glutaraldehyde solution be supplied at acid pH, for prolonged shelf life, with a vial containing the additives in a powdered state. It was in this form that, Ethicon first marketed glutaraldehyde as a chemo-sterilizer in 1963 under the name "Cidex". Indeed, this is perhaps still the most common form supplied today, although alkaline glutaraldehyde formulations have been developed with prolonged shelf life. In 1980 the Ethicon patent expired and since then many other formulations have been introduced ie. acid/alkaline and activated /non-activated glutaraldehyde.

Stonehill *et al.* (1963) concluded from their studies that this 2% buffered glutaraldehyde solution fulfilled nearly all the criteria for the ideal instrument disinfectant as described by Spaulding in1957. The solution was bactericidal within 2 minutes of immersion, tuberculocidal in 10 minutes and sporicidal in 3 hours. It was non-corrosive to metal instruments, non-damaging to lensed instruments or their cement systems and was only slightly to moderately toxic.

Their results on the efficacy of alkaline glutaraldehyde have been confirmed by numerous studies, over the past 30 years. Alkaline glutaraldehyde remains a very useful disinfectant because it has this broad spectrum of activity and is non-corrosive and non-damaging. At present, 2% glutaraldehyde is the first line agent for the disinfection of heat labile equipment such as, flexible endoscopes. However, glutaraldehyde is currently classed as a hazardous substance in the UK. Contrary to the earlier belief, it is toxic, irritant and allergenic and problems may arise from contact with the liquid or its vapour.

The Control of Substances Hazardous to Health Regulations 1988 (COSHH) requires employers to assess the risks to the health of staff from exposure to hazardous substances such as glutaraldehyde. This means a completely enclosed process must be used or there must be adequate extraction and ventilation to keep glutaraldehyde vapour levels to a minimum. Even with these complications in the use of glutaraldehyde, it will remain the recommended first line disinfectant for endoscopes until a disinfectant is available that can match its effectiveness within a short contact time and will not damage endoscopic equipment.

It is now well established that the presence of free aldehyde groups is essential for microbicidal activity. Therefore its efficacy as a disinfectant/sterilant is particularly dependant on the state of the molecule which in turn is greatly influenced by time, temperature and pH (Fig 1.3.7.2). At acid pH, glutaraldehyde exists as a monomeric dialdehyde in equilibrium with its cyclic hemiacetal and polymers of its cyclic hemiacetal (Fig.1.3.7.3). This equilibrium results in a very stable solution with a long shelf-life; however, its microbicidal activity is reduced, due to the absence of free aldehyde groups. At neutral and basic pH, glutaraldehyde undergoes an aldol condensation with itself followed by loss of water resulting in significant amounts of α,β unsaturated aldehydes (Fig. 1.3.7.4). With time and increasing pH these progress further to higher polymeric forms resulting in loss of free aldehyde groups. Therefore alkaline glutaraldehyde is a very effective microbicidal agent but loss of activity is observed with time (i.e. on storage) and increase in pH. Activity of alkaline glutaraldehyde is also greatly reduced through application of heat but acidic solutions become more microbicidal with increasing temperature. This can be

explained by loss of reactive aldehyde groups through polymerization in alkaline glutaraldehyde and displacement of equilibrium in acid solutions towards the monomer (Scott & Gorman, 1991).

Fig.1.3.7.1 Synthesis of glutaraldehyde

(Russell & Hopwood, 1976)

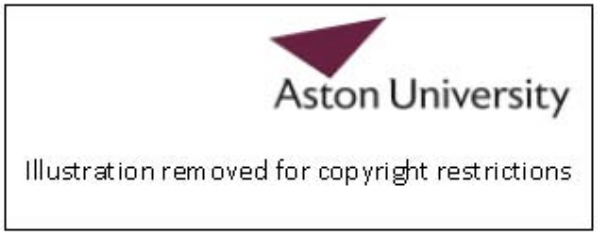


Fig 1.3.7.2 Influence of temperature, pH and storage time on the activity of glutaraldehyde (Scott & Gorman, 1991)

Fig. 1.3.7.4 Glutaraldehyde in acid media
(Scott & Gorman, 1991)

1.4: METHODS FOR TESTING DISINFECTANTS; PAST, PRESENT & FUTURE

The purpose of testing disinfectants is to establish if these products fulfil their objectives, i.e. elimination or destruction of microorganisms (Reybrouck, 1992). In theory, this is a simple task; microorganisms are exposed to disinfectants at in-use concentrations for a specified time, then removed and the number of survivors assessed. In practice, however, disinfectant test methods are fraught with complications due to the number and variability of factors which can influence the accuracy and reproducibility of the test. According to Crowshaw (1981), if a disinfectant test method is to provide useful information three general principles must apply. It should: 1) give information that can be interpreted in terms of practical use; 2) give repeatable and reproducible results; and 3) be adequately controlled. To meet these requirements it is necessary to standardize every aspect of the method including the test organism and its maintenance, neutralization of disinfectant residues, recovery of survivors etc. The need to standardize was recognized as early as 1903 (Rideal & Walker, 1903) and is still being debated today.

For over a century researchers have devoted much time and money to the development of disinfectant test methods; however, there is still no internationally accepted technique. Instead, different countries and even different professions within the same country have their own standard methods based on their own regulations (if any) and requirements. The current main "official" methods for assessing disinfectant activity are listed in Table 1.4.1. This is confusing not only for the manufacturers but also for the consumer, as a

disinfectant which is accepted for use in one country, is not necessarily recommended for the same use in another country.

Table 1.4.1 Main "Official" Methods for assessing disinfectant activity

BSI (UK)	**1960**	BS3286: Disinfectant activity of QACs - *Suspension*
	1984	BS6471: Antimicrobial value of QAC disinfectant formulations - *Suspension*
	1985	BS 541: Rideal-Walker coefficient - *Suspension*
	1986	BS808: Modified Chick-Martin test - *Suspension*
		BS6734: Antimicrobial efficacy of disinfectant for veterinary and agricultural use - *Suspension*
	1987	BS6905: Modified Kelsey-Sykes Test (Disinfectants used in dirty conditions in hospitals) - *Suspension*
AFNOR (France)	**1986**	NFT 72-281: Methods of airborne disinfection of surfaces - *Carrier*
	1987	NFT 72-150 + NFT 72-151: Bactericidal activity - *Suspension*
		NFT 72-200 + NFT 72-201: Fungicidal activity - *Suspension*
	1988	NFT 72-230 + NFT 72-231: Sporicidal activity - *Suspension*
		NFT 72-170 + NFT 72-171: Bactericidal activity in the presence of specific interfering substances - *Suspension*
		NFT 72-190: Germicidal carrier method - *Carrier*
	1989	NFT 72-300 + NFT 72-301: Suspension test
		NFT 72-180: Virucidal activity (viruses of vertebrates)
		NFT 72-181: Virucidal activity (bacteriophages)
AOAC 14th ed USA	**1984**	Phenol coefficient methods - *Suspension*
		Use-dilution Germ carrier test - *Carrier*
		Use-dilution methods Chlorine (av) in disinfectants - *Capacity*
		Fungicidal activity of disinfectants - *Suspension*
		Germicidal spray for products as disinfectants - *Carrier*
		Germicidal and detergent sanitizing action of disinfectants - *Suspension*
		Sporicidal activity of disinfectants - *Carrier*
		Tuberculocidal activity o disinfectants - *Carrier*
		Disinfectants for swimming pools - *Suspension*
DGHM (Germany)	**1981**	Bacteriostatic and fungistatic activities
		Bactericidal and fungicidal activities
		Influence of proteins and detergents
		Carrier method (tuberculous bacilli, bacteria and fungi)
		Control of disinfection of hands, clothes an surfaces
		Control of disinfection of tuberculous sputum, faeces and instruments
Dutch		5-5-5 suspension test method (bactericidal, fungicidal and sporicidal activity)
Council of Europe	**1987**	Test methods for the antimicrobial activity of disinfectants in food hygiene - *Suspension*

Cremieux & Fleurette (1991)

Adding to this confusion is the number of tests necessary to assess bactericidal, sporicidal, virucidal, tuberculocidal and fungicidal activity of the disinfectants. No single method can be used for all these test organisms due to the need for individual considerations depending on the type of test organism. Hugo (1991) subdivided the classification of disinfectant test methods in an attempt to clarify the situation (Table 1.4.2).

Table 1.4.2: Classification of disinfectant tests

A. Classification according to test organism

1. Determination of antibacterial activity: non-acid fast vegetative bacteria: bactericidal tests, acid-fast bacteria: tuberculocidal tests
2. Determination of antifungal activity: fungicidal tests
3. Determination of antiviral activity: virucidal tests

B. Classification according to the type of action

i.e. static vs cidal tests

C. Classification according to test structure

1. In-vitro tests: suspension, capacity and carrier tests
2. Practical tests: tests determining the efficacy of the disinfection of surfaces, rooms, instruments, skin, fabric etc
3. In-use tests

D. Classification according to the aim of the test

1. First testing stage: preliminary tests, screening tests: tests determining whether a chemical substance or preparation possesses antibacterial properties
2. Second testing stage: test determining the use-dilution of a disinfectant for a specific application
3. third testing stage: tests in the field *in loco* or *in situ* determining the usability of the disinfectant in practice-clinical effectiveness studies

Taken from Hugo (1991)

Cremieux & Fleurette (1991) present the current status as the evolution of test methods along two parallel and complementary lines: 1) the development of in-vitro tests (specific activity of the product); and 2) development of methods that simulate in the laboratory, the conditions of practical use. From the 1800s to the present day, disinfectant test methods have developed by progressive improvements to those first described by Koch in 1881 and Rideal & Walker in 1903.

Although disinfectants were being evaluated long before the "golden age of bacteriology", Koch is credited with the first extensive article on chemical disinfection (Chick & Martin, 1908; Hugo, 1991). *Bacillus anthracis* spores were dried onto silk threads, which were then soaked in mercuric chloride solutions for varying lengths of time. He determined whether spores survived by inoculating broths with the threads. Not only is this the first description of a carrier test, it is also the first example of inaccurate results due to failure to neutralize disinfectant residues. In 1889 and 1890 Geppert published two papers introducing another approach to disinfectant testing (Sykes, 1965). He demonstrated a qualitative bacterial suspension test in which aliquots of the disinfectant and bacterial suspensions were mixed, allowing removal of samples from the same mixture after various contact times. He also introduced the use of neutralizing agents by employing ammonium sulphide to neutralize the mercuric chloride residues carried over to the recovery media. In doing so, he obtained more realistic values than those of Koch. The simple structure of this method allowed for several concentrations of disinfectant to be assessed at several exposure times.

The work of Geppert is often overshadowed by the Rideal-Walker suspension test of 1903. Their "phenol coefficient method" is currently described as a major advance in the development of a standard test method. In this method, the disinfectant under test was compared with phenol which was used in every test as a reference standard, thereby increasing the reproducibility of the method. Dilutions of the unknown disinfectant were compared with 5 standard dilutions of phenol for their activity against *Salmonella typhi* NCTC 786. At time intervals of 2.5, 5, 7.5 and 10 minutes, a standard loopful of the mixture was removed to 5ml recovery broth. The phenol coefficient was calculated by dividing the dilution of the test disinfectant which allows growth at 2.5 and 5 minutes but not at 7.5 and 10 minutes by the dilution of phenol giving the same response.

Interestingly almost 125 years prior to this Rideal-Walker method, John Pringle (1750) published a report on the evaluation of salts as preservatives for meat (Block, 1991). He added pieces of meat to glass jars containing solutions of different salts, which he then incubated. As this was before the scientific identification of microbial species, he judged his end point to be the presence or absence of smell. He also used sea salt as a reference standard, with which he compared the relative efficiency of the salts under test. This method is very similar, in structure to the Rideal-Walker method.

Although all details of this phenol coefficient method were specified and standardized, Crowshaw (1981) points out that its disadvantages far outweighed its advantages. No neutralization system was employed, the effect of interfering substances was not considered, *Salmonella typhi* was an unpopular and unsuitable test organism and

the use of phenol allowed comparison only with similar disinfectants, i.e. like with like.

In 1908 Chick & Martin modified the method by extending the contact times to 30 minutes and introducing an organic soil which they felt provided a more realistic test. The organic soil originally used was 3% dried sterilized human faeces but this was later replaced by 5% w/v yeast. No other aspects of the test were changed and therefore the Chick-Martin method incorporated all the disadvantages of the original Rideal-Walker. Both the British Standard Institute (BSI, 1961) and the American Association of Official Analytical Chemists (AOAC, 1960) adopted and published the phenol coefficient method of Rideal and Walker as a standard test method and its numerous modifications and improvements. In the US the Rideal-Walker method was used for several years until in 1911 Anderson and McClintic endeavoured to overcome some of its recognized defects and published a modified method which became known as the Hygienic Laboratory Method of 1912 and subsequently 1921 (Sykes, 1965). This method successfully eliminated some of the faults in the Rideal-Walker method but introduced others. Hence the method soon fell into disrepute. Following on from the experiences of Rideal & Walker and the Hygienic laboratory Method, Shippen & Reddish produced a new method which was later adopted as the Food and Drugs Agency (FDA) official method (Ruehle & Brewer, 1931). This was later superseded by the AOAC official phenol coefficient method of 1960. The AOAC (1984) and BSI (1985) still publish a phenol coefficient method in their list of standard disinfectant test methods.

In 1960 BSI published another standard method under the title of the Method for Laboratory Evaluation of Disinfectant Activity of Quaternary Ammonium Compounds. This method is the first example of a quantitative suspension test. It was designed because QACs could not be evaluated using the standard phenol coefficient method. Three test organisms were used; *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*, in the presence of milk as an organic load. Different contact times (up to 30 minutes) and temperatures were tested and a neutralizer was added to the recovery medium. Different elements in the method can be varied depending on the aim of the test. Many quantitative suspension tests have since been described some of which have been adopted as national standard test methods.

The Dutch standard suspension test (Van Klingeren *et al.*, 1977) is known as the 5-5-5 suspension test. The name arose because originally 5 test organisms (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Saccharomyces cerevisiae*) were exposed to the disinfectant under test for 5 minutes and a 5 $\log_{10}$ reduction was used as an indication of efficacy. Originally the test was designed for evaluating disinfectants in the food industry, hence the organic soil chosen to simulate dirty conditions was albumin. The test varies slightly depending on whether the disinfectant is intended for use in hospitals, the food industry or for veterinary use, e.g. in the tests for disinfectants for use in hospitals, the test organisms are type strains of *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella typhimurium* and *Staphylococcus aureus*. France and Germany also publish standard suspension tests in the form of AFNOR and DGHM guidelines.

In 1970, a European committee for the standardization of disinfectants was formed. With little progress, this committee was disbanded in 1978 and the task was taken up by the Council of Europe. Under its auspices, a new European suspension test was developed for use within the food industry, which could easily be modified for application in other areas (Council of Europe, 1987; Ayliffe, 1989).

Even as the quantitative suspension tests were developing and being made more stringent, e.g. presence of an organic soil, these tests were not sufficiently realistic to simulate practical use conditions of the disinfectants. A well recognized "practical test" is that of Kelsey & Sykes. They originally published their "capacity test" in 1965 (Kelsey & Sykes, 1965) and a modified test in 1969 (Kelsey *et al.*, 1969). This was further improved by Kelsey & Maurer in 1974. The test was designed to assess the ability of a disinfectant to retain its activity in the presence of an increasing organic load, e.g. mop in a bucket. The Kelsey-Sykes test as described by Kelsey & Maurer in 1974 is still widely used in the UK and Europe. Four test organisms i.e. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Proteus vulgaris* are suspended in standard hard water (clean conditions) or yeast (dirty conditions). Successive additions of the bacterial suspensions are added to the disinfectant, each addition increasing the organic and microbial load.

Although suspension tests give information on the activity of disinfectants under varied conditions, they give no indication of the effectiveness of the disinfectants against microorganisms dried onto surfaces. From the first carrier tests of Koch in 1881, many considered

this type of test to be more realistic, therefore carrier/surface tests were developed in parallel with the suspension tests.

The thread method of Koch (1881) was used by many workers to assess the value of numerous disinfectants. In 1890, Behring used it to assess the efficacy of a series of disinfectants against anthrax spores and also vegetative bacteria including streptococci and staphylococci (Sykes, 1965; Chick & Martin, 1908). Esmarch in 1887 used this method to compare the activity of creolin, a new emulsified disinfectant against carbolic acid using *Bacillus cholerae*, streptococci and anthrax spores (Chick & Martin, 1908).

In 1897 Kronig and Paul carried out similar tests to those of Koch, but the spores were dried onto garnets instead of silk threads (Sykes, 1965). This method is of particular importance historically as they made the important observation that bacteria were not killed simultaneously but were destroyed at a measurable and orderly rate. They also noted the importance of the concentration and temperature of the disinfectant. The work carried out with the help of the Japanese physical chemist Ikeda, established the science of disinfection dynamics (Sykes, 1965). As a result of their studies they laid down the general laws, that in any comparison of disinfectants, close regard must be paid to the following conditions;

1. Constancy of number and species of bacterium used
2. Constancy of temperature
3. Constancy of nutrient medium for test cultures
4. Absence of other organic matter during disinfection

These laws are repeated time and again in even the most current publications dealing with disinfectant test methods.

Delepine (1907) adapted Koch's method to assess the activity if disinfectants against vegetative bacteria, e.g. *Salmonella typhi* and *Escherichia coli*, instead of spores. After this, it was not until 1933 that interest in carrier test methods was stimulated again by Jensen & Jensen. They published a report of tests performed by drying *Staphylococcus aureus* onto cover slips at 37°C for 30 minutes, immersing them in a series of dilutions of the disinfectant for 2 minutes, then rinsing twice with sterile water and inoculating into 10ml nutrient broth (Sykes, 1965). In 1945, Mallman & Hanes published their test which used glass cylinders as the carriers. They also incorporated appropriate neutralizers in the subculture medium. After slight modifications this method was adopted as the official AOAC use dilution confirmation test (Mallman & Hanes, 1945). Authorities in Belgium, France, The Netherlands and Germany all currently recommend standard quantitative surface tests, most of which were developed by improving on the original DGHM qualitative surface test.

In 1989, a Central European de Normalisation (CEN) committee (TC216) was established to produce harmonized methods for disinfectants and antiseptics used in food hygiene, medicine, agriculture and veterinary practice (Bloomfield *et al.*, 1995). The committee have decided that tests should comprise a number of phases:

phase1 - suspension tests to define minimum standards for bactericidal, fungicidal and sporicidal activity.

phase 2 - suspension and surface tests carried out under conditions simulating practical use.

Phase 1 tests have been agreed and are published as "provisional European Norms", i.e. prEN 1275 and pr EN 1650 and phase 2 tests are in the final stages of preparation

Table 1.4.3 lists a sample of some of the disinfectant test methods currently under the guidance of CEN TC216 (Simpson RA., personal communication, 1997)

Table 1.4.3: Disinfectant test methods for food hygiene, domestic and institutional use currently under the guidance of CEN TC216

Reference	Title
216 004	Chemical disinfectants and antiseptics - Quantitative suspension tests for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas - Test method and requirements
216 005	Chemical disinfectants and antiseptics - Quantitative suspension tests for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas - Test method and requirements
216 006	Food, domestic and institutional areas - Specific quantitative suspension test for the evaluation of sporicidal activity of antiseptics and disinfectants
216 027	Food, domestic and institutional areas - Specific quantitative suspension test for the evaluation of virucidal activity - Phages
216 028	Food, domestic and institutional areas - Specific quantitative surface test for the evaluation of bactericidal, fungicidal, sporicidal activity
216 021	Chemical disinfectants and antiseptics - Medical instrument disinfection - Test method and requirements (Phase 2/step 1)
216 022	Chemical disinfectants and antiseptics in the medical area: Virucidal activity (Phase 2/step 1)
216 023	Chemical disinfectants and antiseptics in the medical area: Fungicidal activity (Phase 2/step 1)

1.5: GLUTARALDEHYDE RESISTANT *MYCO. CHELONAE*

Mycobacterium chelonae, is being isolated with increasing frequency from decontaminated (i.e. cleaned and disinfected) flexible endoscopes and endoscope washer disinfectors (Nye *et al.*, 1990; Fraser *et al.*, 1992; Spach *et al.*, 1993).

Automated systems are now used for decontaminating endoscopes in most hospitals as they are more convenient than manual processing and protect staff from skin and eye contact and, in some instances, from respiratory exposure to glutaraldehyde vapour (Bradley & Babb, 1995). In a recent study of gastro-intestinal endoscopy units in the UK, 98.6% were reported to be using 2% glutaraldehyde (Wicks, 1994). Two% glutaraldehyde is recommended by the Department of Health and professional societies as the most suitable disinfectant for endoscope disinfection.

Reports of contaminated endoscopes have highlighted the need to destroy or remove atypical mycobacteria, including *Myco. chelonae*, present in instrument rinse water and automated systems. If these organisms are not removed or destroyed, they may be deposited in, or on endoscopes, during processing. In bronchoscopy, this has, on occasions, led to misdiagnosis of tuberculosis as acid-fast bacilli are deposited in the channel of the bronchoscope and these are transferred to bronchial lavage samples for ZN staining (Uttley *et al.*, 1990).

Van Klingeren & Pullen (1993) showed that machine-associated isolates of *Myco. chelonae var. abscessus* were far more tolerant to 2% glutaraldehyde than a laboratory strain of *Myco. chelonae* and *Myco.*

terrae ATCC 15755 the official test organism for mycobactericidal testing in Germany and the Netherlands. Two strains of *Myco. chelonae var. chelonae* which were being consistently isolated from 2 separate endoscope washer disinfectors and processed endoscopes were included in this study to assess their sensitivity to glutaraldehyde and other disinfectants,

Both these strains were found to be very resistant to 2% alkaline glutaraldehyde and further tests were carried out in an attempt to understand the mechanism of this reduced affinity for glutaraldehyde. These tests included MICs, resistance to other aldehydes and heat, fatty acid and mycolic acid analysis and others.

1.6: AIMS AND OBJECTIVES

Much work has been done on the resistance of *Myco. tuberculosis* to chemical agents; however, variations in test protocols have resulted in variable efficacy data. There is a wide range of test methods in use, some of which have been adopted by official bodies including the AOAC in the USA, DGHM in Germany, AFNOR in France and BSI in the UK. However, there is no one test or series of tests accepted internationally. Flaws in current protocols for mycobactericidal activity include a lack of proper quantitation, unrealistically long contact times at higher than ambient temperatures, ineffective neutralizer/recovery systems, absence of a suitable organic load and unsuitable surrogates for *Myco. tuberculosis* (Ascenzi *et al.*, 1991; Sattar *et al.*, 1995).

The aim of this study was to review current methods for establishing mycobactericidal activity and having done that, to select or create a practical, accurate, realistic, reproducible and meaningful test method. This would then be used to determine the sensitivity of *Myco. tuberculosis* and other mycobacteria of increasing clinical importance, to a wide variety of disinfectants.

Concern has been expressed at the increasing frequency of isolation of *Myco. chelonae* from endoscopes and endoscope washer disinfectors (Nye *et al.*, 1990; Fraser *et al.*, 1992; Spach *et al.*, 1993; Griffiths *et al.*, 1997). Two known glutaraldehyde resistant washer disinfector isolates were included in this study to assess their resistance to the selected disinfectants. In addition, their hydrophobicity, resistance to other aldehydes, heat and antibiotics would be assessed and their short chain fatty acids and mycolic acids analyzed to determine if their phenotypic characteristics differ from those of the type strain of *Myco. chelonae* NCTC 946

2.0 MATERIALS AND METHODS

2.1 TEST ORGANISMS

Seven test organisms were chosen for inclusion in this study:

Mycobacterium chelonae var. chelonae NCTC 946

Mycobacterium chelonae var. chelonae (machine isolate x2)

Mycobacterium fortuitum NCTC 10394

Mycobacterium terrae NCTC 10856

Mycobacterium avium-intracellulare (clinical isolate)

Mycobacterium tuberculosis H37 Rv NCTC 7416

All type strains of the test organisms were obtained freeze dried from the National Collection of Type Cultures, Colindale Public Health Laboratory. The clinical isolate of MAI was supplied by Dr. John Holton, University College Hospital London and the machine isolates of *Myco. chelonae* were obtained from two "KeyMed Autodisinfector" endoscope washer disinfector machines at the Epping and Harefield hospitals. The machine isolates were typed at the Mycobacteria Reference Laboratory, Cardiff (now situated at Dulwich Public Health Laboratory).

2.2 SAFETY NOTE

The current "Hazard Groups" for the mycobacterial species used in this study are listed in Table 2.2.1 as endorsed by the Advisory Committee on Dangerous Pathogens in their most recent publication on the "Categorisation of Pathogens according to hazard and categories of containment" (ACDP, 1995). *Myco. terrae*, a hazard group 1 organism,

is "an organism that is most unlikely to cause human disease". *Myco. chelonae* and *Myco. fortuitum*, placed in hazard group 2, "may cause human disease and might be a hazard to laboratory workers but are unlikely to spread to the community". Laboratory exposure to hazard group 2 organisms rarely produce infection and effective prophylaxis is usually available. *Myco. tuberculosis* H37 Rv (the virulent strain known to cause human tuberculosis), *Myco. avium* and *Myco. intracellulare* are all classed as hazard group 3 organisms, i.e. "may cause severe human disease and present a serious hazard to laboratory workers". There may be a risk of spread to the community but treatment is usually available. Hazard group 1 and 2 organisms may be used on an open bench in a laboratory by trained laboratory personnel, however the category group 3 organisms require special containment facilities. The work must be carried out under strict supervision by person(s) trained in handling pathogenic organisms and in the use of safety equipment and controls.

Myco. tuberculosis, *Myco. avium* and *Myco. intracellulare* must NOT be used outside containment level 3 facilities (Appendix A).

Table 2.2.1 Hazard Groupings for mycobacterial species used in this study

Organism	Hazard Group (Category)
Myco. chelonae	2
Myco. fortuitum	2
Myco. terrae	1
Myco. avium-intracellulare	3
Myco. tuberculosis H37 Rv	3

2.3 CULTURE MEDIA

There are three main types of culture media currently in use for the growth of *Myco. tuberculosis* (BBL manual, 1985; Collins *et al.*, 1985; Difco manual, 1988; Wayne, 1994).

1. Liquid media, e.g. Middlebrook 7H9, Proskauer-Beck
2. Egg based solid media, e.g. Lowenstein Jensen, Petragnani
3. Agar based, e.g. Middlebrook 7H11, 7H10,

The **liquid media** used in much of the early research with *Myco. tuberculosis*, were all very similar in their simplicity and clearly defined constituents. They all contained glycerol as a carbon source, either asparagine or an ammonium salt as a nitrogen source, phosphate salts, citrate, magnesium sulphate and trace elements as impurities in the defined ingredients (Wayne, 1994). The media were supplemented with iron. Although these media were suitable to give very high yields of *Myco. tuberculosis* for chemical analysis, they were unsuitable for quantitative studies of growth. Due to the very high amount of clumping, plate counts and optical measurements were neither accurate nor reproducible (Wayne, 1994).

In the 1940s Dubos and co-workers showed that this clumping could be minimized by adding a detergent to the medium (Wayne, 1994). His detergent of choice was Tween 80, a polyoxyethylene derivative of sorbitan mono-oleate. Addition of this particular detergent to the medium resulted in well-dispersed suspensions or at least minimal clumping which could be dispersed easily with gentle agitation. However, Tween 80 releases traces of oleate into the medium which is toxic to *Myco. tuberculosis*. Dubos solved this problem with the

addition of albumin, which complexes with the oleate and removes the possible toxicity (Wayne, 1994). The result of Dubos' research is commercially available today as a dehydrated basal medium, the Dubos broth base to which a sterile glucose albumin supplement is added, commonly known as Dubos Tween-albumin broth. For various reasons it is sometimes unsuitable to use a Tween 80 containing medium and other detergents such as Triton may be used.

Glycerol is commonly added to liquid medium to produce large numbers of *Myco. tuberculosis*. This carbohydrate source is known to stimulate the growth of *Myco. tuberculosis*, however care must be taken if using glycerol, for 2 reasons: 1) it is inhibitory to *Myco. bovis*; and 2) the presence of glycerol greatly stimulates oxygen consumption by *Myco. tuberculosis* and may actually lead to cell death if supplies of dissolved oxygen become severely depleted (Wayne, 1994).

A relatively recent addition to the range of liquid media for the primary isolation of *Myco. tuberculosis* is the Middlebrook 7H12 broth. This is a system manufactured as BACTEC 12B vials by Becton Dickinson Diagnostic Instrument Systems for the radiometric detection of growth in the BACTEC TB-460 system (Heifets & Good, 1994). The BACTEC system is currently the most popular, reliable and sensitive method for the isolation and recovery of *Myco. tuberculosis* from clinical specimens (Roberts *et al.*, 1991; Heifets & Good, 1994). The 7H12 is supplied in 4ml vials which also contain antibiotics and a ^{14}C labelled substrate. As the mycobacteria metabolize, $^{14}CO_2$ is liberated and detected by the BACTEC 460 instrument as a growth index. A growth index of ≥ 10 is considered significant. This system has been shown to increase the recovery rate and reduce the time of recovery of *Myco. tuberculosis* from

19.4 days (conventional media) to 8 days (BACTEC). However, the growth of all other organisms is not completely suppressed and it is necessary to perform an acid-fast stain from the culture vial and subculture to conventional media. It is recommended that Middlebrook 7H10 or 7H11 be inoculated and incubated in parallel with the BACTEC system. The BACTEC TB system is also being used for antimicrobial susceptibility testing of *Myco. tuberculosis* and a number of recent reports on the efficacy of *Myco. tuberculosis* against disinfectants have used this system (Broadley *et al.*, 1993; Cutler, 1995). However, in an unpublished study by Rallings (1991) to assess the system for assessing disinfectant efficacy, it was shown to be less efficient in recovering small numbers of survivors after exposure to disinfectants than 7H11 agar.

The **agar based media** usually comprise semi-synthetic basal media, which are enriched with supplements. Cohn & Middlebrook formulated a series of defined culture media, i.e. the 7H series during the 1950s. These merely involved small modifications to the earlier media of Dubos. Dubos oleic albumin agar is almost identical to his liquid medium except the basal medium is solidified with agar and Tween 80 is omitted. Also, oleic acid is substituted for glucose in the supplement. Cohn and Middlebrook improved on this formulation in a bid to enhance repair of possible damaged organisms. 7H10 is a modified formulation of oleic acid-albumin agar and 7H11 agar is a further modification of this with the addition of 1g of pancreatic digest of casein per litre. This digest was shown to enhance the growth of strains of *Myco. tuberculosis* that were observed to grow poorly or not at all on 7H10 (BBL manual, 1985; Difco manual, 1988).

The **egg-based media** are widely used for primary isolation of *Myco. tuberculosis* from clinical specimens. Krasnow & Wayne (1969) advised that these media tend to yield a higher proportion of positives from direct clinical specimens than do semi-synthetic agar media. Collins *et al.* (1985) recommend that egg-based media should be the first choice for culturing sputum and both egg-based and liquid media containing antibiotics should be used for non-repeatable samples, e.g. CSF. The egg-based media are very rich and complex. It is believed that the phospholipids and proteins they contain tend to bind to and/or neutralize toxic products in clinical specimens, hence their success in primary isolation. They are, however, very complex and not very reproducible, as the quality of constituents can vary from batch to batch and they are therefore not recommended for research purposes.

Although some mycobacteria, e.g. *Myco. chelonae* and *Myco. fortuitum* will grow very rapidly on blood agar, the recommended Middlebrook media for *Myco. tuberculosis* were used throughout this study in order to maintain standard, reproducible methods.

Solid media: 7H11 Agar Base supplemented with Middlebrook OADC (i.e. oleic acid, albumin, dextrose and catalase) enrichment and glycerol. (Becton Dickinson UK Limited, Between Towns Road, Cowley, Oxford)) was used for cultivation of test organisms. This medium plus supplements contains a variety of inorganic salts that provide substances essential for the growth of mycobacteria (See Table 2.3.1).

Liquid Media: Middlebrook 7H9 containing ADC (i.e. albumin, dextrose, and catalase) enrichment (Difco Laboratories, PO Box, 14B

Central Avenue, East Molesey, Surrey) was used as the liquid medium (Table 2.3.2)

Slopes : Lowenstein-Jensen slopes (Difco Laboratories), an egg based medium was used for storage of test organisms (Table 2.3.3). This medium supports the growth of a wide variety of mycobacteria.

Table 2.3.1: Middlebrook 7H11 Agar

7H11 Agar Base Ingredients g / litre	
Pancreatic Digest of Casein	1.0
Magnesium sulphate	0.05
Ferric Ammonium Citrate	0.04
Sodium Citrate	0.4
Ammonium Sulphate	0.5
Monosodium glutamate	0.5
Disodium Phosphate	1.5
Monopotassium Phosphate	1.5
Agar	13.5
Sodium chloride	0.85
Pyridoxine	0.001
Zinc sulphate	0.001
Copper sulphate	0.001
Biotin	0.0005
Malachite green	0.0005
Calcium chloride	0.00025
OADC Enrichment	
Oleic Acid	0.05
Bovine albumin Fraction 5	5.0
Dextrose	2.0
Catalase (Beef)	0.0004
Sodium chloride	0.85
Distilled water	100ml

Table 2.3.2: Middlebrook 7H9 Broth

7H9 Broth Ingredients g / litre	
Ammonium sulphate	0.5
L-Glutamic Acid	0.5
Sodium Citrate	0.1
Pyridoxine	0.001
Biotin	0.0005
Disodium Sulphate	2.5
Monopotassium Phosphate	1
Ferric Ammonium Citrate	0.04
Magnesium sulphate	0.05
Calcium chloride	0.0005
Zinc sulphate	0.001
Copper sulphate	0.001
ADC Enrichment	
Bovine albumin Fraction 5	0.5
Dextrose	2.0
Catalase (Beef)	0.003
Distilled water	100ml

Table 2.3.3 Lowenstein Jensen slopes

Lowenstein Jensen media Base Ingredients g/600ml	
Asparagine	**3.6**
Monopotassium phosphate	**2.4**
Magnesium sulphate	**0.24**
Magnesium citrate	**0.6**
Potato Flour	**30**
Malachite Green	**0.4**
Lowenstein Jensen slopes g/1600ml final solution	
Medium Base	**37.2**
Glycerol	**12ml**
Distilled water	**588ml**
Homogenized egg	**1litre**

2.4 RECONSTITUTION OF FREEZE-DRIED ORGANISMS

The glass vial was carefully broken in a category 3 safety cabinet. A small amount of 7H9 broth was added to the tube and mixed well to rehydrate the organisms. This was transferred to 100ml 7H9 broth and incubated at the appropriate temperatures, as a stock suspension. Also, 100µl was spread onto 7H11 agar plates and similarly incubated. (Table 2.4.1). Figure 2.4.1 shows growth of the test organisms on Middlebrook 7H11 agar after the appropriate incubation at the appropriate time and temperature.

Table 2.4.1 Incubation Times and Temperatures on Solid Media

Test Organism	Temp (^{0}C)	Time (days)*
Myco. chelonae	30	5
Myco. fortuitum	37	5
Myco. terrae	37	14
Myco. avium-intracellulare	37	21
Myco. tuberculosis	37	21

*Time needed to produce visible colonies on 7H11 agar as shown in Fig. 2.4.1

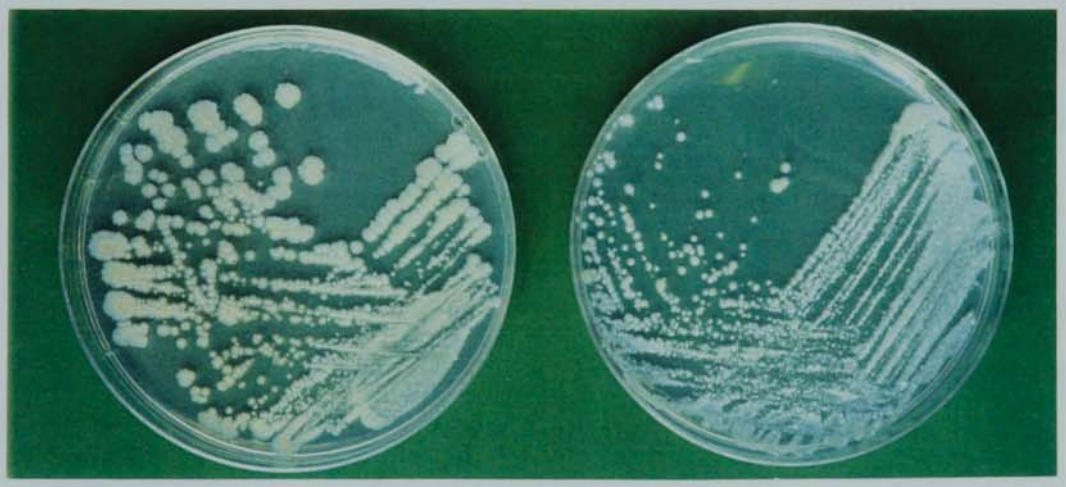
2.5 STORAGE AND MAINTENANCE OF TEST ORGANISMS

All test organisms were initially stored on Lowenstein-Jensen slopes, until sufficient data were obtained to allow reliable storage of early stationary phase suspensions in 7H9 broth at -70^{0}C

Figure 2.4.1 a) *Myco. chelonae* NCTC 946 and *Myco. chelonae*, washer disinfector isolate. b) *Myco. fortuitum* NCTC 10394 and *Myco. terrae* NCTC 10856. c) *Myco. tuberculosis* H37 Rv and *Myco. avium-intracellulare*, clinical isolate. All cultures grown on 7H11

a)

b)



c)

2.6 MEASUREMENT OF BACTERIAL GROWTH IN LIQUID MEDIA

Total Viable Counts (TVC) were used to monitor mycobacterial growth in liquid media. Spectrophotometric measurement was considered but discarded after initial tests as light-scattering is dependent on cell size and, if the cells in a given suspension change due to ageing or laboratory manipulation, they will exhibit alterations of their light scattering properties and appear to be changing in numbers (Carlberg, 1986). Spectrophotometric measurement is particularly unsuitable for mycobacterial suspensions which exhibit various degrees of clumping and leads to inaccurate spectrophotometric measurement. It is also unsuitable in that it gives no indication of contamination of the suspension, an important consideration with mycobacterial suspensions due to their prolonged incubation.

100ml amounts of 7H9 broth were inoculated with one colony of each test organism and incubated at the appropriate temperatures in duplicate. Growth was measured using total viable counts.

2.6.1 Total Viable Counts

The suspension was subjected to mild ultrasonic agitation by immersion of the bottle in an ultrasonic bath (Ultrawave U300) operating at 50-60 Hertz for 10 minutes to break up clumps without damaging the organisms (Piroli *et al.*, 1985) and then shaken gently. 100µl of this suspension was removed to 900µl Ringers solution and serially diluted to 10^{-3}. 100µl of the appropriate dilutions were then spread onto 7H11 agar plates in duplicate, using sterile plastic disposable spreaders and incubated as indicated in Table 2.4.1

2.7 MICROSCOPIC EXAMINATION

Cultures were examined microscopically using the Ziehl-Neelsen stain for acid-alcohol fast bacilli. Microscopic examination gave an indication of the degree of clumping in the test suspensions. A drop of culture was placed on a microscope slide and allowed to air dry at room temperature. When dry, the slide was exposed to ultraviolet light within the cabinet for at least 1 minute. Ultraviolet is highly effective in killing tubercle bacilli suspended in the air (Rubin, 1991). Exposure of the slide to UV light was carried out as a safety procedure, as the slide has to be heat-fixed and viewed under the microscope outside the safety cabinet. The slide was then heat-fixed over a Bunsen burner flame and flooded with hot carbol fuschin. After 5 minutes the stain was washed off and replaced with acid alcohol (3% Hydrochloric acid and 97% ethyl alcohol). This was left for 3 minutes and washed off with water. Malachite green was then added for 1 minute and washed. The slide was dried carefully using blotting paper and viewed under oil immersion at x100 magnification.

2.8 DISINFECTANTS

Several of the most widely used instrument and equipment disinfectants were tested. These are listed in Table 2.8.1 with suppliers, brand names and concentrations

Table 2.8.1: Disinfectants

Disinfectant	Brand Name	Conc.	Supplier
Sodium-dichloroisocyanurate * (Chlorine-releasing agent)	Sanichlor	1,000ppm 10,000ppm	G.H. Wood
Glutaraldehyde †	Asep	2% v/v	Galen
Industrial Methylated Spirits † **	Alcohol	70% v/v	Pharmacy Dept City Hosp. Trust
Peracetic Acid †	NuCidex	3,500ppm	J & J Medical
Peroxygen † *	Virkon	1%, 3%w/v	Antec International
Succinedialdehyde and formaldehyde mixture†	Gigasept	10% v/v	Sanofi Winthrop
Chlorine dioxide † (Chlorine releasing agent)	Tristel	1,100ppm	Hayman MediChem

† = Instrument, * = Environmental, ** = Skin Disinfectants

2.9 DISINFECTANT TEST METHODS

In choosing a test method for use throughout this study, the primary aim was to achieve consistent, reproducible results, without losing sight of the practical applications of the disinfectants. There are numerous reports of "standard" test methods for mycobactericidal activity of disinfectants, using either quantitative suspension or quantitative carrier tests. Following a review of the literature, and in view of the developments of the CEN TC 216 committee, it was decided to use suspension and carrier tests based on the techniques developed by Best *et al.* (1988).

2.9.1 Suspension Test (Fig. 2.9.1.1)

Tests were performed using a water bath to maintain a constant temperature of 20°C. 100µl of the test suspension (See section 2.10.2) was added to 900µl of freshly prepared disinfectant for contact times of 1, 4, 10, 20 and 60 minutes. After the required contact time, 10µl was removed to 990µl neutralization/recovery system, vortex mixed for 20 seconds and serially diluted in Ringers solution to 10^{-3}. 100µl of the initial neutralized samples and subsequent dilutions was spread onto 7H11 agar in duplicate using sterile spreaders. The plates were incubated at the appropriate temperature for the recommended duration (Table 2.4.1). Colonies were enumerated visually and the number of survivors calculated. All tests were carried out in duplicate.

2.9.2 Carrier Test (Figs. 2.9.2.1 and 2.9.2.2)

10µl of the test suspension was placed onto the centre of the base of a sterile glass cup (capacity 600µl - see Fig. 2.9.2.1) supported in a 24 cell well culture plate and left to dry at 25°C for 90 minutes (and no longer than 2 hours). Care was taken to ensure the culture was only placed onto the base of each cup, without touching the side walls. The base of the cups was overlaid with 60µl of freshly prepared disinfectant, which had been allowed to equilibrate to 20°C, and left for contact times of 1, 4, 10, 20 and 60 minutes. After the required contact times the glass cup was removed using sterile forceps and placed aseptically into 2940µl of the neutralization/recovery medium. The mixture was then vortex mixed for 20 seconds and serially diluted in Ringers solution to 10^{-3}. 100µl of the initial neutralized suspension

and subsequent dilutions were spread onto 7H11 agar in duplicate using sterile spreaders. Plates were incubated at the appropriate temperature for the recommended time (Table 2.4.1). Colonies were enumerated visually and the number of survivors calculated. All tests were carried out in duplicate.

Fig. 2.9.2.1 Glass cups used in the carrier test

FIG 2.9.1.1 Diagrammatic Representation of the Suspension Test

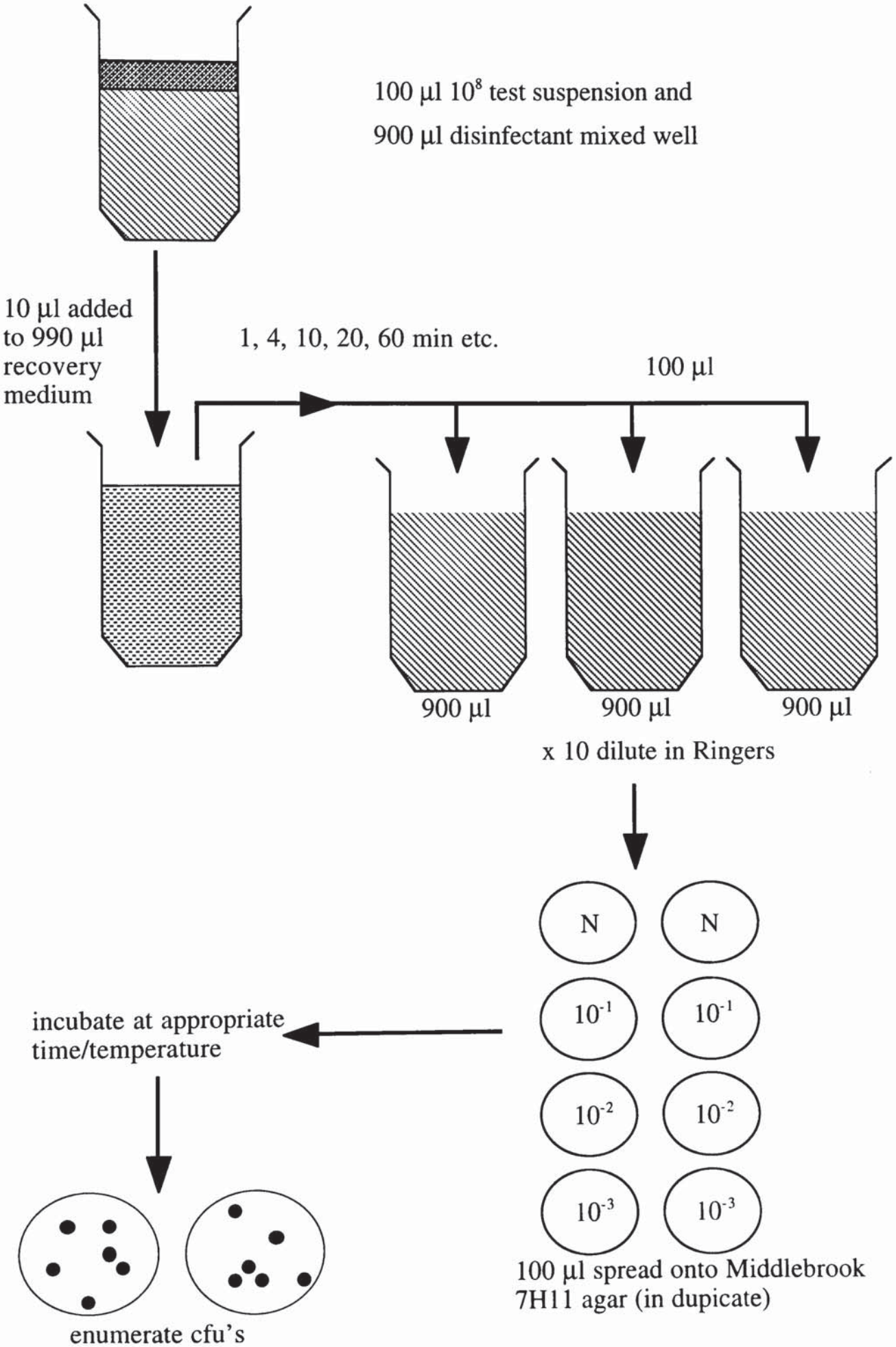
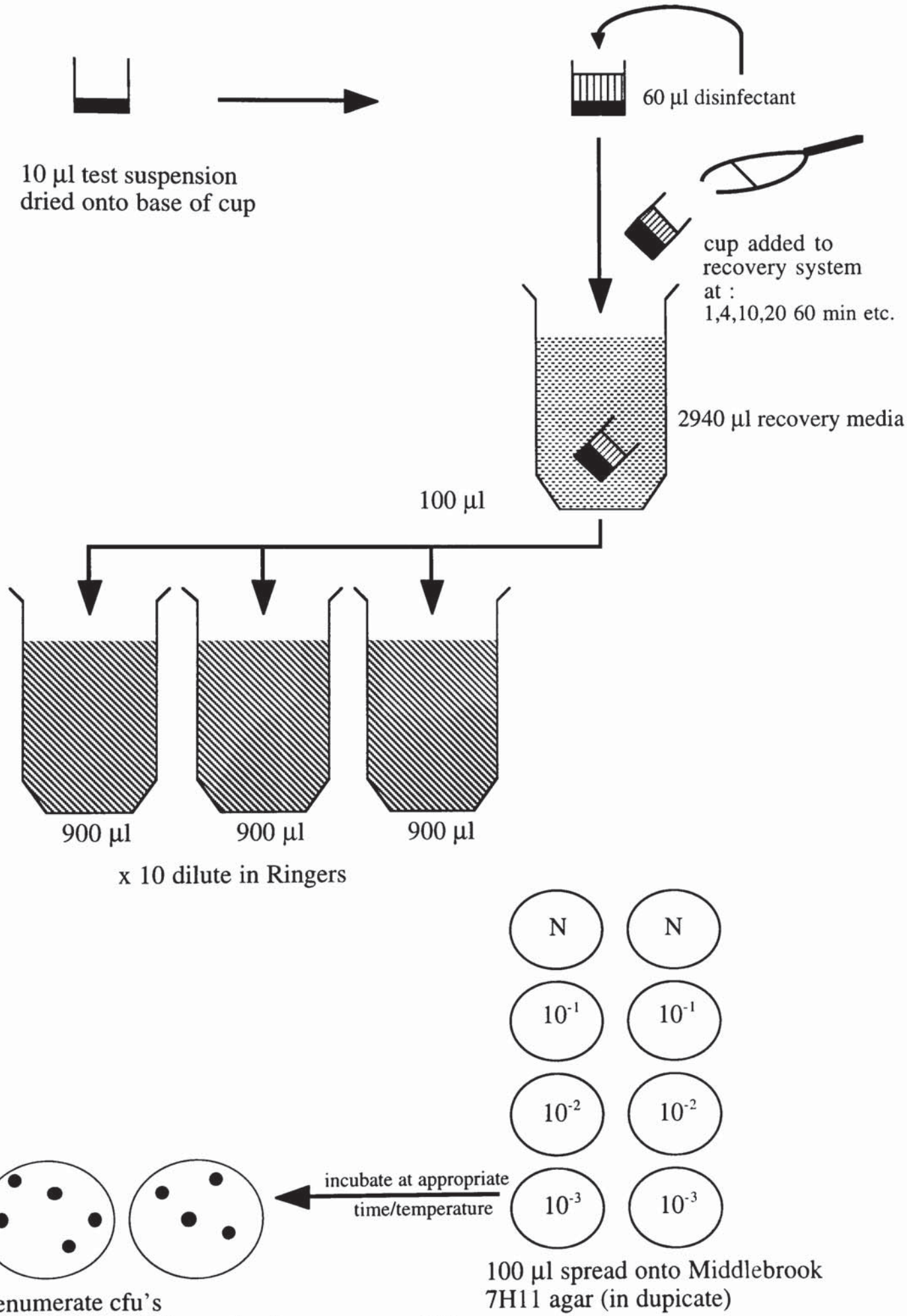


FIG 2.9.2.2 Diagrammatic Representation of the Carrier Test



2.9.3 Controls

Controls were carried out in duplicate for both the suspension and carrier tests using sterile distilled water instead of the disinfectant at contact times of 1 and 60 minutes. All controls were plated in triplicate. The mean of the control counts was taken to be the initial challenge for calculation of disinfectant efficacy.

Note: If there were any discrepancies in results of disinfectant tests and controls, for whatever reason, tests were repeated.

2.9.4 Recovery Efficiency

It was necessary to establish the recovery efficiency of the test organisms using the proposed suspension and carrier test methods. This was to confirm that there was no loss of viable organisms through manipulation during the test methods, i.e. drying onto carriers, failure to recover completely from suspension or from the carriers.

Total viable counts were performed on a test suspension of *Myco. tuberculosis* to establish the titre of the initial inoculum (pre-count). Simulated suspension and carrier tests were carried out using water instead of disinfectant and a contact time of 60 minutes (i.e. the longest exposure time). Colony forming units were enumerated after 3 weeks at 37^0C. All counts were transposed to $\log_{10}$. There should be little or no difference in the counts obtained from the simulated disinfectant tests and the pre-counts (allowing for experimental error).

This recovery efficiency estimation procedure was repeated 5 times for both the suspension and carrier tests.

2.9.5 Calculation of Disinfectant Efficacy

The efficacy of the disinfectants in both suspension and carrier tests was established by converting the pre- and post- disinfection counts to the Log_{10} system and subtracting the mean Log_{10} post-disinfection count from the mean Log_{10} pre-disinfection count. The pre-disinfection count was that obtained from the mean of the controls, i.e. water substituted for disinfectant for contact with test organisms for 1 and 60 minutes. In summary:-

Log_{10} pre-disinfection count - Log_{10} post-disinfection count
= Log_{10} reduction (reduction factor, RF)

The neutralization/recovery system largely based on dilution has a limit of sensitivity of Log_{10} reductions less than 3.00 (dilution of $^1/_{1000}$) in the suspension test and less than 3.48 (dilution of 1/3000) in the carrier test. As a >5.00 Log_{10} reduction is often used as an indication of acceptable efficacy (Council of Europe, 1987; Ayliffe, 1993), the initial Log_{10} challenge must therefore be >8.00 in the suspension tests and > 8.48 in the carrier tests.

2.10 STANDARDIZATION OF TEST METHOD

In the absence of an internationally recognized standard test method for assessing the activity of disinfectants against *Myco. tuberculosis*, many recent publications have highlighted a number of areas in the current disinfectant test methods which require particular attention. They are: 1) preparation of the initial inoculum; 2) use of a suitable organic load; 3) effective neutralization of disinfectant residues and recovery of all surviving test organisms; and 4) potential use of standard hard water as a diluent for the disinfectants.

2.10.1 Initial Inoculum

Four methods, i.e. 2 broth and 2 plate methods currently in use, or under proposal, were evaluated in an effort to obtain a standard reproducible test suspension of a high titre with minimal clumping. Ideally an initial inoculum should have a titre of 10^8-10^9 colony forming units /ml to enable the assessor to demonstrate at least a 5 $\log_{10}$ (99.999%) reduction in numbers in disinfectant tests. Safety and ease of manipulation must also be a prime concern, due to the hazardous nature of the mycobacterial test organisms. The broth method was also evaluated for the preparation of a seed pool of each of the test organisms.

Total viable counts were carried out to assess if the method of preparation of the initial inoculum would provide a high enough titre and disinfectant tests performed to determine the ease and accuracy with which the inoculum could be used.

Method A - Broth method

Each test strain was plated onto 7H11 agar for single colonies. One colony of each of the test strains was then taken from these plates and inoculated into 7H9 broth. The broths were subjected to ultrasonics agitation for 10 minutes (see section 2.6.1) and inverted several times to homogenize the suspension. The broths were then incubated without shaking at 30°C (*Myco. chelonae*) or 37°C (*Myco. fortuitum, Myco. terrae, Myco. tuberculosis* and *Myco. avium-intracellulare*). Growth was measured at time zero, after 1 day and thereafter every second day for 21 days using total viable counts. The test was carried out at least in duplicate. With these data obtained, it was possible to calculate the number of days necessary to achieve a high inoculum titre in the stationary phase.

A fresh batch of cultures was then prepared. After the established number of days, the suspension was mixed with 10% glycerol to give a homogeneous suspension. The glycerol was added as a preservative and helped to maintain an even suspension. 1ml amounts of this suspension were placed into microcentrifuge tubes and stored at -70°C until required.

Prior to use as a test suspension, one of the suspensions was removed from the freezer and left to thaw at room temperature. This was centrifuged, washed and resuspended in sterile distilled water.

Method B - Plate method

One colony of the test organism was plated onto 7H11 agar and incubated at 30°C for 5 days (*Myco. chelonae*), 37°C for 5 days (*Myco. fortuitum*), 37°C for 14 days (*Myco. terrae*) or 37°C for 21 days (*Myco. tuberculosis* and *Myco. avium-intracellulare*). The microbial lawn was harvested into 2ml of sterile distilled water, confined in a bijou bottle with glass beads and subjected to ultrasonic agitation at 50-60 Hz for 10 minutes. The suspension was then vortex mixed to give an even suspension equivalent to a McFarland standard no. 5 (as explained below) (Carlberg, 1986). This was used as the test suspension.

Preparation of the McFarland Standard No. 5

Five ml of 0.048M barium chloride (1.175 w/v) was added to 95ml of 0.36M sulphuric acid (H_2SO_4 :1% v/v) and mixed with a magnetic stirrer. This was distributed into 2ml amounts in clean bijou bottles. The bottles were sealed tightly and stored at room temperature in the dark. They were vigorously agitated on a vortex mixer prior to use

Method C - Plate method

This plate method for preparing the initial inoculum was based on a method proposed at the first meeting of an ad hoc group on mycobactericidal testing CEN/TC216/WG1.

The test organism was spread onto 7H11 agar and incubated at the appropriate temperature for the established time (as described in the

previous section). 7-10 loopfuls of the microbial lawn were harvested into a 100ml sterile glass bottle containing a layer of glass beads ie. just enough to cover the bottom of the vessel. Previously the beads were moistened with a few drops of sterile distilled water. The bacterial growth was then dispersed over the surface of the beads by rotational shaking of the bottle on the bench surface for 5 minutes. Subsequently 10ml sterile distilled water was added and a suspension prepared by gentle swirling. After 30 minutes sedimentation, the supernatant was transferred to a fresh sterile universal (20ml capacity) and allowed to stand for 2 hours. This supernatant was subjected to ultrasonic agitation for 10 minutes at 50-60Hz in an ultrasonic water bath and vortex mixed for 20 seconds prior to use as the test suspension.

Method D - Broth method

(based on a method published by Ascenzi *et al.*, 1991)

One ml of previously frozen *Myco. tuberculosis* H37 Rv culture was added to 10ml 7H9 broth and 0.1% Tween 80 and incubated at 37^{0}C for 21 days with daily manual shaking. The culture was added to 100ml 7H9 broth and incubated as above. After a further 21 days incubation, the 100ml culture was added to a 1 litre 7H9 broth in a 2 litre bottle and incubated at 37^{0}C for 21 days. One day before harvesting 1ml Tween 80 was added to the culture.

2.10.2 Neutralization And Recovery

Whatever the procedure used to evaluate a disinfectant, exposed mycobacteria must be transferred to a recovery medium, which means some of the disinfectant is also carried over (Russell, 1991). Neutralization of the disinfectant residues carried over to the recovery system and selection of a suitable recovery system which supports the growth of surviving test organisms after exposure to the disinfectant are essential for an accurate assessment of efficacy (Cremieux & Fleurette, 1991). If the disinfectant is not completely neutralized and the recovery system not effective in supporting the growth of small numbers of surviving organisms, it may give false results, leading to inaccurate efficacy claims.

Recently in a test method developed by Best *et al.* (1988) to assess the activity of disinfectants against viruses and then modified for use with mycobacteria, a combination of dilution and Tween 80 was used as the neutralization system. In the hope of obtaining a standard system which could be used universally for all disinfectants, neutralization tests were carried out to assess the efficacy of this system. Before the activity of each disinfectant was tested, a neutralization test was carried out to ensure this neutralizer/recovery system would neutralize the disinfectant residues carried over without inhibiting the growth of small numbers of test organisms, i.e. that there was no reduction in the numbers, growth rate or colony size of the test organism.

2.10.2.1 Neutralization System

Preliminary neutralization tests were carried out using *Myco. chelonae* NCTC 946. To mimic test conditions, 100µl of sterile distilled water was added to 900µl of the disinfectant at the highest concentration, mixed and left for 1 minute. 10µl of the mixture was then added to 990µl of Ringers solution containing 0.5% Tween 80. 10µl of the undiluted test suspension of *Myco. chelonae* was added to this mixture (stock) and serially diluted to 10^{-5} in Ringers solution only. 100µl of the stock and subsequent dilutions were spread onto 7H11 agar in duplicate, using sterile spreaders. The plates were incubated at 30^{0}C for up to 5 days. (in plastic bags to prevent drying out due to prolonged incubation) and colony forming units enumerated. The undiluted test suspension was used as the initial count. The test was repeated using water instead of the disinfectant as the control.

Further tests were then carried out using *Myco. tuberculosis* as the test organism. Using the method already described, the effectiveness of a combination of dilution and Tween 80 as a neutralization system was assessed. Controls were again carried out using water instead of disinfectant.

If the system failed to neutralize the disinfectant, 0.5% sodium thiosulphate was added and the test repeated or a neutralizer recommended by the manufacturer was assessed.

2.10.2.2 Recovery Media

The choice of recovery media following a disinfectant test is equally as important as the neutralization. Middlebrook 7H11 agar was the choice for this study, a solid medium being required for quantitative purposes. It has been shown to be more effective than Lowenstein Jensen medium and others in its ability to recover mycobacteria after exposure to disinfectants (Ascenzi *et al.*, 1991).

2.10.3 Organic Load

In an attempt to select a suitable soil to simulate dirty conditions in suspension and surface tests, four representative soils have been investigated (Table 2.10.3.1). As the test soils are protein based, a protein estimation was carried out to determine the protein content of each of the soils. The activity of 1,000 ppm av Cl NaDCC and 2% alkaline glutaraldehyde against *Myco. tuberculosis* in the presence and absence of the 4 organic loads was also tested to assess the comparative stringency of the soils. NaDCC and glutaraldehyde were chosen as examples of an oxidizing agent and a fixative respectively.

Table 2.10.3.1 Organic Loads and Concentrations Tested

Organic Material	Concentration	Supplier
Defibrinated Horse Serum	10% v/v	Tissue Culture Services Ltd
Bakers Yeast	3% w/v	Hopkins and Williams
Bovine Albumin	1%w/v	Sigma Chemical Co.
Yeast extract +	0.5%w/v +	Sigma Chemical Co.
Bovine serum albumin	0.5% w/v	Sigma Chemical Co.

2.10.3.1 Assessment of the effect of the organic soils on the activity of 1,000ppm NaDCC and 2% alkaline glutaraldehyde against *Myco. tuberculosis* H37 Rv

Suspension tests were carried out as detailed in 2.9.1 using *Myco. tuberculosis* as the test organism. NaDCC at a concentration of 1,000ppm and 2% glutaraldehyde were tested in the presence and absence of the 4 "organic loads" and mean log reductions of survivors calculated. The soil posing the greatest challenge to the efficacy of the disinfectants was deemed to be the most stringent. With the data obtained from the disinfectant tests and the protein assay, it was possible to correlate the amount of protein present with the degree of stringency each soil imposed on the disinfectants.

2.10.3.2 Protein Estimation

The total protein content of each of the organic soils at their in-use concentrations was measured by the Clinical Chemistry Department at the City Hospital, Dudley Road. Results were expressed as grams/litre.

2.10.4 Standard Hard Water

Sterile distilled water was the diluent of choice for this study, but comparative tests were carried out with standard hard water, to determine if there were any differences in disinfectant activity.

Water of standard hardness was prepared as follows:

Solution A: 19.84g of anhydrous magnesium chloride ($MgCl_2$) and 46.24g of anhydrous calcium chloride ($CaCl_2$) was dissolved in glass distilled water, diluted to 1 litre and sterilized at 121°C for 20 minutes. Solution B: 35.02g of sodium carbonate ($NaHCO_3$) was dissolved in glass distilled water, diluted to 1 litre and sterilized by passing through a filter (0.45µm pore size)

600ml of sterile distilled water was added to 6ml solution A in a sterile 1 litre flask. 80ml of solution B was added to this and diluted to 1 litre with sterile glass distilled water.

The disinfectants, 1,000ppm NaDCC, 1% Virkon, 70% alcohol, Tristel and 10% Gigasept, were made up to the required concentration using either standard hard water or sterile distilled water. Clean suspension tests were carried out as described in 3.6.1 using *Myco. tuberculosis* as the test organism. Results of the activity of the disinfectants made up in sterile distilled water were compared with those of disinfectants diluted using the standard hard water.

2.11 GLUTARALDEHYDE RESISTANT *MYCO. CHELONAE*

In addition to disinfectant tests, a series of additional tests were performed to assess the surface properties and lipid composition of the three strains of *Myco. chelonae*, in an attempt to understand the observed increased resistance of the two washer disinfector isolates to glutaraldehyde.

2.11.1 Minimum Inhibitory Concentration of 2% glutaraldehyde

Two ml of 2% alkaline glutaraldehyde was added to the first of 8 sterile glass tubes (3 x0.5 inch, round bottomed) and 1 ml of 7H9 broth to the remainder. One ml of 2% alkaline glutaraldehyde was removed from the first one, into the second and vortex mixed with the 7H9 broth. One ml of this was then removed to the next tube and again mixed well. This 1:2 dilution was carried out along the series of tubes. The final 1ml was discarded. A positive and negative control was set up using 1ml 7H9 broth with and without culture. A tube containing only 2% glutaraldehyde was also used. A suspension of *Myco. chelonae* NCTC 946 was prepared using the plate method C and diluted 1:20. One drop of this suspension was added to each of the tubes using a sterile disposable pipette, each drop equivalent to 0.02ml. This procedure was repeated using *Myco. chelonae* Harefield and Epping as the test organisms. All tubes were incubated at 30^{0}C for up to 5 days and examined daily for growth. Russell & Munton (1974) have reported the inaccuracies of such a minimum inhibitory concentration (MIC) test for glutaraldehyde due to the possible strong interactions between the glutaraldehyde and the proteins in the media, thereby depleting the available glutaraldehyde. In view of this, the test was

also carried out using water instead of 7H9 broth in the series of tubes. Carson *et al.* (1978) and Wolinsky (1979) reported the ability of *Myco. chelonae* to grow and survive in water. Carson *et al.* (1978) also reported that acid fast cells grown in commercial distilled water do not produce visible turbidity at levels of 10^5-10^6 cells/ml. Therefore the tests were taken one stage further. After 5 days incubation, 1 loopful was removed from each tube (broths and water) and used to inoculate 7H11 plates. The inocula were spread using disposable sterile spreaders and the plates were incubated for 5 days at 30°C.

The MIC of the disinfectant was indicated by the lowest concentration which gave no growth of the organism in broth ie no turbidity

The minimum mycobactericidal concentration was indicated by the lowest concentration of disinfectant to produce no visible colonies on 7H11 agar plates.

2.11.2 Susceptibility to aldehydes other than 2% alkaline glutaraldehyde

Neutralization tests followed by suspension tests were carried out under clean conditions using the methods previously described (Sections 4.6 & 4.7). The activity of a number of aldehydes was assessed and compared to earlier results achieved with 2% alkaline glutaraldehyde and 10% succinedialdehyde and formaldehyde mixture (Gigasept), i.e.

1. 4% formaldehyde
2. 10% glyoxal
3. 2% acid glutaraldehyde

2.11.3 Hydrophobicity Tests

The hydrophobicity of the three tests organisms was assessed using two methods, bacterial adherence to hydrocarbons (BATH) and resistance to phenolics.

The adherence of the three strains of *Myco. chelonae* to octane and hexadecane was compared using a method based on Rosenberg *et al.* (1980). A standard test suspension was prepared in PUM buffer instead of water. PUM buffer comprised 22.2g $K_2HPO_4.3H_2O$, 7.26g KH_2PO_4, 1.8g urea, 0.2g $MgSO_4.7H_2O$ and distilled water up to 1 litre. The pH was adjusted as necessary to 7.1. 1.2ml of the test suspension in PUM buffer was added to 5 glass tubes (3 x 0.5 inch, round bottomed). 0.05ml, 0.1ml, 0.15ml and 0.2ml hydrocarbon was added to 4 tubes, the final tube was left as a control i.e. no hydrocarbon was added. The tubes were incubated in a 30^{0}C incubator for 10 minutes, removed and agitated uniformly for 2 minutes. They were then left on the bench at room temperature for 15 minutes to allow the hydrocarbon phase to completely rise. The aqueous phase (bottom layer) was carefully removed and placed in a disposable 1ml cuvette. Absorbance at 400nm was measured using a Perkin Elmer Lambda 2 uv/visual spectrophotometer. The absorbance of the suspension exposed to the hydrocarbon was expressed as a % of the absorbance of a suspension without hydrocarbon and illustrated graphically. A

decrease in the absorbance of the aqueous phase was taken as a measure of hydrophobicity.

In addition to the BATH method, neutralization tests followed by suspension tests (See sections 4.6 and 4.7) were carried out to determine the activity of a clear soluble phenolic disinfectant (Stericol) at 1, 1.5 & 2% against the three strains.

2.11.4 Fatty Acid Analysis (using Gas Chromatography)

(White *et al.*, 1988; Welch, 1991)

The 3 test organisms were spread onto 7H11 plates and incubated at 30°C. One loopful of growth was scraped from the plates into the bottom of a sealed test tubes. 1ml of 3.75M (15%) NaOH in 50% methanol was added to the tubes, mixed and heated in a beaker of boiling water (100°C) for 30 minutes with vortexing after 5 minutes. The tubes were allowed to cool to room temperature. 2ml of a 1:1 6M HCl/50% methanol solution was added, mixed, heated to 80°C for 10 minutes and cooled rapidly under a cold running tap. 1ml of a 1:1 solution of hexane/diethyl ether was then added and the mixtures shaken for 10 minutes. The lower aqueous phase was very carefully removed and discarded. 3ml of a 0.3M (1.2%) NaOH was added to the remaining solution in the tubes and shaken for 5 minutes. The resulting extract (i.e. the upper layer containing the fatty acid methyl esters, FAMEs) was removed to a clean tube, dried under air at 45°C and stored at -20°C until required.

Each sample was resuspended in hexane (100µl) and 1µl was loaded onto a Hewlett Packard HP-1 capillary column (crosslinked methyl

silicone gum, ID 0.32mm, film thickness 0.17μm, length 25m) on a Unicam 610 series GC operating with 1:100 sample splitting and flame ionization detector. The gas phase comprised helium at 6.5psi with nitrogen as the makeup gas. The flame ionization detector used hydrogen and air, the injector temperature was set at 200°C and the detector temperature was set at 280°C. The GC was controlled by Microsoft Windows-compatible software (Unicam) that permitted integration of the peaks and storage of both the integration table and the detector trace. Fatty acids were identified by comparison of retention times with those of a standard bacterial FAME mix containing 25 bacterial FAMEs prepared according to manufacturers instructions (chain length C12-C20, Hewlett-Packard).

2.11.5 Mycolic acid analysis

(Butler & Kilburn, 1990)

Myco. chelonae was grown on 7H11 plates for 5 days at 30°C. 1-2 loopfuls of colonial growth was placed directly into a teflon-lined screw-cap glass test tube containing 2ml of 50% solution of ethanolic potassium hydroxide (25%), the saponification reagent ie. 50% solution of ethanol containing 25% w/v potassium hydroxide (KOH). The whole cells were then saponified for 2 hours at 100°C in a water bath. The tubes were cooled to room temperature and the mycolic acids were acidified by adding 1.5ml of 50% concentrated HCl in water. 2ml HPLC grade chloroform was then added and the solutions were mixed thoroughly for 20 seconds on a rotamixer. The layers were left to separate, and the lower layer was carefully removed to a clean tube and air dried at 45°C. To the dried layer 100μl of 2mg/ml potassium bicarbonate (in water) was added and mixed. This was again

evaporated to dryness at 45°C. One ml of chloroform containing 50µl of *p*-bromophenacyl-8 reagent (Pierce Chemical Company) was added. Derivatization was carried out for 20 minutes at 85°C before the tubes were cooled to room temperature. After cooling, the samples were acidified by the addition of 0.5ml of 50% concentrated HCl in water and 0.5ml methanol. The solutions were mixed well using a vortex mixer and the solutions were left at room temperature to allow separation of the two layers. The lower, chloroform layer was removed to a small, clean, screw-top glass tube and air dried at 45°C. The mycolic acid bromophenacyl esters were separated by reverse phase liquid chromatography as described by Butler & Kilburn (1990) using acetonitrile/dichloromethane instead of acetonitrile/chloroform for the solvent gradient. A Hewlett Packard series 1100 HPLC system was used with an ODS-Hypersil column (5µm, 200 x 2.1mm) operating with UV detection at 254nm. Peaks eluted from the column were analyzed using the Hewlett Packard HP 59987A API-electrospray LC/MS integrated system for the mass spectral analysis of liquid samples. This enabled accurate molecular weights of each peak to be determined.

2.11.6 Resistance to heat

The resistance of the three strains of *Myco. chelonae* to 65°C, 71°C, 80°C and 90°C was assessed in sterile distilled water using water at room temperature as control. 990µl of sterile distilled water was placed in a number of screw-capped plastic microcentrifuge tubes. The tubes were then placed in a heating block, which had been pre-set at the appropriate temperature, and allowed to equilibrate at that temperature. A type "T" thermocouple was inserted into one tube and

attached to a Chessell chart recorder to allow continuous monitoring of temperatures (Fig.2.11.6.1). When the temperature in the tubes had stabilized, 10µl of a standard test suspension of *Myco. chelonae* NCTC 946 was added and mixed. After contact times of 1, 3, 5, 10, 20 and 30 minutes at 65°C, 71°C and 80°C and 1, 30, 60 and 90 seconds at 90°C, the tube was removed from the block and placed in a bowl of iced water to cool rapidly. The suspensions were then diluted to 10^{-4} and 100µl of the dilutions and neat suspension plated onto 7H11 plates in duplicate. Each test was carried out in duplicate. The entire procedure was repeated using the two washer disinfector strains. Total viable counts of each test suspension were performed and used as pre-counts.

Fig 2.11.6.1: Apparatus used in the heat resistance tests

2.11.7 Glutaraldehyde uptake by *Myco. chelonae*

Standard test suspensions of the three *Myco. chelonae* strains were prepared using the plate method C and exposed to 2% alkaline glutaraldehyde using a suspension test under clean conditions. Controls were carried out at 1 and 60 minutes. After the required contact times of 1, 4, 10, 20 and 60 minutes, the culture/disinfectant mixture was filtered using a syringe filter into sterile 20ml universals. The amount of glutaraldehyde remaining in the filtrate was measured using the following colourimetric method (Sawicki *et al.*, 1961; Barnes, 1996)

As the samples contained approximately 20,000ppm glutaraldehyde, it was necessary to dilute all of them to an approximate 20ppm glutaraldehyde concentrate. Each sample (5ml) was then pipetted into 50 ml volumetric flasks in duplicate and diluted to volume with an aqueous solution of 0.5% w/v 3-methyl-2-benzothiazoline hydrazone hydrochloride (MBTH) and mixed. The solutions were left to stand at room temperature for 20 minutes. Fifteen ml aliquots were transferred from each flask to separate 20ml/25ml stoppered flasks. Two ml of the colour developing reagent was added and the flasks were incubated at 37^{0}C in a water bath for 20 minutes. The colour developing reagent was prepared by weighing approximately 1.0g ferric chloride and 1.6g sulphamic acid into a 100ml volumetric flask. They were dissolved in water and diluted to volume with mixing. The flasks were shaken occasionally to remove gas bubbles. On removal of the flasks from the water bath they were left to cool by standing in cold water for 10 minutes. The entire determination was also carried out using two 5ml water samples as blanks and a set of 5ml glutaraldehyde standards.

containing 5, 10, 15 and 20ppm glutaraldehyde. A 2% glutaraldehyde solution was used to prepare the standards (i.e. 20,000ppm). The absorbance of the blanks, standard solutions and samples was measured at 628nm in a Perkin Elmer Lambda 2 uv/visual spectrophotometer. If the absorbance was >1.2, it was necessary to further dilute the original solution and repeat the entire determination. Absorbance was measured immediately upon transfer of sample to spectrophotometer.

2.11.8 Resistance to isoniazid and ethambutol

The susceptibility of the three strains of *Myco. chelonae* to isoniazid (INH) and ethambutol (ETM) was assessed using an agar dilution MIC method. Stock solutions of the antibiotics were prepared in sterile distilled water at 10,000 mg/L and 1,000 mg/L. Table 2.11.8.1 shows the concentrations of antibiotic required and the appropriate dilutions of the stock solutions, per 100ml agar. Doubling dilutions from 1024 mg/L to 0.5 mg/L were tested.

Table 2.11.8.1: Concentrations of antibiotic required and the appropriate dilutions of stock solutions, per 100ml agar

Conc. required (mg/L)	Stock solution (mg/L)	Amt. of antibiotic required in 100ml agar (µl)
0.5	100	500
1	1000	100
2	1000	200
4	1000	400
8	1000	800
16	1000	1600
32	1000	3200
64	10,000	640
128	10,000	1280
256	10,000	2560
512	10,000	5120
1024	10,000	10240

90ml aliquots of 7H11 agar were prepared in 150ml screw capped bottles. 10ml OADC supplement and the appropriate volume of antibiotic solution was aseptically added to each bottle of molten agar and mixed well. (Note: the agar was left to cool to approximately 50°C prior to the addition of the supplements). The agar was then carefully decanted in 20-25ml amounts into 9cm sterile petri dishes (i.e. approximately 5 plates per bottle). The plates, labelled with the appropriate antibiotic and concentration were left to set at room temperature. Plates without antibiotic were prepared as controls. Prior to inoculation, the plates were dried for 15 minutes in a drying cabinet.

Standard test suspensions of the three strains of *Myco. chelonae* were prepared using the plate method C and diluted 1:1000 to give a concentration of approx. 10^5 cfu/ml. Total viable counts were performed on each suspension. Each plate was inoculated with twelve 10 µl spots of suspension, which were allowed to dry prior to

incubation at 30°C for 5 days. Plates were checked for surviving organisms on removal from the incubator, the MIC being determined as the lowest concentration to demonstrate no growth of the organism.

In addition to these tests, the three strains were forwarded on L-J slopes to the Regional Mycobacterial Reference Laboratory for routine antibiotic screening at Birmingham Heartlands Hospital.

3.0 RESULTS

3.1 STANDARDIZATION OF TEST METHOD

3.1.1 % Recovery efficiency of the disinfectant test methods

Table 3.1.1.1 shows the $\log_{10}$ counts (cfu/ml) obtained using the suspension and carrier tests and the % recovery efficiency. Recovery efficiency using the suspension test method was never <99% which seemed acceptable allowing for experimental error. However, an unacceptable recovery efficiency was observed using the carrier test method (86.09-97.36%). It was noticed that on a number of occasions some of the test organisms remained on the base of the cup even after vortexing. Therefore this test was repeated and when the cup was placed in the neutralization system, a sterile loop was used to remove any growth still adhering to the base of the cup by gentle scraping. This was shown to increase the recovery efficiency of the method to an acceptable level (Table 3.1.1.2).

Table 3.1.1.1: % Recovery efficiency using the proposed suspension and carrier test methods

Suspension Test Method			Carrier Test Method		
Pre count $\log_{10}$ cfu/ml	Test $\log_{10}$ cfu/ml	**% Recovery efficiency**	Pre count $\log_{10}$ cfu/ml	Test $\log_{10}$ cfu/ml	**% Recovery efficiency**
8.35	8.35	**100**	7.98	7.00	**87.72**
8.35	8.27	**99.04**	8.20	7.56	**91.46**
8.48	8.48	**100**	7.19	6.19	**86.09**
7.99	7.99	**100**	8.01	7.20	**89.89**
8.52	8.51	**99.88**	7.19	7.00	**97.36**

Table 3.1.1.2: % Recovery efficiency of the carrier test method incorporating the use of a sterile loop

Carrier test method		
Pre count log_{10} cfu/ml	Test log_{10} cfu/ml	% **Recovery efficiency**
7.72	7.72	**100**
8.84	8.63	**97.62**
7.72	7.68	**99.48**
8.85	8.79	**99.32**
8.23	8.21	**99.76**

3.1.2 Initial Inoculum

Method A

Tables 3.1.2.1-7 show the counts obtained when one colony of each organism was grown in a batch culture of 7H9 at 30°C or 37°C as appropriate, over a period of 21 days. Counts were obtained in duplicate and the mean calculated. The washer disinfector isolates (Harefield and Epping) and the type strain (NCTC 946) of *Myco. chelonae* and *Myco. fortuitum* NCTC 10394 reached maximum titres at 14-15 days. *Myco. terrae* required approximately 17 days and *Myco. tuberculosis* H37 Rv and *Myco. avium-intracellulare* 21 days. However, the stationary phase (i.e. a constant cell count) was maintained in all cultures for up to 21 days.

Table 3.1.2.1: Reproducibility of total viable counts of *Myco. chelonae* NCTC 946 in 7H9 broth

Days	*Myco. chelonae* 1*	*Myco. chelonae* 2*	Mean
0	5.93	5.09	5.51
1	6.52	5.70	6.11
3	6.31	6.19	6.25
5	6.27	6.20	6.24
7	7.42	7.16	7.29
9	6.78	6.49	6.64
11	7.27	7.15	7.21
13	7.23	7.19	7.21
15	7.46	7.32	7.39
17	6.93	7.09	7.01
19	7.14	6.99	7.07
21	7.31	7.24	7.28

*1 and 2 = duplicate cultures

Table 3.1.2.2: Reproducibility of total viable counts of *Myco. chelonae* (Harefield isolate) in 100ml 7H9 broth

Days	*Myco. chelonae* 1*	*Myco. chelonae* 2*	Mean
0	5.65	5.72	5.69
1	6.07	5.88	5.98
3	5.93	6.10	6.02
5	6.46	6.69	6.58
7	7.27	6.85	7.06
9	6.31	6.36	6.34
11	6.90	6.69	6.80
13	7.23	7.19	7.21
15	7.53	7.26	7.40
17	6.74	6.60	6.67
19	7.00	7.32	7.16
21	7.23	7.22	7.23

* 1 and 2 = duplicate cultures

Table 3.1.2.3: Reproducibility of total viable counts of *Myco. chelonae* (Epping isolate) in 7H9 broth

Days	*Myco. chelonae* 1*	*Myco. chelonae* 2*	Mean
0	5.38	5.28	5.33
1	5.64	5.63	5.64
3	5.88	5.71	5.80
5	6.33	6.42	6.38
7	6.62	6.85	6.74
9	6.38	6.32	6.35
11	6.88	6.83	6.86
13	7.04	7.00	7.02
15	7.15	7.11	7.13
17	6.34	6.62	6.48
19	6.91	6.73	6.82
21	7.62	7.54	7.58

* 1 and 2 = duplicate cultures

Table 3.1.2.4: Reproducibility of total viable counts of *Myco. fortuitum* NCTC 10394 in 7H9 broth

Days	*Myco. fortutium* 1*	*Myco. fortuitum* 2*	Mean
0	5.35	5.34	5.35
1	6.37	6.70	6.54
3	6.83	7.00	6.92
5	6.80	7.20	7.00
7	7.11	6.90	7.01
9	7.46	7.37	7.43
11	7.45	7.43	7.44
13	7.20	7.20	7.20
15	7.42	7.32	7.37
17	7.40	7.50	7.45
19	7.00	7.04	7.02
21	7.08	7.01	7.05

* 1 and 2 = duplicate cultures

Table 3.1.2.5: Reproducibility of total viable counts of *Myco. terrae* NCTC 10856 in 7H9 broth

Days	*Myco. terrae* 1*	*Myco. terrae* 2*	Mean
0	5.06	5.34	5.20
1	5.85	5.60	5.73
3	6.48	6.28	6.38
5	6.50	5.99	6.25
7	6.98	7.08	7.03
9	7.10	7.10	7.10
11	6.93	6.81	6.87
13	7.16	6.93	7.05
15	7.65	7.02	7.24
17	7.46	7.24	7.34
19	7.23	7.19	7.21
21	7.23	6.91	7.07

* 1 and 2 = duplicate cultures

Table 3.1.2.6: Reproducibility of total viable counts of *Myco. tuberculosis* H37 Rv NCTC 7416 in 7H9 broth

Days	*Myco. tuberculosis* 1*	*Myco. tuberculosis* 2*	Mean
0	4.66	4.19	4.43
1	4.81	4.48	4.65
3	5.06	5.01	5.04
5	6.00	6.00	6.00
7	6.20	5.98	6.09
9	6.41	6.29	6.35
11	6.79	6.95	6.87
13	6.94	6.96	6.95
15	6.94	6.89	6.92
17	6.96	6.85	6.91
19	7.10	7.11	7.11
21	7.19	7.14	7.17

* 1 and 2 = duplicate cultures

Table 3.1.2.7: Reproducibility of total viable counts of *Myco. avium-intracellulare* (clinical isolate) in 7H9 broth

Days	MAI 1*	MAI 2*	Mean
0	5.52	4.85	5.19
1	6.05	5.52	5.79
3	6.93	6.40	6.67
5	7.43	7.11	7.27
7	7.48	7.40	7.44
9	7.55	7.64	7.60
11	7.95	7.75	7.85
13	7.69	7.67	7.68
15	7.79	7.84	7.82
17	7.66	7.54	7.60
19	7.62	7.56	7.59
21	7.64	7.70	7.67

* 1 and 2 = duplicate cultures

The titres obtained using the plate method A to prepare the test suspensions are presented in Table 3.1.2.8. The counts (log_{10} 6.28 - 7.95) were lower than required for the initial inoculum in a disinfectant test. This was confirmed by the results of the activity of 2% alkaline glutaraldehyde and 1,000ppm NaDCC against *Myco. tuberculosis* in suspension under clean conditions (Table 3.1.2.9). Also, colonies were very uneven in size and distribution and counts varied from plate to plate and from test to test.

Table 3.1.2.8: Total viable counts/ml of suspension obtained using the broth method A

Test Organism	Log_{10} Counts/ml			Mean
Myco. chelonae				
NCTC	7.43	7.01	7.09	7.17
Harefield	7.33	7.94	7.56	7.61
Epping	7.56	7.50	7.72	7.59
Myco. fortuitum	7.31	7.87	7.95	7.71
Myco. terrae	7.01	6.68	7.09	6.93
Myco. tuberculosis	7.11	6.28	6.50	6.63
MAI	7.04	7.19	7.13	7.12

Table 3.1.2.9: Activity of 2% glutaraldehyde and 1,000ppm NaDCC against *Myco. tuberculosis* H37 Rv using the broth method A to obtain the test suspension

Disinfectant	Log initial count	Mean $\log_{10}$ reduction factor				
		1 min	4 min	10 min	20 min	60 min
Suspension Test Clean conditions						
2% glutaraldehyde	7.54	1.22	2.02	>4.54	>4.54	>4.54
1,000ppm NaDCC	6.78	>3.48	>3.48	>3.48	>3.48	>3.48
Dirty conditions						
2% glutaraldehyde	7.77	2.57	>4.77	>4.77	>4.77	>4.77
1,000ppm NaDCC	7.41	>4.41	>4.41	>4.41	>4.41	>4.41

Method B - Plate method

Quite high titres ($\log_{10}$ 7.89-$\log_{10}$ 8.5) were obtained using method B, as can be seen in Table 3.1.2.10.

Table 3.1.2.10: Total viable counts/ml of suspension obtained using the plate method B

Test Organism	Log_{10} Counts/ml			Mean
Myco. chelonae				
NCTC	8.13	8.20	8.22	8.18
Harefield	8.19	8.22	8.29	8.23
Epping	8.24	8.26	8.17	8.22
Myco. fortuitum	8.50	8.48	8.50	8.49
Myco. terrae	7.89	8.11	8.20	8.07
Myco. tuberculosis	8.0	8.15	8.09	8.08
MAI	8.48	8.44	8.46	8.46

Its value as an initial inoculum in a disinfectant test was assessed by testing the activity of 2% glutaraldehyde and 1,000ppm NaDCC under clean and dirty conditions against *Myco. tuberculosis* in suspension. The results, as shown in Table 3.1.2.11, were accurate and

reproducible. The colonies were evenly sized and distributed and appeared to grow much more luxuriantly than in the previous method.

Table 3.1.2.11 Activity of 2% glutaraldehyde an 1,000ppm NaDCC against *Myco. tuberculosis* H37 Rv using the plate method B to obtain the test suspension

Disinfectant	Log Initial Count	Mean Log_{10} Reduction Factor				
		1 min	4 min	10 min	20 min	60 min
Suspension Test Clean Conditions						
2% Glutaraldehyde	8.14	0.40	1.40	>5.14	>5.14	>5.14
1,000ppm NaDCC	8.03	>5.03	>5.03	>5.03	>5.03	>5.03
Dirty Conditions						
2% Glutaraldehyde	8.00	0.50	2.22	>5.00	>5.00	>5.00
1,000ppm NaDCC	8.15	1.29	>5.15	>5.15	>5.15	>5.15

Method C - Plate method

This plate method also gave very high titres of evenly sized colonies (log_{10} 8.2-9.98) which were evenly dispersed on the plates. Table 3.1.2.12 shows the counts obtained from test suspensions of all test organisms prepared using the plate method C.

Table 3.1.2.12: Total viable counts/ml of suspension obtained using the plate method C

Test Organism	Counts /ml (log_{10})			Mean
Myco. chelonae				
NCTC 946	9.72	9.75	9.51	9.66
Harefield	9.77	9.68	9.71	9.72
Epping	9.66	9.70	9.75	9.70
Myco. fortuitum	9.44	9.34	9.14	9.31
Myco. terrae	9.38	9.71	9.13	9.41
Myco. tuberculosis	8.20	8.45	8.61	8.42
MAI	9.93	9.98	9.84	9.92

To assess its value as an initial inoculum suspension tests under clean and dirty conditions were carried out using 2% glutaraldehyde and 1,000ppm NaDCC and *Myco. tuberculosis* as the test organism. The results are presented in Table 3.1.2.13

Table 3.1.2.13 Activity of 2% glutaraldehyde an 1,000ppm NaDCC against *Myco. tuberculosis* H37 Rv using the plate method C to obtain the test suspension

Disinfectant	Log Initial Count	Mean Log_{10} Reduction Factor				
		1 min	4 min	10 min	20 min	60 min
Suspension Test Clean Conditions						
2% Glutaraldehyde	8.00	1.28	2.83	4.60	>5.00	>5.00
1,000ppm NaDCC	8.18	>5.18	>5.18	>5.18	>5.18	>5.18
Dirty Conditions						
2% Glutaraldehyde	8.00	0.50	2.22	>5.00	>5.00	>5.00
1,000ppm NaDCC	8.00	1.10	>5.00	>5.00	>5.00	>5.00

Broth Method D

Method D was attempted but it was decided not to use it for reasons of safety as the 2 litre bottle containing 1 litre of culture (approximate density 10^8 cfu/ml) was very awkward to manipulate in the safety cabinet. Table 3.1.2.14 shows the initial counts obtained in 10ml and 100ml. No count was taken on the 1 litre suspension. This method is currently used in the new "AOAC" disinfectant test as proposed by Ascenzi *et al.* (1991). The test organism, *Myco. bovis* BCG is a category 2 organism which does not require the use of a class 1 safety cabinet and other safety constraints necessary when using *Myco. tuberculosis*. No disinfectant tests were carried out.

Table 3.1.2.14 Total viable counts/ml in varying amounts of 7H9 broth using method D

	Count / ml (log_{10})
1 colony in 10 ml	3.93
10ml @ 21 days	7.00
100ml @ 21 days	8.10

As a result of the investigations of the various methods of preparation of an initial inoculum, it was decided to use the broth method A for preparing a seed pool of each of the test organisms. To prepare the seed pool, suspensions were incubated as described for the Broth method A at 30°C for 14-15 days (*Myco. chelonae*), 37°C for 14-15 days (*Myco. fortuitum*), 17 days (*Myco. terrae*), or 21 days (*Myco. tuberculosis* and *Myco. avium-intracellulare*) prior to storage at -70°C. This allowed the storage and use of the same mycobacterial culture for 12 months.

The plate method C was used for the preparation of the initial inoculum for all disinfectant tests.

3.1.3 Neutralization and Recovery

In the preliminary tests using *Myco. chelonae*, initial counts (i.e. total viable counts of test suspensions) were compared with the controls to determine if the presence of 0.5% Tween 80 was in itself inhibitory. The counts were very similar allowing for experimental error confirming that this concentration of Tween 80 does not inhibit the recovery of the test organisms. The test and controls were then compared to

determine the success of the neutralization system. Similarities in the test and control systems (i.e. no differences in the numbers, growth rate or colony size of the test organisms) confirmed that the combination of dilution and Tween 80 was successful in neutralizing all the disinfectant residues with two exceptions, Tristel and NuCidex (Table 3.1.3.1).

There was a >1 $\log_{10}$ difference between the test and control counts for Tristel and NuCidex which implies that the neutralization/recovery system was inappropriate for these particular agents. The findings in the tests using *Myco. tuberculosis* were similar. Again a combination of dilution and Tween 80 were ineffective in neutralizing Tristel and NuCidex (Table 3.1.3.2). Due to the results obtained, it was necessary to assess other neutralization systems for these two agents. Further tests were carried out using a combination of dilution, 0.5% Tween 80 and 0.5% thiosulphate (Tristel, Table 3.1.3.3) and a combination of dilution, 0.025% catalase and 5% sodium thiosulphate (NuCidex, Table 3.1.3.4).

Table 3.1.3.1: Preliminary neutralization tests using a combination of dilution and 0.5% Tween 80 and *Myco. chelonae* as the test organism

Disinfectant	Initial Count $\log_{10}$ cfu/ml	Test $\log_{10}$ cfu/ml	Control $\log_{10}$ cfu/ml
10,000ppm NaDCC	7.57	7.11	7.60
2% Glutaraldehyde (Asep)	7.80	8.08	8.02
10% Gigasept	8.99	8.87	8.89
70% Alcohol	7.98	7.74	7.61
1% Per oxygen (Virkon)	8.01	7.89	7.77
Tristel	8.34	6.41	8.40
0.35% Peracetic acid (NuCidex)	7.84	6.02	7.79

Table 3.1.3.2: Neutralization tests using a combination of dilution and Tween 80 with *Myco. tuberculosis* as the test organism

Disinfectant	Initial Count $\log_{10}$ cfu/ml	Test $\log_{10}$ cfu/ml	Control $\log_{10}$ cfu/ml
10,000 NaDCC	8.75	8.91	8.96
2% Glutaraldehyde	8.62	8.48	8.52
10% Gigasept	8.94	8.96	8.94
70% Alcohol	8.96	9.05	9.05
1% Virkon	8.74	8.88	8.91
Tristel	8.74	6.69	8.91
NuCidex	8.76	7.20	8.63

Table 3.1.3.3 Neutralization test for Tristel using a combination of dilution and Tween 80 with sodium thiosulphate and *Myco. tuberculosis* as the test organism.

Disinfectant	Initial Count $\log_{10}$ cfu/ml	Test $\log_{10}$ cfu/ml	Control $\log_{10}$ cfu/ml
Tristel	9.15	9.41	9.36

Table 3.1.3.4 Neutralization of NuCidex using a combination of dilution, catalase and sodium thiosulphate and *Myco. tuberculosis* as the test organism

Disinfectant	Initial Count $\log_{10}$ cfu/ml	Test $\log_{10}$ cfu/ml	Control $\log_{10}$ cfu/ml
NuCidex	8.21	8.13	8.17

On the basis of the results achieved in the neutralization tests a combination of dilution and 0.5% Tween 80 was used to neutralize all disinfectants with the exception of chlorine dioxide and peracetic acid. A combination of dilution, 0.025% catalase and 5% sodium thiosulphate was used for the peracetic acid compound (NuCidex) and a combination of dilution, 0.5% Tween 80 and 0.5% sodium thiosulphate for chlorine dioxide (Tristel).

3.1.4 Organic Load

3.1.4.1 Assessment of the effect of the organic soils on the activity of 1,000ppm NaDCC and 2% alkaline glutaraldehyde using *Myco. tuberculosis* as the test organism

The effect of different organic loads on the activity of 1,000ppm av. Cl (NaDCC) and 2% alkaline glutaraldehyde against *Myco. tuberculosis* in suspension is shown in Table 3.1.4.1. All organic loads tested adversely affected the activity of NaDCC. A >5 $\log_{10}$ reduction was achieved in 1 minute under clean conditions, in 4 minutes in the presence of serum, CEN soil (a mixture of 0.5% yeast and 0.5% serum) and yeast at 3% and in 10 minutes in the presence of 1% albumin. Albumin at 1% was shown to be more stringent than the other three.

Results against glutaraldehyde were different. Tests under clean conditions and in the presence of 3% yeast were most stringent, requiring 20 minutes to achieve a >5 $\log_{10}$ reduction. In the presence of 10% serum a >5 $\log_{10}$ reduction was achieved in 10 minutes and in the presence of the yeast and albumin mixture and albumin at 1% in 4 minutes. This would suggest that of the protein based organic loads tested, 10% serum provides the most stringent test for glutaraldehyde.

3.1.4.1: Assessment of the effect of different organic loads on the activity of NaDCC and glutaraldehyde using *Myco. tuberculosis* as the test organism

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
1,000ppm NaDCC						
clean conditions	8.18	**>5.18**	>5.18	>5.18	>5.18	>5.18
10% serum	8.00	1.10	**>5.00**	>5.00	>5.00	>5.00
0.5% albumin + 0.5% yeast	8.13	>5.13	**>5.13**	>5.13	>5.13	>5.13
3% yeast	8.00	>5.00	**>5.00**	>5.00	>5.00	>5.00
1% albumin	8.12	0.5	2.75	**>5.12**	>5.12	>5.12
2% glutaraldehyde						
clean conditions	8.00	1.28	2.83	4.60	**>5.00**	>5.00
10% serum	8.00	0.50	2.22	**>5.00**	>5.00	>5.00
0.5% albumin + 0.5% yeast	8.08	1.10	**>5.08**	>5.08	>5.08	>5.08
3% yeast	8.11	1.31	2.54	4.87	**>5.11**	>5.11
1% albumin	8.10	1.13	**>5.10**	>5.10	>5.10	>5.10

3.1.4.2 Protein estimation

Table 3.1.4.2 shows the total protein present in each of the organic soils at their in-use concentration

Table 3.1.4.2 Total protein present (g/L) in each of the organic soils

Organic soil	Total Protein content (g/L)
10% serum	4.67
0.5% albumin + 0.5% yeast	0.51
3% "Bakers" yeast	<0.10
1% bovine albumin	21.6

1% albumin exerted the biggest effect on activity of NaDCC. However, all organic loads apparently increase the activity of glutaraldehyde,

with the exception of 3% yeast, which was the organic load with the least amount of protein. To simulate realistic in-use conditions of organic soiling, e.g. blood, serum, it was felt that a protein based organic load should be used. Therefore, although 3% yeast was most stringent against glutaraldehyde, it was rejected for use in this study due to its exceptionally low protein content. 10% serum was chosen as the organic load for use in this study.

3.1.5 Standard Hard Water

The activity of the disinfectants diluted to in-use concentration with standard hard water compared favourably with those made up in distilled water, with the exception of the lower concentration of NaDCC ie. 1,000ppm av Cl. The mean $\log_{10}$ reductions obtained against *Myco. tuberculosis* in a suspension test in the absence of an organic load are presented in Table 3.1.5.1.

Table 3.1.5.1 Comparison of standard hard water and distilled water as a diluent for disinfectants

Disinfectant	Mean Log_{10} Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
70% alcohol						
hard water	8.35	**>5.35**	>5.35	>5.35	>5.35	>5.35
distilled water	8.01	**>5.01**	>5.01	>5.01	>5.01	>5.01
1% Virkon						
hard water	8.27	0	0	0	0.21	0.12
distilled water	8.06	0.31	0.19	0.33	0.14	0.47
1,000 NaDCC						
hard water	8.01	0.85	**>5.01**	>5.01	>5.01	>5.01
distilled water	8.18	**>5.18**	>5.18	>5.18	>5.18	>5.18
Tristel						
hard water	8.32	**>5.32**	>5.32	>5.32	>5.32	>5.32
distilled water	8.23	**>5.23**	>5.23	>5.23	>5.23	>5.23
10% Gigasept						
hard water	8.41	0.07	0.61	1.70	**>5.41**	>5.41
distilled water	8.12	0.34	0.34	3.14	4.72	**>5.12**

The use of sterile hard water to dilute the disinfectants affected the activity of 1,000ppm av Cl NaDCC only.

Sterile distilled water was used as the diluent during this study as following a review of the literature and in light of these results it was felt that hard water should be treated as an interfering substance and as such should not be incorporated universally into all tests. Tests similar to those used for organic loads should be carried out to establish the activity of a disinfectant in the presence and absence of standard hard water as part of a standard set of tests.

The standard test method which was chosen for all experiments in this study on the basis of the standardization tests is detailed in Appendix B

3.2 RESISTANCE OF MYCOBACTERIA TO DISINFECTANTS

3.2.1 Suspension test

Results of the suspension tests have been presented as mean $\log_{10}$ reductions obtained in the presence and absence of 10% serum as an organic load. Tables 3.2.1.1 -7 illustrate the results of the suspension tests by test organism.

Myco. chelonae NCTC 946 (Table 3.2.1.1) was sensitive to most of the disinfectants tested after 1 minute exposure in the presence and absence of 10% serum. The exceptions were Virkon, the peroxygen compound and Gigasept, the succinedialdehyde-formaldehyde mixture. Gigasept was effective after 10 minutes exposure under clean and dirty conditions. However 1 hour exposure to 1% Virkon was required to achieve a $\log_{10}$ reduction of >6.07 in the presence of 10% serum and 20 minutes to achieve a $\log_{10}$ reduction of >5.43 in its absence. The same product at 3% was ineffective after 60 minute exposure achieving at best, a $\log_{10}$ 3.22 reduction.

The results obtained using the two washer disinfector isolates of *Myco. chelonae*, i.e. Harefield and Epping can be seen in Tables 3.2.1.2 and 3.2.1.3 respectively. Both were very resistant to 2% alkaline glutaraldehyde. No significant reduction was achieved after 60 minutes exposure to the disinfectant. Both organisms were comparable to the type strain in their sensitivities to some of the other disinfectants although they appeared to be slightly more resistant to

the lower concentrations of NaDCC, Virkon, Gigasept and NuCidex than the type strain, *Myco. chelonae* NCTC 946. NaDCC at 10,000ppm, 70% alcohol and Tristel were all effective in achieving a >5 $\log_{10}$ reduction in both test organisms, under both clean and dirty conditions, after 1 minute exposure. NuCidex was effective in 4 minutes against both strains and Virkon was totally ineffective ie <1 $\log_{10}$ reduction in 60 minutes. Under clean conditions 1,000ppm NaDCC required 4 minutes against the Harefield and Epping strains, but in the presence of 10% serum, the Epping isolate appeared to be slightly more resistant than Harefield, which required a 20 minute exposure as opposed to 60 minutes to achieve a >5 $\log_{10}$ reduction.

With so many similarities between *Myco. chelonae* and *Myco. fortuitum*, to the extent they are both often considered together as the *Myco. fortuitum-chelonae* complex, it is not surprising they exhibit similar sensitivity patterns to some of the disinfectants tested. Table 3.2.1.4 shows that 10,000ppm NaDCC, 70% alcohol, 2% alkaline glutaraldehyde and Tristel were all effective in 1 minute under clean and dirty conditions. Virkon at both concentrations was ineffective in 60 minutes again achieving <1 $\log_{10}$ reduction. Gigasept and 1,000ppm NaDCC achieved a >6 $\log_{10}$ reduction in 10 minutes and NuCidex in 4 minutes, under clean and dirty conditions.

Myco. terrae (Table 3.2.1.5) was effectively destroyed after 1 minute exposure to 10,000ppm NaDCC, 70% alcohol and Tristel, under clean and dirty conditions. NuCidex achieved a >6.16 $\log_{10}$ reduction in 4 minutes under clean conditions and a >6.07 $\log_{10}$ reduction in 4

minutes in the presence of 10% serum. 1,000ppm NaDCC achieved a >6.02 $\log_{10}$ reduction in 4 minutes in the absence of an organic load and a 5.33 $\log_{10}$ reduction in 10 minutes in its presence. Gigasept required 60 minutes exposure under clean and dirty conditions to achieve a >6 $\log_{10}$ reduction and Virkon was again completely ineffective, i.e. <1 $\log_{10}$ reduction. 2% alkaline glutaraldehyde produced an unusual result. A 5.48 $\log_{10}$ reduction was achieved in 60 minutes under clean conditions and 5.30 reduction in 10 minutes under dirty conditions. It would appear that 2% alkaline glutaraldehyde is more effective under dirty conditions than under clean.

The results of the tests against *Myco. tuberculosis* are presented in Table 3.2.1.6. As *Myco. terrae* is being proposed as a surrogate for *Myco. tuberculosis* in disinfectant test methods, it was expected to find comparable sensitivities to the disinfectants. *Myco. tuberculosis* appears, in fact, to be slightly more sensitive to some of the products tested than *Myco. terrae.* Under clean conditions, 1,000ppm 10,000ppm NaDCC, 70% alcohol, NuCidex and Tristel all achieved at least a >5 $\log_{10}$ reduction after 1 minute exposure. In the presence of 10% serum, 10,000ppm, 70% alcohol and Tristel remained effective at 1 minute but NuCidex and 1,000ppm NaDCC required the slightly longer contact time of 4 minutes. Gigasept required 60 minutes under clean and 20 minutes under dirty conditions and Virkon at both concentrations was again totally ineffective in 1 hour. 2% alkaline glutaraldehyde achieved >5.14 $\log_{10}$ reduction in 20 minutes in the absence of serum and >5 $\log_{10}$ reduction in 10 minutes in its presence.

Myco. avium intracellulare (Table 3.2.1.7) was by far the most resistant of all the test organisms. Tristel was the only disinfectant to achieve a >5 $\log_{10}$ reduction with 1 minute exposure under both clean and dirty conditions. Although 10,000ppm NaDCC was effective in 1 minute under clean conditions, 10 minutes were required in the presence of 10% serum. 70% alcohol and NuCidex were both effective in 4 minutes and 1,000ppm NaDCC in 60 minutes in the presence and absence of an organic load. Gigasept and Virkon were totally ineffective in 60 minutes.

Table 3.2.1.1: Resistance of *Myco. chelonae* NCTC 946 in suspension to various disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	8.27	**>5.27**	>5.27	>5.27	>5.27	>5.27
10,000ppm NaDCC	8.45	**>5.45**	>5.45	>5.45	>5.45	>5.45
2% v/v Asep	8.64	**>5.64**	>5.64	>5.64	>5.64	>5.64
70% v/v IMS	8.76	**>5.76**	>5.76	>5.76	>5.76	>5.76
1% w/v Virkon	8.42	0.87	2.56	4.48	**>5.43**	>5.43
3% w/v Virkon	9.20	1.49	2.51	2.64	2.77	3.22
10% v/v Gigasept	8.67	0	0.51	**>5.67**	>5.67	>5.67
NuCidex	8.76	**>5.76**	>5.76	>5.76	>5.76	>5.76
Tristel	9.30	**>6.30**	>6.30	>6.30	>6.30	>6.30
Dirty Conditions						
1,000ppm NaDCC	8.13	**>5.13**	>5.13	>5.13	>5.13	>5.13
10,000ppm NaDCC	8.07	**>5.07**	>5.07	>5.07	>5.07	>5.07
2% v/v Asep	8.82	**>5.82**	>5.82	>5.82	>5.82	>5.82
70% v/v IMS	8.64	**>5.64**	>5.64	>5.64	>5.64	>5.64
1% w/v Virkon	9.07	0.45	1.78	4.06	4.83	**>6.07**
3% w/v Virkon	9.24	1.71	2.56	2.69	2.85	3.02
10% v/v Gigasept	8.27	0.18	0.19	**>5.27**	>5.27	>5.27
NuCidex	8.47	**>5.47**	>5.47	>5.47	>5.47	>5.47
Tristel	9.26	**>6.26**	>6.26	>6.26	>6.26	>6.26

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.2: Resistance of *Myco. chelonae* (Harefield) in suspension to various disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	8.54	2.90	**>5.54**	>5.54	>5.54	>5.54
10,000ppm NaDCC	9.00	**>6.00**	>6.00	>6.00	>6.00	>6.00
2% v/v Asep	8.43	0.24	0.30	0.35	0.51	0.64
70% v/v IMS	9.00	**>6.00**	>6.00	>6.00	>6.00	>6.00
1% w/v Virkon	8.18	0	0	0	0.07	0.07
3% w/v Virkon	9.35	0	0	0	0.02	0.34
10% v/v Gigasept	8.44	0	0	2.69	**>5.44**	>5.44
NuCidex	8.06	4.06	**>5.06**	>5.06	>5.06	>5.06
Tristel	9.43	**>6.43**	>6.43	>6.43	>6.43	>6.43
Dirty Conditions						
1,000ppm NaDCC	8.50	2.17	4.02	4.10	**>5.50**	>5.50
10,000ppm NaDCC	8.75	**>5.75**	>5.75	>5.75	>5.75	>5.75
2% v/v Asep	8.83	0.81	0.98	0.97	0.94	1.08
70% v/v IMS	8.69	**>5.69**	>5.69	>5.69	>5.69	>5.69
1% w/v Virkon	8.00	0	0.14	0.14	0.10	0.34
3% w/v Virkon	9.42	0.08	0.11	0.06	0.36	0.99
10% v/v Gigasept	8.67	0	0	1.15	**>5.67**	>5.67
NuCidex	8.07	4.07	**>5.07**	>5.07	>5.07	>5.07
Tristel	9.42	**>6.42**	>6.42	>6.42	>6.42	>6.42

Numbers in bold indicate the time taken to achieve a >5 log_{10} reduction

Table 3.2.1.3: Resistance of *Myco. chelonae* (Epping) in suspension to various disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	8.80	1.79	**>5.80**	>5.80	>5.80	>5.80
10,000ppm NaDCC	9.18	**>6.18**	>6.18	>6.18	>6.18	>6.18
2% v/v Asep	9.10	0	0.12	0.09	0.33	0.29
70% v/v IMS	9.04	**>6.04**	>6.04	>6.04	>6.04	>6.04
1% w/v Virkon	8.30	0.03	0.06	0.08	0.18	2.25
3% w/v Virkon	9.22	0	0	0	0.01	0.03
10% v/v Gigasept	8.35	0.09	0.16	0.06	0.17	0.13
NuCidex	9.12	4.03	**>6.12**	>6.12	>6.12	>6.12
Tristel	9.14	**>6.14**	>6.14	>6.14	>6.14	>6.14
Dirty Conditions						
1,000ppm NaDCC	9.47	0.03	0.81	3.20	4.82	**5.59**
10,000ppm NaDCC	8.19	**>5.19**	>5.19	>5.19	>5.19	>5.19
2% v/v Asep	9.16	0	0	0.01	0.02	0.04
70% v/v IMS	9.30	**>6.30**	>6.30	>6.30	>6.30	>6.30
1% w/v Virkon	9.36	0	0	0	0	0
3% w/v Virkon	9.28	0	0	0.06	0.11	0.24
10% v/v Gigasept	9.28	0	0	0.14	0.24	0.88
NuCidex	9.43	4.15	**>6.43**	>6.43	>6.43	>6.43
Tristel	9.26	**>6.26**	>6.26	>6.26	>6.26	>6.26

Numbers in bold indicate the time taken to achieve a >5 log_{10} reduction

Table 3.2.1.4: Resistance of *Myco. fortuitum* NCTC 10394 in suspension to various disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	9.17	3.92	3.93	**>6.17**	>6.17	>6.17
10,000ppm NaDCC	9.30	**>6.30**	>6.30	>6.30	>6.30	>6.30
2% v/v Asep	9.43	**>6.43**	>6.43	>6.43	>6.43	>6.43
70% v/v IMS	9.51	**>6.51**	>6.51	>6.51	>6.51	>6.51
1% w/v Virkon	9.18	0	0	0	0	0
3% w/v Virkon	9.38	0.06	0.13	0.13	0.18	0.35
10% v/v Gigasept	9.41	0	0.04	4.50	**>6.41**	>6.41
NuCidex	9.34	3.24	**>6.34**	>6.34	>6.34	>6.34
Tristel	9.44	**>6.44**	>6.44	>6.44	>6.44	>6.44
Dirty Conditions						
1,000ppm NaDCC	9.06	2.65	4.41	**5.18**	>6.06	>6.06
10,000ppm NaDCC	9.40	**>6.40**	>6.40	>6.40	>6.40	>6.40
2% v/v Asep	9.10	**>6.10**	>6.10	>6.10	>6.10	>6.10
70% v/v IMS	9.54	**>6.54**	>6.54	>6.54	>6.54	>6.54
1% w/v Virkon	9.30	0.15	0.06	0.17	0.14	0.15
3% w/v Virkon	9.40	0.06	0.09	0.09	0.06	0.09
10% v/v Gigasept	9.47	0.08	0.45	**>6.47**	>6.47	>6.47
NuCidex	9.00	4.91	**>6.00**	>6.00	>6.00	>6.00
Tristel	9.27	**>6.27**	>6.27	>6.27	>6.27	>6.27

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.5: Resistance of *Myco. terrae* NCTC 10856 in suspension to various disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	9.02	1.74	**>6.02**	>6.02	>6.02	>6.02
10,000ppm NaDCC	8.95	**>5.95**	>5.95	>5.95	>5.95	>5.95
2% v/v Asep	9.00	1.61	3.75	4.82	4.76	**5.48**
70% v/v IMS	9.08	**>6.08**	>6.08	>6.08	>6.08	>6.08
1% w/v Virkon	8.82	0.36	0.60	0.66	0.68	0.85
3% w/v Virkon	8.85	0.47	0.54	0.73	0.74	0.79
10% v/v Gigasept	9.32	0	0.1	0.78	1.83	**>6.32**
NuCidex	9.16	3.00	**>6.16**	>6.16	>6.16	>6.16
Tristel	9.15	**>6.15**	>6.15	>6.15	>6.15	>6.15
Dirty Conditions						
1,000ppm NaDCC	9.43	0.68	2.70	**5.33**	>6.43	>6.43
10,000ppm NaDCC	9.15	**>6.15**	>6.15	>6.15	>6.15	>6.15
2% v/v Asep	9.40	3.00	4.37	**5.30**	6.00	>6.40
70% v/v IMS	9.36	**>6.36**	>6.36	>6.36	>6.36	>6.36
1% w/v Virkon	8.88	0.41	0.68	0.81	0.82	1.53
3% w/v Virkon	8.84	0.50	0.67	0.77	0.79	0.80
10% v/v Gigasept	9.46	0.04	0.37	1.40	1.69	**>6.46**
NuCidex	9.07	2.55	**>6.07**	>6.07	>6.07	>6.07
Tristel	9.30	**>6.30**	>6.30	>6.30	>6.30	>6.30

Numbers in bold indicate the time taken to achieve a >5 log_{10} reduction

Table 3.2.1.6: Resistance of *Myco. tuberculosis* H37 Rv NCTC 7416 in suspension to various disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	8.18	**>5.18**	>5.18	>5.18	>5.18	>5.18
10,000ppm NaDCC	8.01	**>5.01**	>5.01	>5.01	>5.01	>5.01
2% v/v Asep	8.00	1.28	2.83	4.60	**>5.00**	>5.00
70% v/v IMS	8.01	**>5.01**	>5.01	>5.01	>5.01	>5.01
1% w/v Virkon	8.06	0.31	0.19	0.33	0.14	0.47
3% w/v Virkon	8.29	0	0.13	0.10	0.14	1.66
10% v/v Gigasept	8.12	0.34	0.34	3.14	4.72	**>5.12**
NuCidex	8.10	**>5.10**	>5.10	>5.10	>5.10	>5.10
Tristel	8.23	**>5.23**	>5.23	>5.23	>5.23	>5.23
Dirty Conditions						
1,000ppm NaDCC	8.00	1.10	**>5.00**	>5.00	>5.00	>5.00
10,000ppm NaDCC	8.12	**>5.12**	>5.12	>5.12	>5.12	>5.12
2% v/v Asep	8.00	0.50	2.22	**>5.00**	>5.00	>5.00
70% v/v IMS	8.00	**>5.00**	>5.00	>5.00	>5.00	>5.00
1% w/v Virkon	8.42	0.27	0.42	0.25	0.35	0.39
3% w/v Virkon	8.26	0.23	0.08	0.05	0.10	0.59
10% v/v Gigasept	8.35	0.04	0.68	2.20	**>5.35**	>5.35
NuCidex	8.38	1.82	**>5.38**	>5.38	>5.38	>5.38
Tristel	8.30	**>5.30**	>5.30	>5.30	>5.30	>5.30

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.7: Resistance of *Myco. avium-intracellulare* (clinical isolate) in suspension to various disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	9.92	0.01	1.47	3.06	3.66	**5.22**
10,000ppm NaDCC	9.60	**>6.60**	>6.60	>6.60	>6.60	>6.60
2% v/v Asep	9.90	0.35	1.32	1.99	3.73	**>6.90**
70% v/v IMS	9.58	2.83	**>6.58**	>6.58	>6.58	>6.58
1% w/v Virkon	9.94	0	0	0	0	0
3% w/v Virkon	9.99	0.03	0.03	0	0	0.03
10% v/v Gigasept	9.90	0.05	0.13	0.15	0.21	1.89
NuCidex	9.87	2.08	**5.24**	>6.87	>6.87	>6.87
Tristel	9.98	**>6.98**	>6.98	>6.98	>6.98	>6.98
Dirty Conditions						
1,000ppm NaDCC	9.54	0.14	0.32	0.52	1.47	**>6.54**
10,000ppm NaDCC	9.55	3.56	4.79	**>6.55**	>6.55	>6.55
2% v/v Asep	9.62	1.24	3.80	**>6.62**	>6.62	>6.62
70% v/v IMS	9.80	4.32	**>6.80**	>6.80	>6.80	>6.80
1% w/v Virkon	9.04	0	0	0	0	0
3% w/v Virkon	9.74	0.08	0.07	0.09	0.08	0.11
10% v/v Gigasept	9.88	0	0.11	0.34	0.46	1.29
NuCidex	9.68	1.12	**>6.68**	>6.68	>6.68	>6.68
Tristel	9.40	**>6.40**	>6.40	>6.40	>6.40	>6.40

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Tables 3.2.1.8 - 3.2.1.16 present the same data but the format has been changed for clarity of presentation. These tables show the activity of each of the disinfectants against the various mycobacteria

Table 3.2.1.8: Activity of 1,000 ppm av. Cl NaDCC against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.27	**>5.27**	>5.27	>5.27	>5.27	>5.27
Myco. chel Harefield	8.54	2.90	**>5.54**	>5.54	>5.54	>5.54
Myco. chel Epping	8.80	1.79	**>5.80**	>5.80	>5.80	>5.80
Myco. fortuitum	9.17	3.92	3.93	**>6.17**	>6.17	>6.17
Myco. tuberculosis	8.18	**>5.18**	>5.18	>5.18	>5.18	>5.18
MAI clinical	9.92	0.01	1.47	3.06	3.66	**5.22**
Myco. terrae	9.02	1.74	**>6.02**	>6.02	>6.02	>6.02
Dirty Conditions						
Myco. chel NCTC 946	8.13	**>5.13**	>5.13	>5.13	>5.13	>5.13
Myco. chel Harefield	8.50	2.17	4.02	4.10	**>5.50**	>5.50
Myco. chel Epping	9.47	0.03	0.81	3.20	4.82	**5.59**
Myco. fortuitum	9.06	2.65	4.41	**5.18**	>6.06	>6.06
Myco. tuberculosis	8.00	1.10	**>5.00**	>5.00	>5.00	>5.00
MAI clinical	9.54	0.14	0.32	0.52	1.47	**>6.54**
Myco. terrae	9.43	0.68	2.70	**5.33**	>6.43	>6.43

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.9: Activity of 10,000ppm av. Cl NaDCC against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.45	**>5.45**	>5.45	>5.45	>5.45	>5.45
Myco. chel Harefield	9.00	**>6.00**	>6.00	>6.00	>6.00	>6.00
Myco. chel Epping	9.18	**>6.18**	>6.18	>6.18	>6.18	>6.18
Myco. fortuitum	9.30	**>6.30**	>6.30	>6.30	>6.30	>6.30
Myco. tuberculosis	8.01	**>5.01**	>5.01	>5.01	>5.01	>5.01
MAI clinical	9.60	**>6.60**	>6.60	>6.60	>6.60	>6.60
Myco. terrae	8.95	**>5.95**	>5.95	>5.95	>5.95	>5.95
Dirty Conditions						
Myco. chel NCTC 946	8.07	**>5.07**	>5.07	>5.07	>5.07	>5.07
Myco. chel Harefield	8.75	**>5.75**	>5.75	>5.75	>5.75	>5.75
Myco. chel Epping	8.19	**>5.19**	>5.19	>5.19	>5.19	>5.19
Myco. fortuitum	9.40	**>6.40**	>6.40	>6.40	>6.40	>6.40
Myco tuberculosis	8.12	**>5.12**	>5.12	>5.12	>5.12	>5.12
MAI clinical	9.55	3.56	4.79	**>6.55**	>6.55	>6.55
Myco. terrae	9.15	**>6.15**	>6.15	>6.15	>6.15	>6.15

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.10: Activity of 2% v/v alkaline glutaraldehyde against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.64	**>5.64**	>5.64	>5.64	>5.64	>5.64
Myco. chel Harefield	8.43	0.24	0.30	0.35	0.51	0.64
Myco. chel Epping	9.10	0	0.12	0.09	0.33	0.29
Myco. fortuitum	9.43	**>6.43**	>6.43	>6.43	>6.43	>6.43
Myco. tuberculosis	8.00	1.28	2.83	4.60	**>5.00**	>5.00
MAI clinical	9.90	0.35	1.32	1.99	3.73	**>6.90**
Myco. terrae	9.00	1.61	3.75	4.82	4.76	**5.48**
Dirty Conditions						
Myco. chel NCTC 946	8.82	**>5.82**	>5.82	>5.82	>5.82	>5.82
Myco. chel Harefield	8.83	0.81	0.98	0	0.94	1.08
Myco. chel Epping	9.16	0	0	0.01	0.02	0.04
Myco. fortuitum	9.10	**>6.10**	>6.10	>6.10	>6.10	>6.10
Myco. tuberculosis	8.00	0.50	2.22	**>5.00**	>5.00	>5.00
MAI clinical	9.62	1.24	3.80	**>6.62**	>6.62	>6.62
Myco. terrae	9.40	3.00	4.37	**5.30**	6.00	>6.40

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.11: Activity of 70% v/v alcohol (IMS) against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.76	**>5.76**	>5.76	>5.76	>5.76	>5.76
Myco. chel Harefield	9.00	**>6.00**	>6.00	>6.00	>6.00	>6.00
Myco. chel Epping	9.04	**>6.04**	>6.04	>6.04	>6.04	>6.04
Myco. fortuitum	9.51	**>6.51**	>6.51	>6.51	>6.51	>6.51
Myco. tuberculosis	8.01	**>5.01**	>5.01	>5.01	>5.01	>5.01
MAI clinical	9.58	2.83	**>6.58**	>6.58	>6.58	>6.58
Myco. terrae	9.08	**>6.08**	>6.08	>6.08	>6.08	>6.08
Dirty Conditions						
Myco. chel NCTC 946	8.64	**>5.64**	>5.64	>5.64	>5.64	>5.64
Myco. chel Harefield	8.69	**>5.69**	>5.69	>5.69	>5.69	>5.96
Myco. chel Epping	9.30	**>6.30**	>6.30	>6.30	>6.30	>6.30
Myco. fortuitum	9.54	**>6.54**	>6.54	>6.54	>6.54	>6.54
Myco. tuberculosis	8.00	**>5.00**	>5.00	>5.00	>5.00	>5.00
MAI clinical	9.80	4.32	**>6.80**	>6.80	>6.80	>6.80
Myco. terrae	9.36	**>6.36**	>6.36	>6.36	>6.36	>6.36

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.12: Activity of 1% w/v peroxygen compound (Virkon) against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.42	0.87	2.56	4.48	**>5.43**	>5.43
Myco. chel Harefield	8.18	0	0	0	0.07	0.07
Myco. chel Epping	8.30	0.03	0.06	0.08	0.18	2.25
Myco. fortuitum	9.18	0	0	0	0	0
Myco. tuberculosis	8.06	0.31	0.19	0.33	0.14	0.47
MAI clinical	9.94	0	0	0	0	0
Myco. terrae	8.82	0.36	0.60	0.66	0.68	0.85
Dirty Conditions						
Myco. chel NCTC 946	9.07	0.45	1.78	4.06	4.83	**>6.07**
Myco. chel Harefield	8.00	0	0.14	0.14	0.10	0.34
Myco. chel Epping	9.36	0	0	0	0	0
Myco. fortuitum	9.30	0.15	0.06	0.17	0.14	0.15
Myco. tuberculosis	8.42	0.27	0.42	0.25	0.35	0.39
MAI clinical	9.04	0	0	0	0	0
Myco. terrae	8.88	0.41	0.68	0.81	0.82	1.53

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.13: Activity of 3% w/v peroxygen compound (Virkon) against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	9.20	1.49	2.51	2.64	2.77	3.22
Myco. chel Harefield	9.35	0	0	0	0.02	0.34
Myco. chel Epping	9.22	0	0	0	0.01	0.03
Myco. fortuitum	9.38	0.06	0.13	0.13	0.18	0.35
Myco. tuberculosis	8.29	0	0.13	0.10	0.14	1.66
MAI clinical	9.99	0.03	0.03	0	0	0.03
Myco. terrae	8.85	0.47	0.54	0.73	0.74	0.79
Dirty Conditions						
Myco. chel NCTC 946	9.24	1.71	2.56	2.69	2.85	3.02
Myco. chel Harefield	9.42	0.08	0.11	0.06	0.36	0.99
Myco. chel Epping	9.28	0	0	0.06	0.11	0.24
Myco. fortuitum	9.40	0.06	0.09	0.09	0.06	0.09
Myco. tuberculosis	8.26	0.23	0.08	0.05	0.10	0.59
MAI clinical	9.74	0.08	0.07	0.09	0.08	0.11
Myco. terrae	8.84	0.50	0.67	0.77	0.79	0.80

Table 3.2.1.14: Activity of 10% v/v succinedialdehyde and formaldehyde mixture (Gigasept) against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.67	0	0.51	**>5.67**	>5.67	>5.67
Myco. chel Harefield	8.44	0	0	2.69	**>5.44**	>5.44
Myco. chel Epping	8.35	0.09	0.16	0.06	0.17	0.13
Myco. fortuitum	9.41	0	0.04	4.50	**>6.41**	>6.41
Myco. tuberculosis	8.12	0.34	0.34	3.14	4.72	**>5.12**
MAI clinical	9.90	0.05	0.13	0.15	0.21	1.89
Myco. terrae	9.32	0	0.1	0.78	1.83	**>6.32**
Dirty Conditions						
Myco. chel NCTC 946	8.27	0.18	0.19	**>5.27**	>5.27	>5.27
Myco. chel Harefield	8.67	0	0	1.15	**>5.67**	>5.67
Myco. chel Epping	9.28	0	0	0.14	0.24	0.88
Myco. fortuitum	9.47	0.08	0.45	**>6.47**	>6.47	>6.47
Myco. tuberculosis	8.35	0.04	0.68	2.20	**>5.35**	>5.35
MAI clinical	9.88	0	0.11	0.34	0.46	1.29
Myco. terrae	9.46	0.04	0.37	1.40	1.69	**>6.46**

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.15: Activity of 0.35% peracetic acid (NuCidex) against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.76	**>5.76**	>5.76	>5.76	>5.76	>5.76
Myco. chel Harefield	8.06	4.06	**>5.06**	>5.06	>5.06	>5.06
Myco. chel Epping	9.12	4.03	**>6.12**	>6.12	>6.12	>6.12
Myco. fortuitum	9.34	3.24	**>6.34**	>6.34	>6.34	>6.34
Myco. tuberculosis	8.10	**>5.10**	>5.10	>5.10	>5.10	>5.10
MAI clinical	9.87	2.08	**5.24**	>6.87	>6.87	>6.87
Myco. terrae	9.16	3.00	**>6.16**	>6.16	>6.16	>6.16
Dirty Conditions						
Myco. chel NCTC 946	8.47	**>5.47**	>5.47	>5.47	>5.47	>5.47
Myco. chel Harefield	8.07	4.07	**>5.07**	>5.07	>5.07	>5.07
Myco. chel Epping	9.43	4.15	**>6.43**	>6.43	>6.43	>6.43
Myco. fortuitum	9.00	4.91	**>6.00**	>6.00	>6.00	>6.00
Myco. tuberculosis	8.38	1.82	**>5.38**	>5.38	>5.38	>5.38
MAI clinical	9.68	1.12	**>6.68**	>6.68	>6.68	>6.68
Myco. terrae	9.07	2.55	**>6.07**	>6.07	>6.07	>6.07

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.1.16: Activity of chlorine dioxide (Tristel) against mycobacteria in suspension under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	9.30	**>6.30**	>6.30	>6.30	>6.30	>6.30
Myco. chel Harefield	9.43	**>6.43**	>6.43	>6.43	>6.43	>6.43
Myco. chel Epping	9.14	**>6.14**	>6.14	>6.14	>6.14	>6.14
Myco. fortuitum	9.44	**>6.44**	>6.44	>6.44	>6.44	>6.44
Myco. tuberculosis	8.23	**>5.23**	>5.23	>5.23	>5.23	>5.23
MAI clinical	9.98	**>6.98**	>6.98	>6.98	>6.98	>6.98
Myco. terrae	9.15	**>6.15**	>6.15	>6.15	>6.15	>6.15
Dirty Conditions						
Myco. chel NCTC 946	9.26	**>6.26**	>6.26	>6.26	>6.26	>6.26
Myco. chel Harefield	9.42	**>6.42**	>6.42	>6.42	>6.42	>6.42
Myco. chel Epping	9.26	**>6.26**	>6.26	>6.26	>6.26	>6.26
Myco. fortuitum	9.27	**>6.27**	>6.27	>6.27	>6.27	>6.27
Myco. tuberculosis	8.30	**>5.30**	>5.30	>5.30	>5.30	>5.30
MAI clinical	9.40	**>6.40**	>6.40	>6.40	>6.40	>6.40
Myco. terrae	9.30	**>6.30**	>6.30	>6.30	>6.30	>6.30

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

3.2.2 Carrier Test

Sattar *et al.* (1995) reported that data based on carrier tests are generally preferred to those of suspension tests. It is believed that emphasis should be placed on developing a standard quantitative carrier test rather than a suspension test.

NaDCC at both concentrations and 2% alkaline glutaraldehyde achieved a >5 $\log_{10}$ reduction in *Myco. chelonae* NCTC 946 after 1 minute under clean and dirty conditions (Table 3.2.2.1). NuCidex and Tristel, although effective in 1 minute under clean conditions required 4 minutes in the presence of 10% serum. 70% alcohol required 4 minutes, Gigasept 20 minutes and Virkon at both concentrations, >60 minutes to achieve a >5 $\log_{10}$ reduction in the presence and absence of serum

Tables 3.2.2.2 and 3.2.2.3 show that the Harefield and Epping isolate of *Myco. chelonae* were again very similar in their resistance to the disinfectants. Both test organisms were very resistant to 2% alkaline glutaraldehyde after 60 minutes exposure and also Virkon at both concentrations of 1% and 3%. 10,000ppm NaDCC completely destroyed the organisms after a 1 minute contact time under both clean and dirty conditions and 70% alcohol after 4 minutes. 1,000ppm NaDCC achieved a >5 $\log_{10}$ reduction in *Myco. chelonae* Harefield in 20 minutes and Epping in 10 minutes in the absence of serum, but failed to achieve more than a 2.19 - 3.12 $\log_{10}$ reduction in both organisms in 60 minutes in it's presence. Gigasept at 10% was ineffective against the Epping isolate in 60 minutes but achieved a >5 $\log_{10}$ reduction in the Harefield isolate in 60 minutes under both clean and dirty

conditions. Tristel achieved a >5 $\log_{10}$ reduction in the Harefield strain in 1 minute in clean conditions and 4 minutes under dirty conditions and required 4 minutes under both clean and dirty conditions against the Epping strain. NuCidex was similarly effective in 1-4 minutes against both organisms.

Myco. fortuitum (Table 3.2.2.4) was destroyed in 1 minute by 10,000ppm NaDCC, 2% glutaraldehyde, 70% alcohol and Tristel under clean conditions and 2% glutaraldehyde only under dirty conditions. Tristel, 70% alcohol and 10,000ppm NaDCC all required 4 minutes under dirty conditions to achieve a >5 $\log_{10}$ reduction. Gigasept achieved a >5 $\log_{10}$ reduction in 60 minutes and NuCidex in 4 minutes under both clean and dirty conditions. Virkon was again ineffective after 60 minutes exposure. 1,000ppm NaDCC was effective in 10 minutes under clean conditions and 20 minutes in the presence of serum.

10,000ppm NaDCC and NuCidex both effectively destroyed *Myco. terrae* (Table 3.2.2.5) in 1 minute under clean and dirty conditions. 2% alkaline glutaraldehyde and 70% alcohol although effective in obtaining a >5 $\log_{10}$ reduction in 1 minutes in clean conditions required 4 minutes under dirty conditions. 1,000ppm NaDCC achieved a >5 $\log_{10}$ reduction in 20 minutes in clean conditions but required 60 minutes in the presence of 10% serum and Tristel was effective in 4 minutes in clean conditions and 10 minutes under dirty conditions. Virkon was ineffective in 60 minutes and Gigasept required a full 60 minutes to obtain a ≥5 $\log_{10}$ reduction

Myco. tuberculosis (Table 3.2.2.6) seemed to be slightly more sensitive to the disinfectants than *Myco. terrae*. 10,000ppm NaDCC, 2%

alkaline glutaraldehyde, 70% alcohol, NuCidex and Tristel were all effective in achieving a >5 $\log_{10}$ reduction in *Myco. tuberculosis* in clean conditions, although 10,000ppm and 70% alcohol required 4 minutes in the presence of 10% serum. 1,000ppm NaDCC achieved a >5,20 $\log_{10}$ reduction in 10 minutes under clean conditions and >5.01 reduction in 20 minutes under dirty conditions. 10% Gigasept was effective in 60 minutes under both clean and dirty conditions. Virkon at both concentrations was totally ineffective at 60 minutes achieving <1 $\log_{10}$ reduction.

Myco. avium intracellulare was by far the most resistant of the test organisms (Table 3.2.2.7). Tristel was the only disinfectant to achieve a >5 $\log_{10}$ reduction in 1 minute, and only in the absence of 10% serum. Under dirty conditions 4 minutes was required to achieve a >5 $\log_{10}$ reduction. NuCidex achieved a >5 $\log_{10}$ reduction (ie. >5.85 - >6.23) under clean and dirty conditions in 4 minutes. 1,000ppm NaDCC was ineffective in 60 minutes achieving a 4.75 $\log_{10}$ reduction under clean conditions and 0.38 $\log_{10}$ reduction under dirty conditions. Virkon at both concentration and Gigasept were ineffective in 60 minutes under both clean and dirty conditions. 10,000ppm NaDCC achieved a >6.43 $\log_{10}$ reduction in 4 minutes in the absence of 10% serum and a 5.47 $\log_{10}$ reduction in 10 minutes in it's presence. Similarly 70% alcohol was effective in 4 minutes under clean conditions and 10 minutes under dirty conditions. 2% alkaline glutaraldehyde achieved a 5.05 $\log_{10}$ reduction in the absence of 10% serum and a 5.82 $\log_{10}$ reduction in its presence.

Table 3.2.2.1: Resistance of *Myco. chelonae* NCTC 946 dried on to carriers to several disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	8.48	**>5.00**	>5.00	>5.00	>5.00	>5.00
10,000ppm NaDCC	8.53	**>5.05**	>5.05	>5.05	>5.05	>5.05
2% v/v Asep	8.80	**>5.32**	>5.32	>5.32	>5.32	>5.32
70% v/v IMS	9.30	2.62	**>5.82**	>5.82	>5.82	>5.82
1% w/v Virkon	9.18	0.55	1.86	2.92	4.46	4.47
3% w/v Virkon	9.56	0.61	0.90	1.07	1.58	2.05
10% v/v Gigasept	9.29	0.02	0.25	0.40	**>5.81**	>5.81
NuCidex	8.93	**>5.45**	>5.45	>5.45	>5.45	>5.45
Tristel	9.48	**>6.00**	>6.00	>6.00	>6.00	>6.00
Dirty Conditions						
1,000ppm NaDCC	8.55	**>5.07**	>5.07	>5.07	>5.07	>5.07
10,000ppm NaDCC	9.16	**>5.68**	>5.68	>5.68	>5.68	>5.68
2% v/v Asep	8.90	**>5.42**	>5.42	>5.42	>5.42	>5.42
70% v/v IMS	9.17	2.29	**5.60**	>5.69	>5.69	>5.69
1% w/v Virkon	9.09	0	0.18	1.89	3.62	3.41
3% w/v Virkon	9.34	0.72	1.01	1.09	2.03	2.96
10% v/v Gigasept	9.35	0	0.32	0.35	**>5.87**	>5.87
NuCidex	8.71	3.48	**>5.23**	>5.23	>5.23	>5.23
Tristel	9.11	3.80	**>5.63**	>5.63	>5.63	>5.63

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.2: Resistance of *Myco. chelonae* (Harefield strain) dried onto carriers to several disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	9.21	0.83	0.91	4.18	**5.73**	>5.73
10,000ppm NaDCC	8.52	**>5.04**	>5.04	>5.04	>5.04	>5.04
2% v/v Asep	7.37	0.02	0.02	0	0.04	0.15
70% v/v IMS	8.56	2.01	**>5.08**	>5.08	>5.08	>5.08
1% w/v Virkon	8.56	1.42	1.77	2.28	2.61	2.74
3% w/v Virkon	8.39	2.47	2.73	3.03	3.12	3.17
10% v/v Gigasept	8.90	0.03	0.15	2.71	3.63	**>5.42**
NuCidex	8.80	4.37	**>5.32**	>5.32	>5.32	>5.32
Tristel	8.53	**>5.05**	>5.05	>5.05	>5.05	>5.05
Dirty Conditions						
1,000ppm NaDCC	7.84	0.28	0.54	1.80	3.20	2.46
10,000ppm NaDCC	8.74	**>5.26**	>5.26	>5.26	>5.26	>5.26
2% v/v Asep	7.80	0	0	0	0	0
70% v/v IMS	8.80	1.66	**>5.32**	>5.32	>5.32	>5.32
1% w/v Virkon	9.12	0.05	0.32	0.69	1.11	1.47
3% w/v Virkon	8.48	1.21	1.50	1.67	2.07	1.88
10% v/v Gigasept	9.16	0.04	0.05	0.04	1.72	**>5.68**
NuCidex	8.54	2.73	**>5.06**	>5.06	>5.06	>5.06
Tristel	8.74	3.10	**>5.26**	>5.26	>5.26	>5.26

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.3: Resistance of *Myco. chelonae* (Epping strain) dried onto carriers to several disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	8.82	1.88	3.44	**>5.34**	>5.34	>5.34
10,000ppm NaDCC	8.50	**>5.02**	>5.02	>5.02	>5.02	>5.02
2% v/v Asep	8.59	0.02	0.06	0.06	0.04	0.19
70% v/v IMS	8.80	2.42	**>5.32**	>5.32	>5.32	>5.32
1% w/v Virkon	8.74	0.35	0.84	1.03	0.90	1.18
3% w/v Virkon	8.56	0.21	0.30	0.65	0.79	1.56
10% v/v Gigasept	8.61	0	0	0	0	0.23
NuCidex	8.50	**>5.02**	>5.02	>5.02	>5.02	>5.02
Tristel	9.06	2.82	**5.89**	>5.58	>5.58	>5.58
Dirty Conditions						
1,000ppm NaDCC	8.94	0.23	0.39	0.74	1.27	2.19
10,000ppm NaDCC	8.62	**>5.14**	>5.14	>5.14	>5.14	>5.14
2% v/v Asep	8.69	0	0	0	0	0
70% v/v IMS	9.06	1.98	**>5.58**	>5.58	>5.58	>5.58
1% w/v Virkon	8.74	0.35	0.84	1.03	0.90	1.18
3% w/v Virkon	8.63	0.54	1.04	0.78	1.32	1.76
10% v/v Gigasept	8.80	0	0.02	0	0.16	0.46
NuCidex	8.54	4.47	**>5.06**	>5.06	>5.06	>5.06
Tristel	9.05	1.94	**5.57**	>5.57	>5.57	>5.57

Numbers in bold indicate the time taken to achieve a >5 log_{10} reduction

Table 3.2.2.4: Resistance of *Myco. fortuitum* NCTC 10394 dried onto carriers to several disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	9.32	1.42	4.55	**>5.84**	>5.84	>5.84
10,000ppm NaDCC	9.12	**>5.64**	>5.64	>5.64	>5.64	>5.64
2% v/v Asep	9.03	**>5.55**	>5.55	>5.55	>5.55	>5.55
70% v/v IMS	8.87	**5.04**	>5.39	>5.39	>5.39	>5.39
1% w/v Virkon	8.89	0.09	0.17	0.39	0.34	0.86
3% w/v Virkon	8.02	0.36	0.64	0.60	1.06	1.08
10% v/v Gigasept	9.20	0.10	0.33	0.86	3.46	**>5.72**
NuCidex	8.82	4.43	**>5.34**	>5.34	>5.34	>5.34
Tristel	9.12	**>5.64**	>5.64	>5.64	>5.64	>5.64
Dirty Conditions						
1,000ppm NaDCC	8.98	0.20	0.73	0.80	**>5.50**	>5.50
10,000ppm NaDCC	8.98	1.83	**>5.50**	>5.50	>5.50	>5.50
2% v/v Asep	8.93	**>5.45**	>5.45	>5.45	>5.45	>5.45
70% v/v IMS	8.67	**>5.19**	>5.19	>5.19	>5.19	>5.19
1% w/v Virkon	8.89	0.23	0.38	0.45	0.62	0.72
3% w/v Virkon	8.30	0.87	0.82	0.99	1.30	1.47
10% v/v Gigasept	9.02	0	0.10	0.67	3.34	**>5.54**
NuCidex	9.16	**>5.68**	>5.68	>5.68	>5.68	>5.68
Tristel	9.00	4.78	**>5.52**	>5.52	>5.52	>5.52

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.5: Resistance of *Myco. terrae* NCTC 10856 dried onto carriers to several disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	8.78	0.49	1.56	4.39	**>5.30**	>5.30
10,000ppm NaDCC	9.06	**>5.58**	>5.58	>5.58	>5.58	>5.58
2% v/v Asep	9.03	**5.68**	>5.55	>5.55	>5.55	>5.55
70% v/v IMS	9.07	3.39	**>5.59**	>5.59	>5.59	>5.59
1% w/v Virkon	9.13	0	0.21	0.39	0.20	0.17
3% w/v Virkon	8.95	0.02	0.13	0.13	0.37	0.24
10% v/v Gigasept	8.61	0	0.11	0.50	1.78	**>5.13**
NuCidex	8.66	**>5.18**	>5.18	>5.18	>5.18	>5.18
Tristel	9.36	3.91	**>5.88**	>5.88	>5.88	>5.88
Dirty Conditions						
1,000ppm NaDCC	9.10	0.23	1.67	1.31	2.18	**6.22**
10,000ppm NaDCC	9.06	**>5.58**	>5.58	>5.58	>5.58	>5.58
2% v/v Asep	9.07	4.31	**>5.59**	>5.59	>5.59	>5.59
70% v/v IMS	9.16	2.79	**>5.68**	>5.68	>5.68	>5.68
1% w/v Virkon	9.09	0	0	0.14	0.16	0.27
3% w/v Virkon	9.11	0.04	0.01	0.26	0.22	0.53
10% v/v Gigasept	8.68	0.06	0.32	0.71	1.81	**>5.20**
NuCidex	8.74	**>5.26**	>5.26	>5.26	>5.26	>5.26
Tristel	9.29	1.72	2.78	**>5.81**	>5.81	>5.81

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.6: Resistance of *Myco. tuberculosis* H37 Rv NCTC 7416 dried onto carriers to several disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	8.68	1.29	3.46	**>5.20**	>5.20	>5.20
10,000ppm NaDCC	8.77	**>5.29**	>5.29	>5.29	>5.29	>5.29
2% v/v Asep	8.64	**>5.16**	>5.16	>5.16	>5.16	>5.16
70% v/v IMS	8.49	3.07	**>5.01**	>5.01	>5.01	>5.01
1% w/v Virkon	8.32	0.22	0.41	0.38	0.44	0.59
3% w/v Virkon	7.02	0.70	0.47	0.48	0.52	0.51
10% v/v Gigasept	8.94	0.37	0.52	1.63	3.90	**>5.46**
NuCidex	8.75	**>5.27**	>5.27	>5.27	>5.27	>5.27
Tristel	8.79	**>5.31**	>5.31	>5.31	>5.31	>5.31
Dirty Conditions						
1,000ppm NaDCC	8.49	0.57	1.22	3.76	**>5.01**	>5.01
10,000ppm NaDCC	8.51	4.63	**>5.03**	>5.03	>5.03	>5.03
2% v/v Asep	8.65	**>5.17**	>5.17	>5.17	>5.17	>5.17
70% v/v IMS	8.51	3.22	**>5.03**	>5.03	>5.03	>5.03
1% w/v Virkon	8.39	0.61	0.43	0.49	0.67	0.60
3% w/v Virkon	7.93	0.63	0.60	0.48	0.79	0.66
10% v/v Gigasept	8.76	0	0.44	1.19	3.42	**>5.28**
NuCidex	8.48	**>5.00**	>5.00	>5.00	>5.00	>5.00
Tristel	8.63	**>5.15**	>5.15	>5.15	>5.15	>5.15

Numbers in bold indicate the time taken to achieve a >5 log_{10} reduction

Table 3.2.2.7: Resistance of *Myco. avium-intracellulare* (clinical isolate) dried onto carriers to several disinfectants under clean and dirty conditions

Disinfectant	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
1,000ppm NaDCC	9.80	0.48	0.65	0.69	1.87	4.75
10,000ppm NaDCC	9.91	3.99	**>6.43**	>6.43	>6.43	>6.43
2% v/v Asep	9.91	1.57	3.86	**5.05**	>6.43	>6.43
70% v/v IMS	9.46	3.03	**>5.98**	>5.98	>5.98	>5.98
1% w/v Virkon	8.51	0	0	0	0	0.09
3% w/v Virkon	8.64	0.09	0.10	0.11	0.20	0.17
10% v/v Gigasept	9.99	0.52	0.56	0.58	0.67	2.12
NuCidex	9.33	1.10	**>5.85**	>5.85	>5.85	>5.85
Tristel	9.46	**>5.98**	>5.98	>5.98	>5.98	>5.98
Dirty Conditions						
1,000ppm NaDCC	9.82	0.35	0.37	0.38	0.39	0.38
10,000ppm NaDCC	9.99	1.94	3.90	**5.47**	>6.51	>6.51
2% v/v Asep	9.65	1.28	3.08	4.44	**5.82**	>6.17
70% v/v IMS	9.47	1.94	2.31	**>5.99**	>5.99	>5.99
1% w/v Virkon	8.35	0.02	0.06	0	0.07	0.09
3% w/v Virkon	8.92	0	0.02	0.10	0.12	0.25
10% v/v Gigasept	9.89	0.42	0.41	0.57	0.93	2.52
NuCidex	9.71	1.24	**>6.23**	>6.23	>6.23	>6.23
Tristel	9.47	2.33	**>5.99**	>5.99	>5.99	>5.99

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Tables 3.2.2.8 - 3.2.2.16 present the same data but the format has been changed for clarity of presentation. These tables show the activity of each of the disinfectants against various mycobacteria.

Table 3.2.2.8: Activity of 1,000ppm NaDCC against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.48	**>5.00**	>5.00	>5.00	>5.00	>5.00
Myco. chel Harefield	9.20	0.83	0.91	4.18	**5.73**	>5.73
Myco. chel Epping	8.82	1.88	3.44	**>5.34**	>5.34	>5.34
Myco. fortuitum	9.32	1.42	4.55	**>5.84**	>5.84	>5.84
Myco. tuberculosis	8.68	1.29	3.46	**>5.20**	>5.20	>5.20
MAI clinical	9.80	0.48	0.65	0.69	1.87	4.75
Myco. terrae	8.78	0.49	1.56	4.39	**>5.30**	>5.30
Dirty Conditions						
Myco. chel NCTC 946	8.55	**>5.07**	>5.07	>5.07	>5.07	>5.07
Myco. chel Harefield	7.84	0.28	0.54	1.80	3.20	2.46
Myco. chel Epping	8.94	0.23	0.39	0.74	1.27	2.19
Myco. fortuitum	8.98	0.20	0.73	0.80	**>5.50**	>5.50
Myco. tuberculosis	8.49	0.57	1.22	3.76	**>5.01**	>5.01
MAI clinical	9.82	0.35	0.37	0.38	0.39	0.38
Myco. terrae	9.10	0.23	1.67	1.31	2.18	**6.22**

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.9: Activity of 10,000ppm NaDCC against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.53	**>5.05**	>5.05	>5.05	>5.05	>5.05
Myco. chel Harefield	8.52	**>5.04**	>5.04	>5.04	>5.04	>5.04
Myco. chel Epping	8.50	**>5.02**	>5.02	>5.02	>5.02	>5.02
Myco. fortuitum	9.12	**>5.64**	>5.64	>5.64	>5.64	>5.64
Myco. tuberculosis	8.77	**>5.29**	>5.29	>5.29	>5.29	>5.29
MAI clinical	9.91	3.99	**>6.43**	>6.43	>6.43	>6.43
Myco. terrae	9.06	**>5.58**	>5.58	>5.58	>5.58	>5.58
Dirty Conditions						
Myco. chel NCTC 946	9.16	**>5.68**	>5.68	>5.68	>5.68	>5.68
Myco. chel Harefield	8.74	**>5.26**	>5.26	>5.26	>5.26	>5.26
Myco. chel Epping	8.62	**>5.14**	>5.14	>5.14	>5.14	>5.14
Myco. fortuitum	8.98	1.83	**>5.50**	>5.50	>5.50	>5.50
Myco. tuberculosis	8.51	4.63	**>5.03**	>5.03	>5.03	>5.03
MAI clinical	9.99	1.94	3.90	**5.47**	>6.51	>6.51
Myco. terrae	9.06	**>5.58**	>5.58	>5.58	>5.58	>5.58

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.10: Activity of 2% alkaline glutaraldehyde against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.80	**>5.32**	>5.32	>5.32	>5.32	>5.32
Myco. chel Harefield	7.37	0.02	0.02	0	0.04	0.15
Myco. chel Epping	8.59	0.02	0.06	0.06	0.04	0.19
Myco. fortuitum	9.03	**>5.55**	>5.55	>5.55	>5.55	>5.55
Myco. tuberculosis	8.64	**>5.16**	>5.16	>5.16	>5.16	>5.16
MAI clinical	9.91	1.57	3.86	**5.05**	>6.43	>6.43
Myco. terrae	9.03	**5.68**	>5.55	>5.55	>5.55	>5.55
Dirty Conditions						
Myco. chel NCTC 946	8.90	**>5.42**	>5.42	>5.42	>5.42	>5.42
Myco. chel Harefield	7.80	0	0	0	0	0
Myco. chel Epping	8.69	0	0	0	0	0
Myco. fortuitum	8.93	**>5.45**	>5.45	>5.45	>5.45	>5.45
Myco. tuberculosis	8.65	**>5.17**	>5.17	>5.17	>5.17	>5.17
MAI clinical	9.65	1.28	3.08	4.44	**5.82**	>6.17
Myco. terrae	9.07	4.31	**>5.59**	>5.59	>5.59	>5.59

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.11: Activity of 70% v/v alcohol (IMS) against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	9.30	2.62	**>5.82**	>5.82	>5.82	>5.82
Myco. chel Harefield	8.56	2.01	**>5.08**	>5.08	>5.08	>5.08
Myco. chel Epping	8.80	2.42	**>5.32**	>5.32	>5.32	>5.32
Myco. fortuitum	8.87	**5.04**	>5.39	>5.39	>5.39	>5.39
Myco. tuberculosis	8.49	3.07	**>5.01**	>5.01	>5.01	>5.01
MAI clinical	9.46	3.03	**>5.98**	>5.98	>5.98	>5.98
Myco. terrae	9.67	3.39	**>5.59**	>5.59	>5.59	>5.59
Dirty Conditions						
Myco. chel NCTC 946	9.17	2.29	**5.60**	>5.69	>5.69	>5.69
Myco. chel Harefield	8.80	1.66	**>5.32**	>5.32	>5.32	>5.32
Myco. chel Epping	9.06	1.98	**>5.58**	>5.58	>5.58	>5.58
Myco. fortuitum	8.67	**>5.19**	>5.19	>5.19	>5.19	>5.19
Myco. tuberculosis	8.51	3.22	**>5.03**	>5.03	>5.03	>5.03
MAI clinical	9.47	1.94	2.31	**>5.99**	>5.99	>5.99
Myco. terrae	9.16	2.79	**>5.68**	>5.68	>5.68	>5.68

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.12: Activity of 1% w/v peroxygen compound (Virkon) against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	9.18	0.55	1.86	2.92	4.46	4.47
Myco. chel Harefield	8.56	1.42	1.77	2.28	2.61	2.74
Myco. chel Epping	8.74	0.35	0.84	1.03	0.90	1.18
Myco. fortuitum	8.89	0.09	0.17	0.39	0.34	0.86
Myco. tuberculosis	8.32	0.22	0.41	0.38	0.44	0.59
MAI clinical	8.51	0	0	0	0	0.09
Myco. terrae	9.13	0	0.21	0.39	0.20	0.17
Dirty Conditions						
Myco. chel NCTC 946	9.09	0	0.18	1.89	3.62	3.41
Myco. chel Harefield	9.12	0.05	0.32	0.69	1.11	1.47
Myco. chel Epping	8.74	0.35	0.84	1.03	0.90	1.18
Myco. fortuitum	8.89	0.23	0.38	0.45	0.62	0.72
Myco. tuberculosis	8.39	0.61	0.43	0.49	0.67	0.60
MAI clinical	8.35	0.02	0.06	0	0.07	0.09
Myco. terrae	9.09	0	0.	0.14	0.16	0.27

Table 3.2.2.13: Activity of 3% w/v peroxygen compound (Virkon) against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	9.56	0.61	0.90	1.07	1.58	2.05
Myco. chel Harefield	8.39	2.47	2.73	3.03	3.12	3.17
Myco. chel Epping	8.56	0.21	0.30	0.65	0.79	1.56
Myco. fortuitum	8.02	0.36	0.64	0.60	1.06	1.08
Myco. tuberculosis	7.72	0.70	0.47	0.48	0.52	0.51
MAI clinical	8.64	0.09	0.10	0.11	0.20	0.17
Myco. terrae	8.95	0.02	0.13	0.13	0.37	0.24
Dirty Conditions						
Myco. chel NCTC 946	9.34	0.72	1.01	1.09	2.03	2.96
Myco. chel Harefield	8.48	1.21	1.50	1.67	2.07	1.88
Myco. chel Epping	8.63	0.54	1.04	0.78	1.32	1.76
Myco. fortuitum	8.30	0.87	0.82	0.99	1.30	1.47
Myco. tuberculosis	7.93	0.63	0.60	0.48	0.79	0.66
MAI clinical	8.92	0	0.02	0.10	0.12	0.25
Myco. terrae	9.11	0.04	0.01	0.26	0.22	0.53

Table 3.2.2.14: Activity of 10% v/v succinedialdehyde and formaldehyde mixture (Gigasept) against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	9.29	0.02	0.25	0.40	**>5.81**	>5.81
Myco. chel Harefield	8.90	0.03	0.15	2.71	3.63	**>5.42**
Myco. chel Epping	8.61	0	0	0	0	0.23
Myco. fortuitum	9.20	0.10	0.33	0.86	3.46	**>5.72**
Myco. tuberculosis	8.94	0.37	0.52	1.63	3.90	**>5.46**
MAI clinical	9.99	0.52	0.56	0.58	0.67	2.12
Myco. terrae	8.61	0	0.11	0.50	1.78	**>5.13**
Dirty Conditions						
Myco. chel NCTC 946	9.35	0	0.32	0.35	**>5.87**	>5.87
Myco. chel Harefield	9.16	0.04	0.05	0.04	1.72	**>5.68**
Myco. chel Epping	8.80	0	0.02	0	0.16	0.46
Myco. fortuitum	9.02	0	0.10	0.67	3.34	**>5.54**
Myco. tuberculosis	8.76	0	0.44	1.19	3.42	**>5.28**
MAI clinical	9.89	0.42	0.41	0.57	0.93	2.52
Myco. terrae	8.68	0.06	0.32	0.71	1.81	**>5.20**

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.15: Activity of 0.35% v/v peracetic acid (NuCidex) against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	8.93	**>5.45**	>5.45	>5.45	>5.45	>5.45
Myco. chel Harefield	8.80	4.37	**>5.32**	>5.32	>5.32	>5.32
Myco. chel Epping	8.50	**>5.02**	>5.02	>5.02	>5.02	>5.02
Myco. fortuitum	8.82	4.43	**>5.34**	>5.34	>5.34	>5.34
Myco. tuberculosis	8.75	**>5.27**	>5.27	>5.27	>5.27	>5.27
MAI clinical	9.33	1.10	**>5.85**	>5.85	>5.85	>5.85
Myco. terrae	8.66	**>5.18**	>5.18	>5.18	>5.18	>5.18
Dirty Conditions						
Myco. chel NCTC 946	8.71	3.48	**>5.23**	>5.23	>5.23	>5.23
Myco. chel Harefield	8.54	2.73	**>5.06**	>5.06	>5.06	>5.06
Myco. chel Epping	8.54	4.47	**>5.06**	>5.06	>5.06	>5.06
Myco. fortuitum	9.16	**>5.68**	>5.68	>5.68	>5.68	>5.68
Myco. tuberculosis	8.48	**>5.00**	>5.00	>5.00	>5.00	>5.00
MAI clinical	9.71	1.24	**>6.23**	>6.23	>6.23	>6.23
Myco. terrae	8.74	**>5.26**	>5.26	>5.26	>5.26	>5.26

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

Table 3.2.2.16: Activity of chlorine dioxide (Tristel) against mycobacteria dried onto carriers under clean and dirty conditions

Mycobacterium	Mean Log Reductions					
	Log Initial Count	1 min	4 min	10 min	20 min	60 min
Clean Conditions						
Myco. chel NCTC 946	9.48	**>6.00**	>6.00	>6.00	>6.00	>6.00
Myco. chel Harefield	8.53	**>5.05**	>5.05	>5.05	>5.05	>5.05
Myco. chel Epping	9.06	2.82	**5.89**	>5.58	>5.58	>5.58
Myco. fortuitum	9.12	**>5.64**	>5.64	>5.64	>5.64	>5.64
Myco. tuberculosis	8.79	**>5.31**	>5.31	>5.31	>5.31	>5.31
MAI clinical	9.46	**>5.98**	>5.98	>5.98	>5.98	>5.98
Myco. terrae	9.36	3.91	**>5.88**	>5.88	>5.88	>5.88
Dirty Conditions						
Myco. chel NCTC 946	9.11	3.80	**>5.63**	>5.63	>5.63	>5.63
Myco. chel Harefield	8.74	3.10	**>5.26**	>5.26	>5.26	>5.26
Myco. chel Epping	9.05	1.94	**5.57**	>5.57	>5.57	>5.57
Myco. fortuitum	9.00	4.78	**>5.52**	>5.52	>5.52	>5.52
Myco. tuberculosis	8.63	**>5.15**	>5.15	>5.15	>5.15	>5.15
MAI clinical	9.47	2.33	**>5.99**	>5.99	>5.99	>5.99
Myco. terrae	9.29	1.72	2.78	**>5.81**	>5.81	>5.81

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

3.3 COMPARISON OF THE PHENOTYPIC CHARACTERISTICS OF GLUTARALDEHYDE RESISTANT *MYCO. CHELONAE* AND THE TYPE STRAIN NCTC 946

In an attempt to understand the observed reduced affinity of the washer disinfector isolates of *Myco. chelonae* for glutaraldehyde, a number of tests were carried out to investigate the surface properties, lipid composition and the possible increased resistance to other aldehydes and heat of all three strains.

3.3.1 Minimum Inhibitory Concentration

The minimum inhibitory concentration of 2% alkaline glutaraldehyde in 7H9 broth is shown in Table 3.3.1.1. The presence/absence of turbidity was used as an indication of growth. The type strain of *Myco. chelonae* was inhibited by a concentration of 0.063% alkaline glutaraldehyde. A higher concentration of 0.25% was necessary to inhibit the growth of the glutaraldehyde resistant strains. As expected, it was not possible to make any observations when the organisms were grown in water. Results obtained when 1 loopful was removed from each of the tubes and plated onto 7H11 agar can be seen in Table 3.3.1.2

Table 3.3.1.1 Minimum Inhibitory Concentration of 2% alkaline glutaraldehyde against *Myco. chelonae*

Myco. chelonae	Concentration of glutaraldehyde (%) in 7H9 broth								
	2	1	0.5	0.25	0.125	0.063	0.031	0.016	Control
NCTC 946	-	-	-	-	-	-	+	+	+
Harefield	-	-	-	-	+	+	+	+	+
Epping	-	-	-	-	+	+	+	+	+

+ = growth

- = no growth

Table 3.3.1.2 Minimum Mycobactericidal Concentration of 2% glutaraldehyde against *Myco. chelonae*

Myco. chelonae	% glutaraldehyde in tubes from which samples were taken								
	2	1	0.5	0.25	0.125	0.063	0.031	0.016	Control
Water									
NCTC 946	-	-	-	-	-	-	+	+	+
Harefield	-	-	-	-	+	+	+	+	+
Epping	-	-	-	-	+	+	+	+	+
7H9 broth									
NCTC 946	-	-	-	-	+	+	+	+	+
Harefield	-	-	-	+	+	+	+	+	+
Epping	-	-	-	+	+	+	+	+	+

+ = growth

- = no growth

3.3.2 Susceptibility to aldehydes other than 2% glutaraldehyde

The type strain of *Myco. chelonae* and the glutaraldehyde resistant strains i.e. Harefield and Epping are similar in their resistance to 4% formaldehyde. Formaldehyde achieved a >6 $\log_{10}$ reduction in all 3 strains after 20 minutes exposure. Glyoxal was ineffective against the washer disinfector isolates of *Myco. chelonae* in 60 minutes, achieving only a 3 $\log_{10}$ reduction, but was effective against the type strain in 20 minutes, achieving a 5.13 $\log_{10}$ reduction. Aidal Plus, the acid glutaraldehyde was unable to achieve a 1 $\log_{10}$ reduction in the Harefield and Epping strains in 60 minutes but the type strain was more sensitive with a >6.68 $\log_{10}$ reduction in 1 minute. Gigasept, the succinedialdehyde and formaldehyde mixture, achieved a >5.67 $\log_{10}$ reduction in *Myco. chelonae* NCTC 946 in 10 minutes, a >5.44 $\log_{10}$ reduction in the Harefield strain in 20 minutes, but was totally ineffective against the Epping strain in 60 minutes, with <1 $\log_{10}$ reduction being observed. The mean $\log_{10}$ reductions can be seen in Table 3.3.2.1

Table 3.3.2.1 Susceptibility to aldehydes other than 2% glutaraldehyde

Disinfectant	Log Initial Count	Mean log reductions				
		1 min	4 min	10 min	20 min	60 mir
2% Asep*						
Myco. chelonae NCTC 946	8.64	**>5.64**	>5.64	>5.64	>5.64	>5.64
Myco. chelonae Harefield	8.43	0.24	0.30	0.35	0.51	0.64
Myco. chelonae Epping	9.10	0	0.12	0.09	0.33	0.29
10% Gigasept*						
Myco. chelonae NCTC 946	8.67	0	0.51	**>5.67**	>5.67	>5.67
Myco. chelonae Harefield	8.44	0	0	2.69	**>5.44**	>5.44
Myco. chelonae Epping	8.35	0.09	0.16	0.06	0.17	0.13
Aidal Plus @ 20°C						
Myco. chelonae NCTC 946	9.60	**>6.60**	>6.60	>6.60	>6.60	>6.60
Myco. chelonae Harefield	9.82	0	0.01	0.12	0.17	0.31
Myco. chelonae Epping	9.84	0.13	0.16	0.21	0.27	0.42
Aidal Plus @ 25°C						
Myco. chelonae NCTC 946	9.68	**>6.68**	>6.68	>6.68	>6.68	>6.68
Myco. chelonae Harefield	9.76	0.06	0.09	0.25	0.37	0.84
Myco. chelonae Epping	9.69	0.04	0.05	0.18	0.23	0.84
4% Formaldehyde						
Myco. chelonae NCTC 946	9.75	0.18	0.27	1.10	**>6.75**	>6.75
Myco. chelonae Harefield	9.70	0.09	0.30	2.61	**>6.70**	>6.70
Myco. chelonae Epping	9.87	0.15	0.41	2.42	**>6.87**	>6.87
10% Glyoxal						
Myco. chelonae NCTC 946	9.13	0.31	0.50	1.26	**5.13**	>6.13
Myco. chelonae Harefield	9.71	0.03	0.04	0.10	0.34	3.72
Myco. chelonae Epping	9.79	0.10	0.14	0.21	0.55	3.32

* = Results taken from section 4.10

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

3.3.3 Adherence to hydrocarbons

The adherence of the 3 strains of *Myco. chelonae* to octane and hexadecane is illustrated in Figs. 3.3.3.1 and 3.3.3.2 respectively. Table 3.3.3.1 presents the actual % absorbance readings. In both cases, the Harefield and Epping strains appeared to be more adherent than the type strain, i.e. they both adsorbed more of the hydrocarbons and so less of the organisms were detected in the aqueous layer which resulted in a lower % absorbency. This indicates that the glutaraldehyde resistant strains are slightly more hydrophobic in nature than *Myco. chelonae* NCTC 946.

Table 3.3.3.1: % Absorbance of *Myco. chelonae* to hexadecane and octane

Test Strain	**Vol. (ml) hydrocarbon**			
	0.05	**0.10**	**0.15**	**0.20**
Myco. chelonae NCTC 946				
Hexadecane	95.14	94.87	95.45	96.56
Octane	96.78	96.72	96.41	96.25
Myco. chelonae Harefield				
Hexadecane	92.12	88.79	88.18	86.06
Octane	92.12	90.16	88.33	88.64
Myco. chelonae Epping				
Hexadecane	84.22	80.52	82.82	79.02
Octane	85.31	85.11	83.92	81.82

Fig. 3.3.3.1: Adherence of Myco. chelonae to octane

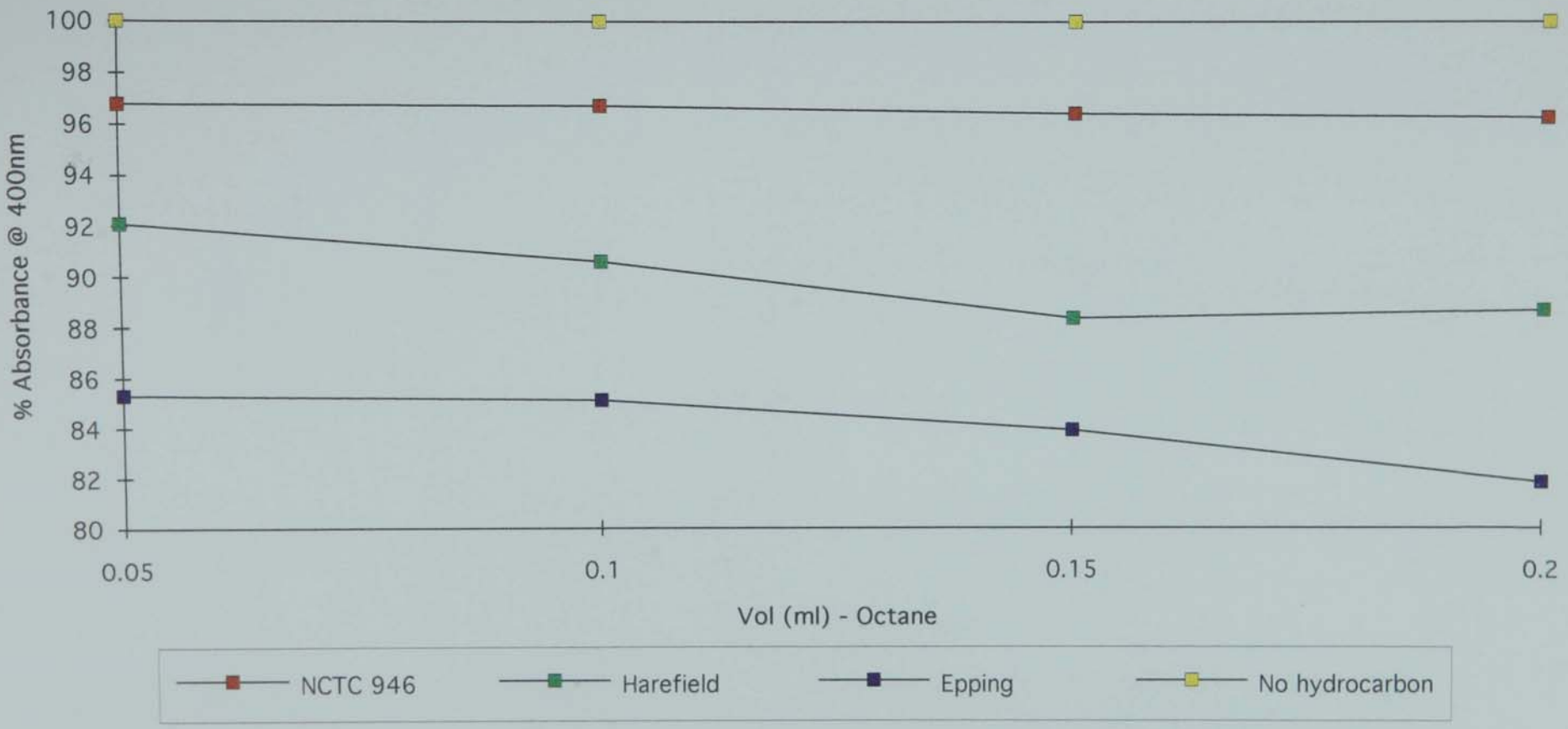
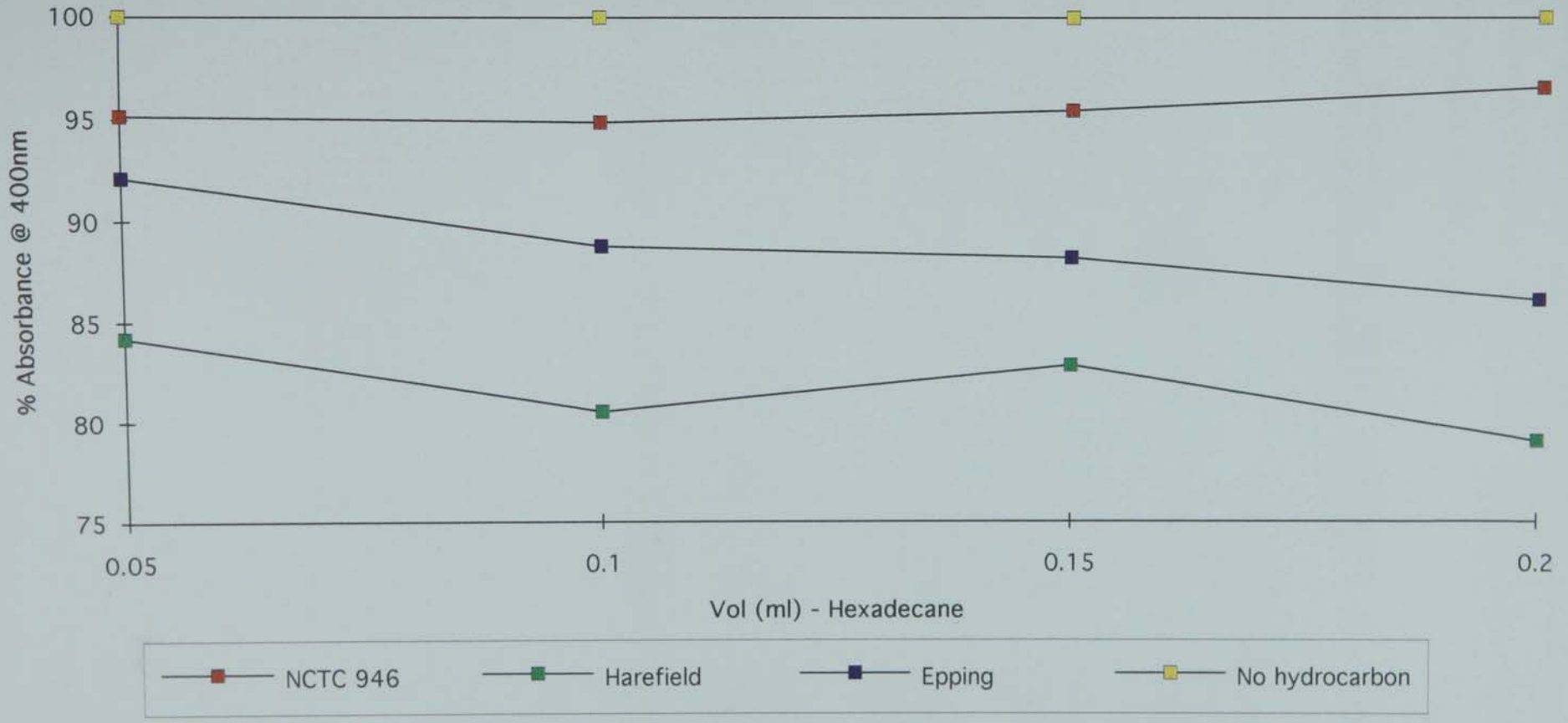


Fig. 3.3.3.2: Adherence of Myco. chelonae to hexadecane



The surface hydrophobicity was matched by the pattern of resistance to Stericol. The more hydrophobic strains were similarly more resistant to killing by this agent. (Table 3.3.3.2)

Table 3.3.3.2: Resistance of *Myco. chelonae* to varying concentrations of phenolic in the form of 'Stericol'

Disinfectant	Log Initial Count	Mean log reductions				
		1 min	4 min	10 min	20 min	60 min
1% Stericol						
Myco. chelonae NCTC 946	9.74	1.64	2.77	3.10	3.61	**>6.74**
Myco. chelonae Harefield	9.44	2.09	2.32	2.90	3.13	3.44
Myco. chelonae Epping	9.39	1.81	2.85	3.23	3.71	3.85
1.5% Stericol						
Myco. chelonae NCTC 946	9.61	1.85	3.81	**5.31**	>6.61	>6.61
Myco. chelonae Harefield	9.18	1.72	2.69	3.92	4.09	**5.30**
Myco. chelonae Epping	9.37	2.34	2.83	3.85	4.26	**5.49**
2% Stericol						
Myco. chelonae NCTC 946	9.64	**>6.64**	>6.64	>6.64	>6.64	>6.64
Myco. chelonae Harefield	9.20	2.71	3.90	4.25	3.98	**>6.20**
Myco. chelonae Epping	9.35	2.60	**5.95**	5.00	5.95	>6.35

Numbers in bold indicate the time taken to achieve a >5 $\log_{10}$ reduction

3.3.4 Fatty Acid Analysis

The fatty acid methyl ester (FAME) profiles of *Myco. chelonae* NCTC 946 and the Epping strain are presented in Fig. 3.3.4.1 together with the standard bacterial FAME mixture (chain length C12-C20, Hewlett Packard). Although it was not possible to identify the major FAMEs, their retention times indicated carbon chain lengths between C14 and C20. The Harefield isolate of *Myco. chelonae* gave a similar profile to Epping, the results showing the retention time (mins) of all the major FAMEs are listed in Table 3.3.4.1 and the relative amounts of each in Table 3.3.4.2. There appears to be no major differences in the relative amounts of short chain fatty acids (C12-C20) of all three strains. This indicates that the observed increased resistance to glutaraldehyde is not associated with changes in the short chain fatty acids.

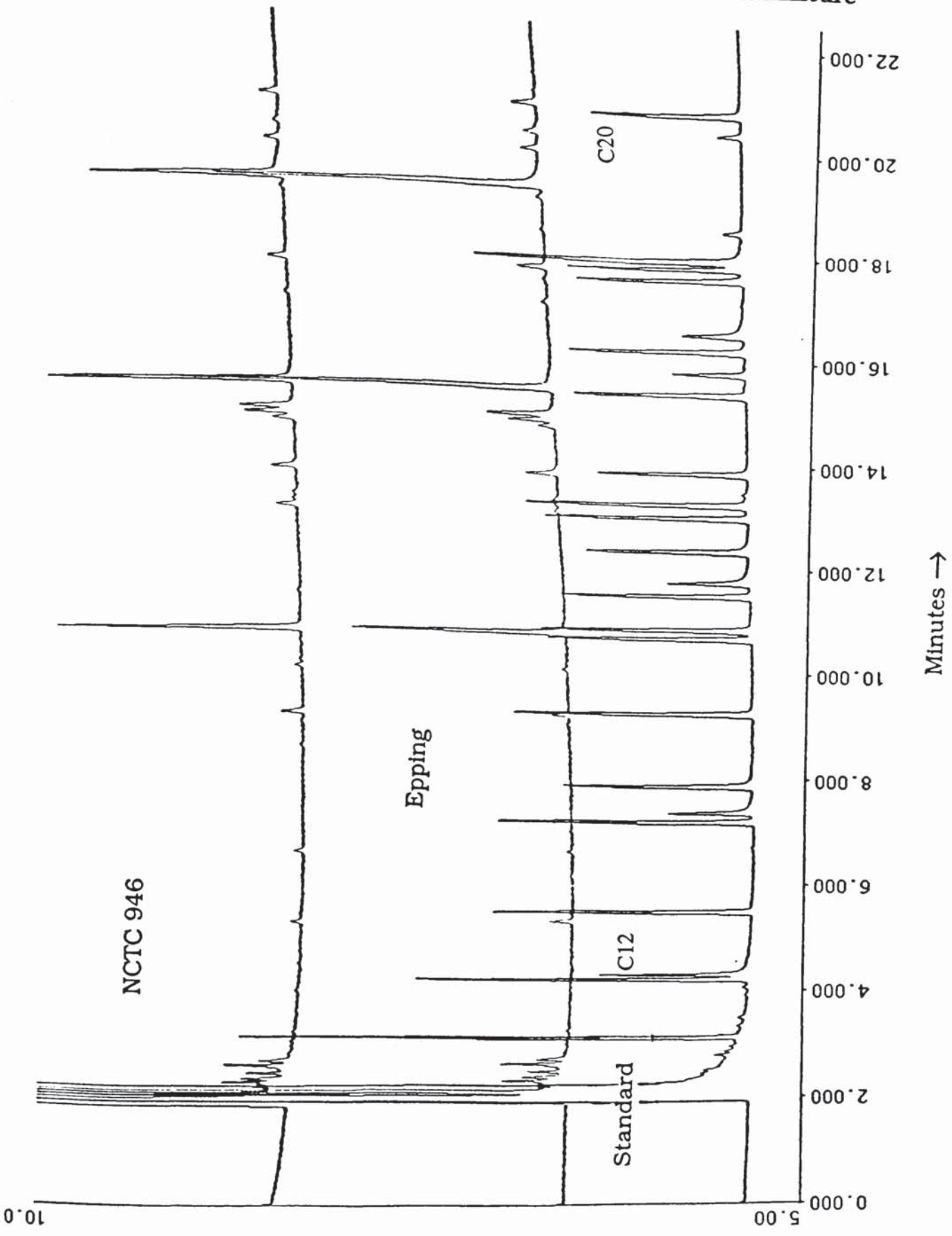
Table 3.3.4.1 Retention time of the major FAMEs

Strain	Retention Time (mins) of the major FAMEs										
NCTC	5.3	-	10.8	13.1	13.8	14.7	14.8	14.9	15.4	17.8	19.3
Epping	5.3	9.2	10.8	-	13.8	14.7	14.8	15.0	15.4	17.8	19.3
Harefield	5.3	9.2	10.9	-	13.8	14.7	14.8	15.0	15.4	17.8	19.3

Table 3.3.4.2. Relative amount of the major FAMEs

Strain	% Relative amount of the major FAMEs										
NCTC	1.3	-	15.9	1.7	2.1	2.2	4.6	6.1	33.1	1.5	23.3
Epping	1.2	1.1	17.2	-	2.3	2.3	4.6	6.1	34.7	2.0	29.5
Harefield	1.2	1.1	17.2	-	2.3	2.3	4.6	6.0	34.7	2.0	29.8

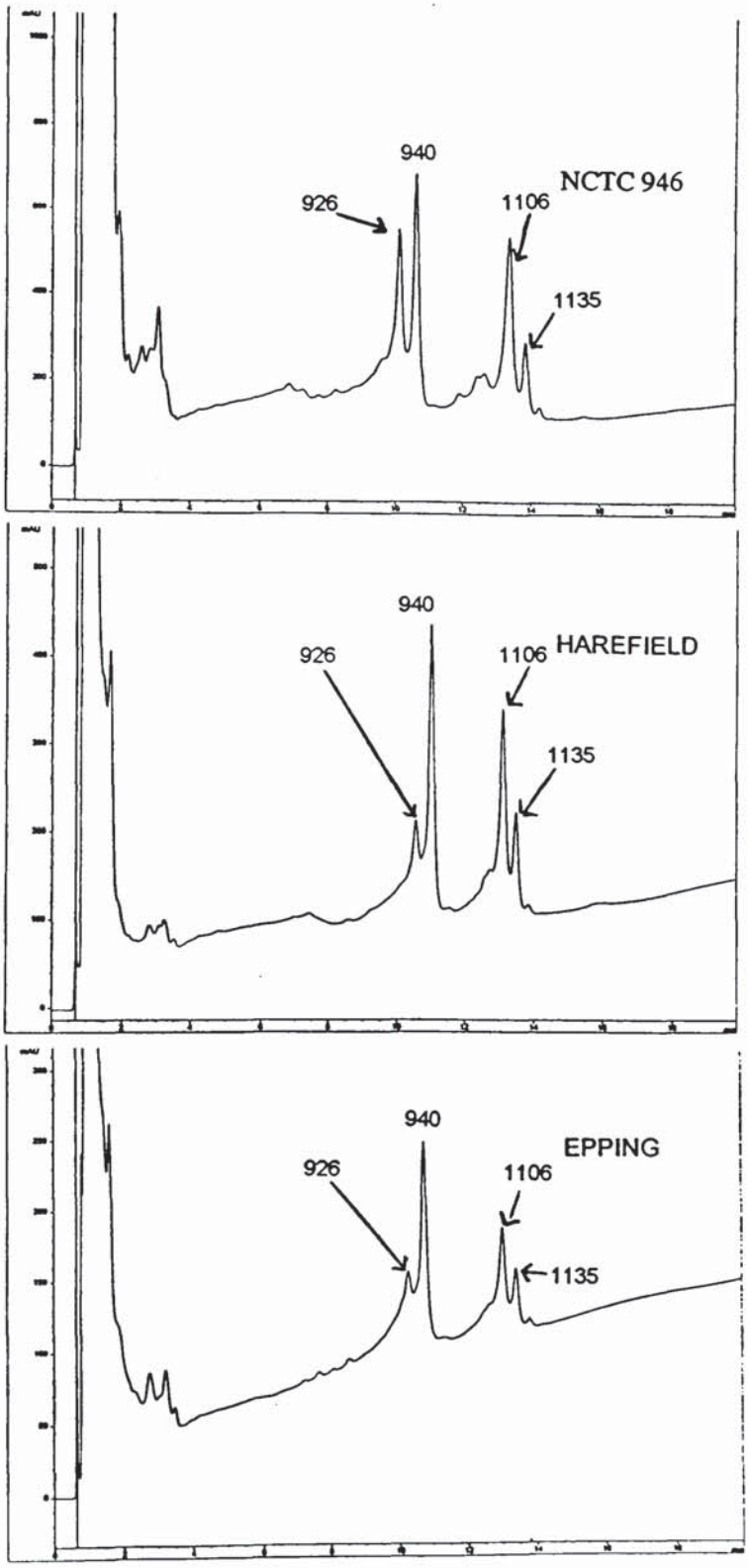
Fig. 3.3.4.1 FAME Profiles of *Myco. chelonae* NCTC 946 and the Epping strain, together with the bacterial FAME standard mixture



3.3.5 Mycolic Acid Analysis

The elution profiles of the mycolic acid bromophenacyl esters are presented in Fig. 3.3.5.1. The profiles are similar to those obtained by Butler & Kilburn (1990). In addition to comparison of HPLC profiles revealed by UV detection, the molecular weights of each of the major peaks were determined using API-electrospray MS. This technique showed that all four major peaks were identical in the three strains of *Myco. chelonae.* This indicates that the chain length and relative amount of mycolic acids present in the glutaraldehyde resistant strains of *Myco. chelonae* do not differ from the sensitive type strain (NCTC 946) and are therefore presumably not directly responsible for the observed increased resistance. It is, however, possible that the conformations of the mycolic acids have altered leading to this resistance and further investigations are required.

Fig. 3.3.5.1 The elution profiles of mycolic acid bromophenacyl esters of the three strains of *Myco. chelonae*. Peaks are measured in milliabsorbance units (mAU) at 254nm, the elution time scale is in minutes. Molecular weights of the four peaks determined by API-electrospray MS are also shown.



3.3.6 Resistance to heat

The mean $\log_{10}$ reductions in the 3 strains of *Myco. chelonae* at 1, 3, 5, 10, 20, and 30 minute time intervals and temperatures of 65°C, 71°C, 80°C and 90°C are presented in Table 3.3.6.1. At 65°C, a 5.47 $\log_{10}$ reduction was achieved in the type strain of *Myco. chelonae* in 20 minutes, but the two washer disinfector strains were not destroyed in 30 minutes. Similarly at 80°C the washer disinfector isolates appeared to be more resistant to heat than the type strain. One minute was sufficient to completely destroy *Myco. chelonae* NCTC 946, but the other two strains required the slightly longer time of 3 minutes. At 71°C all three strains were destroyed in 10 minutes and all were destroyed in 1 second at 90°C.

Table 3.3.6.1: Resistance of *Myco. chelonae* to heat

	Mean $\log_{10}$ reductions					
	1 min	**3 min**	**5 min**	**10 min**	**20 min**	**30 min**
65°C						
Myco. chelonae NCTC 946	0.95	2.32	3.42	4.59	**5.47**	6.37
Myco. chelonae Harefield	2.61	3.12	3.75	3.35	4.52	4.77
Myco. chelonae Epping	2.84	2.91	4.35	2.99	3.94	3.44
71°C						
Myco. chelonae NCTC 946	3.69	4.14	3.83	**6.77**	6.77	6.77
Myco. chelonae Harefield	3.25	3.53	4.97	**7.27**	6.89	6.89
Myco. chelonae Epping	2.71	3.53	3.96	**6.89**	7.27	7.27
80°C						
Myco. chelonae NCTC 946	**6.37**	6.77	6.77	6.77	6.77	6.77
Myco. chelonae Harefield	3.22	**7.27**	7.27	7.27	7.27	7.27
Myco. chelonae Epping	3.75	**5.24**	6.89	6.89	6.89	6.89
90°C	**1 sec**	**30 sec**	**60 sec**	**90 sec**		
Myco. chelonae NCTC 946	**6.77**	6.77	6.77	6.77		
Myco. chelonae Harefield	**7.27**	7.27	7.27	7.27		
Myco. chelonae Epping	**6.89**	6.89	6.89	6.89		

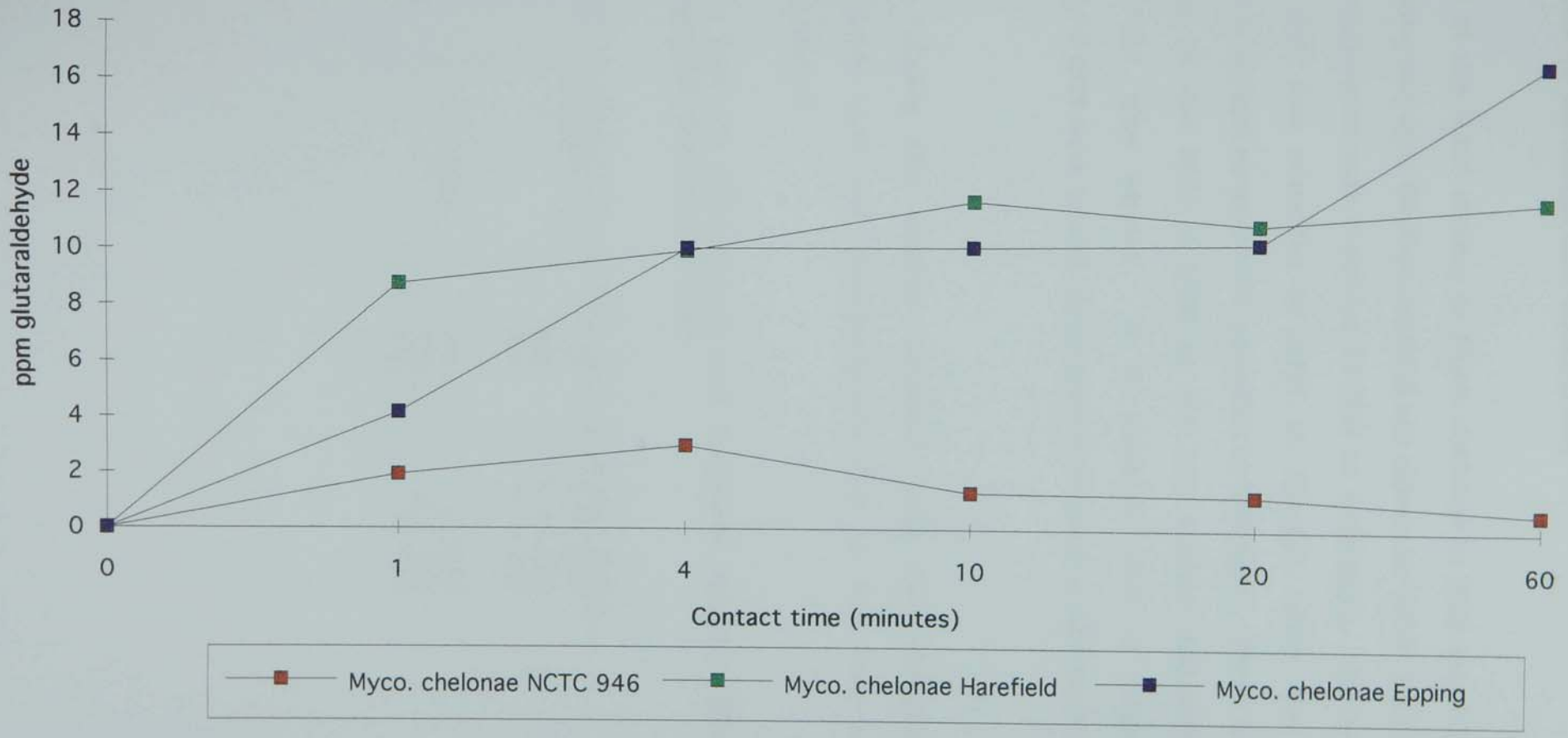
3.3.7 Glutaraldehyde Uptake

A standard curve of the absorbance at 628nm of 0, 5, 10, 15 and 20ppm alkaline glutaraldehyde was prepared, from which the amount of glutaraldehyde remaining in the test suspensions could be read. This allowed calculation of ppm glutaraldehyde and % glutaraldehyde taken up by the cells. The results are presented in Table 3.3.7.1. Fig. 3.3.7.1 is a graphical representation of the results from which it can be clearly seen that *Myco. chelonae* NCTC 946 takes up very little glutaraldehyde over 1-60 minute exposure. However, the glutaraldehyde-resistant organisms appear to take up increasing amounts of glutaraldehyde

Table 3.3.7.1: ppm glutaraldehyde taken up by *Myco. chelonae*

Test Organism	Exposure time	Absorbance @ 628 nm			ppm glutaraldehyde remaining	ppm glutaraldehyde taken up	% glutaraldehyde taken up
		1	2	Mean			
NCTC	1	0.636	0.629	0.633	18.07	1.93	6.69
	4	0.564	0.591	0.578	17.01	2.99	14.96
	10	0.662	0.604	0.633	18.68	1.32	6.61
	20	0.626	0.647	0.637	18.78	1.22	6.08
	60	0.650	0.661	0.656	19.36	0.64	3.22
	Control	-0.048	-0.041	-0.045	-1.72	0	0
Harefield	1	0.390	0.379	0.385	11.20	8.80	44.01
	4	0.310	0.314	0.312	10.00	10.00	50.00
	10	0.291	0.277	0.284	8.17	11.83	59.14
	20	0.297	0.326	0.312	9.00	11.00	55.00
	60	0.260	0.306	0.283	8.14	11.86	59.29
	Control	-0.097	-0.087	-0.092	-3.15	0	0
Epping	1	0.512	0.564	0.538	15.19	4.18	20.91
	4	0.322	0.361	0.342	9.90	10.10	50.48
	10	0.355	0.323	0.339	9.83	10.17	50.86
	20	0.368	0.299	0.334	9.66	10.34	51.69
	60	0.129	0.113	0.121	3.27	16.73	83.67
	Control	-0.094	-0.084	-0.089	-3.06	0	0

Fig. 3.3.7.1: ppm glutaraldehyde taken up by Myco. chelonae



3.3.8 Resistance to isoniazid and ethambutol

The resistance of the three strains of *Myco. chelonae* to INH and ETM as assessed using the agar dilution method are shown in Table 3.3.8.1. All three test organisms were resistant to INH at 1024mg/L. The type strain NCTC 946 was sensitive to ETM at 32mg/L while the 2 glutaraldehyde resistant strains were sensitive at 256mg/L. There is a clear difference in the MIC of ETM to the two washer disinfector isolates and the type strain. It is possible that if higher concentrations of INH were tested, there may have been a difference in this MIC also.

Table 3.3.8.2 shows the results obtained from the Reference Laboratory, which were not discriminatory due to the very low concentrations tested.

Table 3.3.8.1: MIC of ethambutol and isoniazid against *Myco. chelonae* using agar dilution method

Myco. chelonae	Antibiotic	MIC mg/L	Range mg/L
NCTC 946	Ethambutol	32	0.5-1024
Harefield		256	0.5-1024
Epping		256	0.5-1024
NCTC 946	Isoniazid	>1024	0.5-1024
Harefield		>1024	0.5-1024
Epping		>1024	0.5-1024

Table 3.3.8.2: MIC as reported from the Mycobacteria Reference Laboratory

Myco. chelonae	Antibiotic	MIC mg/L
NCTC 946	Ethambutol	>6.4
Harefield		>6.4
Epping		>6.4
NCTC 946	Isoniazid	>0.28
Harefield		>0.28
Epping		>0.28
NCTC 946	Rifampicin	>64
Harefield		>64
Epping		>64

4.0 DISCUSSION

4.0 DISCUSSION

The re-emergence of tuberculosis, the health impact of MDR-TB and the increasing clinical importance of the atypical mycobacteria, are well documented (Sattar *et al.*, 1995), and so is the need for effective infection control procedures. Disinfectants are widely used to destroy microorganisms present on heat-labile equipment and environmental surfaces and accurate data on their mycobactericidal activity is therefore required. However, there can be no national or international system for the approval of disinfectants without first establishing suitable test methods. In theory, this seems a simple task: *Mycobacterium tuberculosis* or a safe surrogate, in suspension or dried onto carriers/test surfaces is exposed to a disinfectant and after specific exposure times, is checked to determine whether or not the test mycobacteria are destroyed (Reybrouck, 1992).

In practice, there are several problems, e.g. choice of a suspension or carrier test, the preparation of a standard challenge, the use of a realistic organic soil to simulate the worst case in-use conditions and the selection of an effective neutralizer and recovery system. All these factors greatly influence efficacy testing. The vast number and diversity of test methods show the lack of agreement on the standardization of such factors within the test methods. With the establishment within the European Community, of the CEN TC216 committee and its various working groups in 1989, it is hoped to have harmonized European disinfectant test methods in the very near future. In the meantime, and, in the absence of an internationally accepted disinfectant test method, it was necessary to decide upon a

practical and reproducible method for establishing mycobactericidal activity.

There has been much debate on the need to use either a suspension or a carrier test or both for testing the efficacy of disinfectants. Many favour a carrier test because it is usually more realistic and stringent. Best *et al.* (1988) indicated that results of suspension vs. carrier tests depend on the type and concentration of the disinfectant being tested and the surface and nature of the carrier. Also, problems have been encountered in carrier tests with the composition of the carrier used, the ability to completely remove or recover the test organism from the carrier after exposure to the disinfectant and enumeration following recovery. Ascenzi *et al.* (1987) pointed out that often, in carrier tests, the carrier (e.g. silk sutures, porcelain cylinders) is not realistic as it does not represent the types of material that usually require disinfection. Several problems were encountered by the AOAC with their carrier tests and these have been extensively documented (Lind *et al.*, 1986; Ascenzi *et al.*, 1986; Ascenzi *et al.*, 1987; Best *et al.*, 1988; Cole *et al.*, 1990; Ascenzi *et al.*, 1991) . Inaccuracies in their methods have led to problems with registration of products in the US. These problems have since been solved and a revised AOAC carrier test published. The AOAC is not the only "official method" to encounter such problems with accuracy and reproducibility of the carrier test, merely the most publicized.

In view of the problems with the standardization of carrier tests, quantitative suspension tests have been recommended (Van Klingeren, 1987). Ideally, to simulate realistic in-use conditions both should be used. A suspension test gives an indication of the effectiveness of

adding a disinfectant to organisms suspended in a liquid, e.g. mop buckets and laboratory discard jars. They also provide a rapid, accurate screening procedure for new products. Carrier tests, in theory, are indicative of the effectiveness of disinfectants used on contaminated surfaces, e.g. heat-sensitive instruments and work surfaces, particularly where the organisms have dried onto the surface. Many disinfectants contain surfactants and are used to remove microorganisms in addition to destroying them.

It is now generally agreed that suspension tests should be used for initial screening tests particularly for new products, and their efficacy should be further confirmed using carrier tests. Best *et al.* (1988) have addressed many of the problems of both suspension and carrier tests with particular emphasis on tests used to assess mycobactericidal activity and have proposed standard carrier and suspension test methods. These tests have been chosen, in principle, as standard, reproducible tests for use throughout this study. Prior to their use, the recovery efficiency of each method was assessed, to ensure there was no loss of inoculum due to manipulation during the tests. There were, however, some modifications. Best *et al.* (1988) used tap water as a diluent, this was rejected and sterile distilled water used as *Myco. chelonae* and other environmental mycobacteria have been isolated often in large numbers, from tap water. Other areas of the method were also assessed and changed if deemed necessary, e.g. organic load, neutralization.

4.1 STANDARDIZATION OF TEST METHOD

Choice of test organisms

One of the main considerations when selecting a standard test method is the choice of test organisms and preparation of the initial inoculum. Several strains have been suggested for use in the evaluation of mycobactericidal agents. Some workers believe that the use of pathogens, particularly those in Hazard Group 3 (ACDP Guidelines, 1995), is inappropriate for safety reasons and slow-growing organisms are impractical for routine disinfectant testing because of very long incubation times required and the possibility of environmental contamination of the recovery system. *Myco. tuberculosis* is therefore unsuitable as a test organism due to its high pathogenicity and slow growth rate. This has led to much research for a "surrogate", which would be safer to use, require shorter incubation times and be similar in its resistance to *Myco. tuberculosis*. As a result a number of possible test strains have been evaluated, including *Myco. bovis* BCG (Ascenzi *et al.*, 1991), *Myco. smegmatis* (Best *et al.*, 1988; 1990) and *Myco. terrae* (Van Klingeren *et al.*, 1987; Borneff *et al.*, 1996). *Myco. bovis* BCG is the test organism currently used in the American standard 'AOAC' method. *Myco. smegmatis*, although initially popular was rejected both in the USA and Europe as it was found to be more sensitive than *Myco. tuberculosis*. *Myco. terrae* has been shown to be similar in its resistance to *Myco. tuberculosis* and it is likely this will be the accepted as a suitable surrogate in the new European standard test methods for mycobactericidal testing.

It must be noted, however, that, with the increasing clinical significance of other mycobacteria, a surrogate must be representative of mycobacteria in general, not just *Myco. tuberculosis*. There is surprisingly little published data on the efficacy of disinfectants against environmental mycobacteria. Much work has been done with *Myco. tuberculosis* but there is no general agreement on the accuracy, reproducibility and reliability of test data. Several species and types of mycobacteria must first be tested before a surrogate can be selected. With this in mind strains of *Myco. tuberculosis*, *Myco. avium-intracellulare*, *Myco. chelonae*, *Myco. fortuitum* and *Myco. terrae* have been chosen as test organisms for use in this study. *Myco. tuberculosis* H37 Rv, the virulent strain was used to develop the standard test method and for comparison with other potential organisms. A type strain with a documented history was chosen because of its clinical relevance and the difficulties likely to be encountered with its manipulation, the extended incubation times necessary for recovery and its tendency to form clumps in suspension.

Of the environmentally associated mycobacteria, *Myco. avium-intracellulare* is currently the one of most clinical significance. Recent reports show an increasing number of infections with this organism, particularly in AIDS patients (Hanson, 1988; Hellyer *et al.*, 1993) and that it is more resistant to glutaraldehyde than *Myco. tuberculosis* (Collins FM., 1986; Hanson, 1988). This study provided an ideal opportunity for a direct comparison between *Myco. tuberculosis* and *Myco. avium-intracellulare* against several widely used disinfectants.

In an attempt to produce a harmonized European test method for assessing the mycobactericidal activity of disinfectants, various surrogates for *Myco. tuberculosis* have been proposed as alternative test organisms. These have been suggested in preference to *Myco. tuberculosis* because they are safer to use and easier to manipulate, yet similar in their resistance to disinfectants. However, one must question the ability of a surrogate to mimic pathogen susceptibility. Is it wise to use a surrogate for the routine assessment of tuberculocidal activity? Lind *et al.* (1988) point out that various mycobacterial species have different patterns/mechanisms of resistance and therefore results obtained with surrogates could not be directly extrapolated to *Myco. tuberculosis* or indeed any of the other mycobacteria. It is interesting to note that the use of a surrogate has never been considered suitable for antibiotic susceptibility testing. Nevertheless a number of possible surrogates have already been evaluated of which *Myco. terrae* is the most popular. It would appear that this would be the test organism of choice for the proposed European test method. The type strain (NCTC 10856) was therefore included in the study to assess if it was, as reported, similar in its resistance to disinfectants as *Myco. tuberculosis*

Myco. chelonae was chosen initially because of an increasing number of reports associated with problems caused by its presence as a contaminant in water supplies, flexible bronchoscopes and endoscope washer disinfectors and the lack of data regarding its susceptibility to disinfectants. During the course of the project, the emergence of two glutaraldehyde resistant washer disinfector strains of *Myco. chelonae*

was highlighted and these were included with the type strain. Both strains were being continuously isolated from 2 endoscope washer disinfectors and appeared to be unaffected by 2% glutaraldehyde which was routinely used to disinfect the endoscopes and the machines.

Myco. fortuitum was chosen because of its close association with *Myco. chelonae*. Both of these mycobacteria are known to be very resistant to antibiotics but there are little or no data available on their susceptibility to disinfectants.

Based on the results of the disinfectant tests carried out in this it is recommended that *Myco. terrae* could be used in initial screening tests using a suspension test under clean conditrions, to assesss the mycobactericidal efficacy (if any) of a new disinfectant formulation. The results obtained in this initial test, would then need to be confirmed using clinically relevant strains of mycobacteria, e.g. *Myco. tuberculosis* or *Myco. avium-intracellulare* using more stringent conditions, e.g. suspension and carrier tests under clean and dirty conditions.

Preparation of test suspensions

The broth method A utilized 100ml 7H9 broth which was inoculated with 1 colony of each test organism and incubated appropriately. The 7H9 supported the growth of all the test organisms in a batch culture system. However, although the initial counts were high ($\log_{10}$ 6.28 - 7.95) the maximum obtainable titres were not quite high enough for

use as test suspensions i.e. to accommodate a 3 $\log_{10}$ loss during recovery and demonstration of at least a 5 $\log_{10}$ reduction in mycobacteria. Problems were encountered with method A in maintaining similar titres in each tube on freezing in 1ml amounts. This was especially so with *Myco. tuberculosis*, in which post freeze counts varied considerably and were much lower than pre freeze counts. Freezing at -70^{0}C has been shown not to affect the viability of mycobacteria, therefore the lower post freeze counts have to be attributed to clumping and a failure to disperse the mycobacteria in the challenge inocula.

With the rapid growers, i.e. *Myco. chelonae* and *Myco. fortuitum*, the titre was increased to $\log_{10}$ 8.7-9.0 by concentrating several tubes together and using the resulting concentrates as the initial inoculum. A similar concentration technique was attempted with *Myco. tuberculosis* but proved unsuccessful again due to clumping. Ziehl-Neelsen stains carried out on post freeze suspensions confirmed this. This would lead to problems with reproducibility in a standard disinfectant test. This was confirmed by the results of suspension tests carried out to assess the activity of 2% alkaline glutaraldehyde and 1,000ppm NaDCC against *Myco. tuberculosis* under clean and dirty conditions. Initial counts were lower than required for the disinfectant test and results were not consistent (i.e. counts varied from plate to plate and from test to test). Colonies were very uneven in size and distribution. This method could not cope with the high degree of clumping in *Myco. tuberculosis* suspensions, and is therefore unsuitable for preparing an initial inoculum for any tests with this

organism. It can, however, be reliably used with the rapid growers, i.e. *Myco. chelonae* and *Myco. fortuitum*, but it does not lend itself well to a standard test method.

This method is not, therefore, recommended for preparing the test suspensions, but it can be used very successfully for preparing a seed pool and storing the test organisms for at least 12 months. This allows the same test strains to be used time and again without excessive subculturing which can affect the susceptibility of the organism to the disinfectant under test (Wayne, 1994).

The plate method B produced test suspensions of higher titres ($\log_{10}$ 7.89-8.50). When used in a disinfectant test the titre was high enough to accommodate a 3 $\log_{10}$ reduction in recovery and show a >5 $\log_{10}$ reduction. Also, the results were accurate and reproducible and the colonies were evenly sized and distributed. They appeared to grow much more luxuriantly than in the previous method. Using the plate method B to obtain the initial test suspension a >5 log reduction was achieved in 10 minutes in both clean and dirty suspensions, colonies appeared more luxuriant and recovery was observed within the 21 days incubation. Although clumped mycobacteria provide a more stringent and realistic challenge, they would be unacceptable in a standard test method.

The plate method C also gave very high titres of evenly sized colonies which were evenly dispersed on the plates. Counts as high as $\log_{10}$ 9.92 were obtained and growth was luxuriant. Disinfectant test results were again accurate and reproducible. Although both of the

plate methods gave similar results, the plate method C was chosen for use in the study. This method originated from a proposal by an ad hoc working group (WG1) of the CEN TC216 committee for use in a European mycobactericidal disinfectant test. It addresses the problem of clumping by using the supernatant of a suspension, prepared by scraping growth from a plate, which has been allowed to settle for 40 minutes, as opposed to the mechanical dispersal by means of 2 nails and a vortex mixer used in plate method B. In theory, the large clumps of test organisms are removed from the suspension by allowing them time to settle due to their heavier weight, leaving the supernatant relatively free from clumping. Modifications were made to the recommended method by increasing the settlement times thus reducing the clumping even further. Also the final supernatant, i.e. that which was to be used as the test inoculum, was subjected to ultrasonics for 10 minutes in the safety cabinet prior to use. This method proved to be very easy and effective with minimal risks of exposure to the worker.

The broth method D is that currently used in the AOAC use dilution test (Ascenzi *et al.*, 1991). However, the AOAC test uses *Myco. bovis* BCG as the test organism which is a category 2 organism and does not require the use of a safety cabinet. This method proved very difficult to perform safely using *Myco. tuberculosis* within the confines of the class I safety cabinet and would not be recommended in any test using a category 3 organism.

Therefore, of the four methods assessed for preparing the test suspensions the plate method C was chosen as the most suitable and the broth method A was used to prepare a seed pool.

Organic Load

The use of an organic load is important in any disinfectant test, as microorganisms are encountered in body fluids such as blood, urine, saliva, faeces, mucus and sputum. The presence of such organic material may mask or inhibit the activity of the disinfectant in use. The need for testing in the presence of an organic load is not a new concept. Chick & Martin (1908) modified the Rideal-Walker phenol coefficient method by introducing an organic load, as they felt it provided a more realistic test. They originally used 3% dried sterilized human faeces, which was later replaced by serum. There is still no general agreement on the type or the amount of organic load to be used under test conditions. Disinfectants may be applied directly to body fluid spills and in this instance perhaps an organic load of 50% blood may be most realistic. However, many disinfectants are used on pre-cleaned surfaces, e.g. surgical instruments and the use of any organic load in these circumstances is probably unnecessary. Hence disinfectant tests are usually performed under both "clean" and "dirty" conditions.

Earlier studies of mycobactericidal activity favoured sputum as the organic load. Sputum naturally contaminated with *Myco. tuberculosis* and pooled mycobacteria-negative sputum have been used (Best *et al.*, 1990; Sattar. 1995). Although this load simulated actual conditions of use, it was difficult to standardize the challenge and consistency and the material was awkward to use. More recent studies prefer soils with a protein base, which are more consistent in performance and are available commercially. Gelinas & Goulet (1983) assessed the effect of 3 types of organic matter (i.e. dried whole milk powder, dried beef blood

and fish meal) on the activity of 8 disinfectants. They used powders in their tests and found a direct relationship between the capacity of the powder to remain in suspension and its interference level. They also believed that the higher the protein content of the organic matter, the greater the interference with the disinfectants. The most commonly used test soils today include yeast, defibrinated horse serum and bovine serum albumin at various concentrations. In an attempt to determine if (as Gelinas & Goulet believe) the type and amount of organic load used significantly influenced results of disinfectant tests, suspension tests were carried out using 1,000ppm NaDCC, as the effectiveness of this agent is known to be greatly reduced in the presence of even small amounts of organic matter. 10% serum, 1% albumin, 3% yeast and a mixture of 0.5% yeast + 0.5% albumin were assessed using *Myco. tuberculosis* as the test organism. All the soils adversely affected the performance of the chlorine-releasing agent as expected. However, the 1% albumin appeared to be the most stringent, followed by 10% serum. This correlates with the amount of protein present on the soils which follows the order 1% albumin > 10% serum > CEN soil (0.5% yeast and 0.5% albumin) > 3% yeast. This test was then repeated using 2% alkaline glutaraldehyde which produced some unusual results. Under clean conditions, the test appeared to be more stringent than tests carried out in the presence of the soils, with the exception of 3% yeast. From its introduction in 1963 it has been documented that the activity of 2% alkaline glutaraldehyde is unaffected by organic matter. This is an unusual finding as glutaraldehyde is known to interact with proteins. Russell & Hopwood (1976) concluded that interaction with proteins in the outer layers of the cell, is at least partially responsible for its efficacy. It would therefore be expected that glutaraldehyde would interact with any

organic matter of a protein base, leaving a much smaller residual concentration of glutaraldehyde for mycobacterial inhibition or destruction. In this instance, the activity of glutaraldehyde appears to be enhanced not reduced in the presence of the protein organic loads. Cremieux (Prof A., Microbiology and Hygiene, Faculty of Pharmacy, University of Marseilles, France, personal communication, 1996) has also observed an apparent increase in the activity of disinfectants (i.e. glutaraldehyde and formaldehyde) in the presence of proteins. Gorman (Dr. Sean P., School of Pharmacy, Queens University, Belfast. personal communication, 1996) reminds us of the difficulty in explaining these results and suggests that the serum or albumin present as organic matter could adsorb to the bacterial cell wall thus presenting a preferential surface for interaction with the glutaraldehyde. However, additional work is necessary to address this peculiarity.

Based on the results of the tests with 1,000ppm NaDCC 1% albumin has been shown to be by far the most stringent of the organic loads investigated, but is it the most realistic? Albumin at 1% has such a high protein content that it reacted with some disinfectants to form a gelatinous mass thus invalidating the test. Also, albumin is supplied in powder form which must be dissolved and sterilized by filtration. Serum on the other hand is obtained as a frozen sterile solution and in our experience has never interacted with the disinfectant solution to form a gel. Therefore, serum would seem a more appropriate and practical organic test soil and was chosen for use throughout this study. Yeast at 3% was rejected as a potential organic soil following the protein estimation tests which showed it to have an exceptionally low protein content. It was felt that a protein-based soil was

necessary to simulate realistic soiling conditions, e.g. blood, serum etc.

Neutralization and Recovery

Neutralization of the disinfectant residues carried over to the recovery system and the selection of a suitable recovery media which supports the growth of surviving test organisms after exposure to the disinfectant, are essential for an accurate assessment of efficacy. If the disinfectant is not completely neutralized and the recovery medium not effective in supporting the growth of small numbers of surviving test organisms, it may give an exaggerated efficacy result.

Three main methods of neutralization are recognized (Cremieux & Fleurette, 1991). They are dilution to sub-inhibitory levels, washing and filtration, and neutralization by chemical means.

Dilution to sub-inhibitory levels may be sufficient for neutralizing the residual activity of disinfectants with high dilution coefficients (i.e. those which rapidly lose their activity on dilution). However, dilution alone is rarely sufficient and a neutralizing agent may also be necessary. Examples of well recognized neutralizers include sodium thiosulphate, Tween 80, lecithin etc. A specific neutralizer will neutralize a particular disinfectant depending on the active agent, but no single chemical will neutralize all disinfectants. Table 4.1.1 lists appropriate and established neutralizers for all the disinfectants used in this study. Although this is generally an effective method of neutralization it does not lend itself well to standardization and in

some cases the neutralizers may themselves exhibit an inhibitory effect on the growth/recovery of test organisms.

The other method, i.e. washing, implies the physical separation of the test organism and the disinfectant. This is usually achieved by rinsing followed by centrifugation or filtration. Centrifugation can destroy fragile organisms following contact with the disinfectant and filtration is generally preferred (Cremieux & Fleurette, 1991). When using filtration, care must be taken to ensure that there are no disinfectant residues fixed or adsorbed onto to the filter membrane which may subsequently be transferred to the recovery medium and inhibit the growth of survivors. All the methods are well established and documented.

Table 4.1.1: Recognized neutralizers for disinfectants used in this study

DISINFECTANT	NEUTRALIZER	COMMENTS
Chlorine-releasing agents (eg. NaDCC and chlorine dioxide)	Sodium thiosulphate	May be inhibitory or lethal to some bacterial species, i.e. staphylococci
Aldehydes (eg. glutaraldehyde. and succine dialdehyde)	Sodium sulphite Glycine Dilution to sub-inhibitory levels	Inhibitory/lethal Preferred method Not always effective
Alcohol	Dilution to sub-inhibitory levels	Effective No other agent necessary
Peracetic acid & Peroxygen	Catalase and Sodium thiosulphate	Recommended by the manufacturer

In this study, neutralization based on previous work by Best *et al.* (1988), i.e. a combination of dilution and Tween 80, were used to neutralize disinfectant residues. It was hoped this could be used as a universal neutralization system; however, it proved ineffective in neutralizing chlorine dioxide and peracetic acid. It soon became evident during the course of this work that the neutralization system had to be assessed for its efficacy with each new disinfectant. Therefore, a neutralization test should be an integral part of a standard disinfectant test method and although no single neuutralization system can be guaranteed for all disinfectants at least the system chosen has been shown to be effective.

Contact times and temperature

One, 4, 10, 20 and 60 minutes were chosen as the contact or exposure times for disinfectants in all tests. They were selected following a review of various professional societies, i.e. British Thoracic Society (BTS, 1989), the British Society of Gastroenterology (BSG, 1988) and the British Association of Urological Surgeons (BAUS) (Cooke *et al*, 1993). These guidelines were largely produced for the for disinfection of heat sensitive rigid and flexible endoscopes (including bronchoscopes) using 2% glutaraldehyde and alternatives. The BTS recommend 20 minutes for disinfection of bronchoscopes between patients and 60 minutes after a patient with known or suspected pulmonary tuberculosis. The BSG recommend 4 minutes as the shortest connect time between patients and 20 minutes at the end of a list of gastrointestinal endoscopies. Ten minutes is the recommendation of the BAUS although, in the latter two cases, mycobacteria are less significant. The disinfectant manufacturers and

the Medical Devices Agency (MDA, 1996) recommend times between 10 minutes and 1 hour for disinfection. One minute has been chosen as suitable for assessing the efficacy of environmental disinfectants, i.e. those used to wipe surfaces as longer contact times are unlikely. 20°C was used as room temperature in all tests.

Standard Hard Water

Some disinfectants are produced at use dilution, i.e. glutaraldehyde, peracetic acid and others require dilution to bring them to their in-use strength. Tap water is normally used as the diluent for routine application of the disinfectants. It is not, however, recommended as the diluent in testing methods because of the regional variability of its chemical composition. It is also particularly unsuitable for use in this study due to the number of the environmentally associated mycobacteria which may be present in the tap water. There is currently a choice of 2 diluents for use in standardized disinfectant testing ie. sterile distilled water and standard hard water. At the European level, i.e. CEN TC216, there appears to be conflict over the use of standard hard water. In the proposed European bactericidal suspension test, standard hard water is recommended as the diluent, however in the sporicidal suspension test sterile glass distilled water is recommended

Recent publications recommend the use of standard hard water in preference to distilled water, arguing that standard hard water provides a more stringent and realistic test (Sattar *et al.*, 1995). The electrolytes in hard water can adversely effect the activity of disinfectants particularly those that form chelates with metallic ions.

In other cases, the electrolytes can react with the disinfectant to form insoluble salts or an inactive complex. Gardner & Peel (1991) report that the Mg^{++} and Ca^{++} ions in standard hard water have been shown to have no effect on chlorine releasing agents. However, tests carried out in this study indicate that the lower concentration of NaDCC was affected by the presence of hard water. When sterile distilled water was used as the diluent, a >5 $\log_{10}$ reduction was achieved in 1 minute in clean suspension tests but 4 minutes was required to achieve the same result when standard hard water was used. If standard hard water is classed as an "interfering substance" it should not be included in the initial tests used to assess the mycobactericidal activity of new disinfectants. However, it could be included as an additional test when the effect of the presence of other interfering substances e.g. organic load is being determined.

4.2 ACTIVITY OF SELECTED DISINFECTANTS AGAINST MYCOBACTERIA

Disinfectants are used to destroy microorganisms present on environmental surfaces, instruments and the skin (Babb, 1990). They are often the only means of disinfecting heat-sensitive equipment including flexible endoscopes and can be placed into one of three categories, i.e. low, intermediate or high level, depending on their spectrum of activity (Rutala, 1990). Low level disinfectants are expected to be effective against most vegetative bacteria, some viruses and fungi, but cannot be relied upon to kill resistant microorganisms, e.g. *Myco. tuberculosis* or bacterial spores. Intermediate level disinfectants kill *Myco. tuberculosis*, vegetative bacteria, most viruses

and fungi but not necessarily bacterial spores. High level disinfectants destroy all microorganisms, with the exception of high numbers of bacterial spores.

Myco. tuberculosis is considered separately from other bacteria due to its above average resistance to disinfectants and much work has been carried out to assess the tuberculocidal activity of disinfectants. Other mycobacteria have also been studied as possible surrogate organisms to qualify intermediate level disinfection claims. It is important to establish the mycobactericidal activity of disinfectants, to prevent the transmission of *Myco. tuberculosis* and other pathogenic or problematic bacteria on instruments, equipment and environmental surfaces. However, when choosing a disinfectant, in addition to microbicidal efficacy, consideration of other factors, e.g. instrument compatability, corrosiveness, user friendliness and cost is equally important.

Results of mycobactericidal suspension and carrier tests performed in this study, with a few notable exceptions, are in agreement with other published findings.

Aldehydes

2% alkaline glutaraldehyde is currently the recommended agent for disinfection of heat-sensitive endoscopes. There have been extensive investigations of its activity. Many early reports suggested that at 2% and a pH of 7.5-8.5, glutaraldehyde was effective in destroying *Myco. tuberculosis* in <10 minutes. Collins & Montalbine (1976) reported a contact time of 5 minutes at 18^{0}C as being effective against *Myco.*

tuberculosis but subsequent work questioned the accuracy of this result and a time of >30 minutes was suggested as being more realistic. More recent work by Collins (1986) showed that, at 20°C, at least 20 minutes was required for effective destruction of 10^5 *Myco. tuberculosis* when using alkaline glutaraldehyde. This was confirmed by Ascenzi *et al.* (1987) using *Myco. bovis* as the test organism. Results in this study show that in a suspension, in the absence of an organic load, 2% alkaline glutaraldehyde requires 20 minutes at 20°C. However, *Myco. avium-intracellulare* and *Myco. terrae* were found to be more resistant to glutaraldehyde than *Myco. tuberculosis* and a 60 minute contact time was required. The type strains of *Myco. chelonae* and *Myco. fortuitum* were very sensitive to 2% glutaraldehyde and a >5 $\log_{10}$ reduction in both was achieved during 1 minute exposure under both clean and dirty conditions, in suspension and dried onto carriers. The two washer disinfector isolates of *Myco. chelonae* on the other hand were extremely resistant to 2% glutaraldehyde. In clean and dirty suspension and carrier tests only a 1 $\log_{10}$ reduction was achieved in 60 minutes. Van Klingeren & Pullen (1993) were first to recognize problematic glutaraldehyde-resistant *Myco. chelonae* from endoscope washer disinfectors. It was because of the interest raised by their paper that these two washer disinfector strains were included in the study. Tests were carried out to compare some of their phenotypic characteristics with the type strain and the results of these tests are discussed in the next section.

Two unusual results were observed during tests with glutaraldehyde. There was an apparent increase in the efficacy of glutaraldehyde when tested in suspension in the presence of a protein load and also when the inoculum was dried onto a glass carrier prior to testing. The effect

of the protein load was first noticed when tests were carried out to assess the most suitable organic load (see section 4.1). As the study progressed, further suspension tests with *Myco. avium-intracellulare* and *Myco. terrae* confirmed the earlier results with *Myco. tuberculosis* in the presence and absence of 10% serum. In the presence of the serum all three organisms were destroyed (>5 $\log_{10}$ reduction) in 10 minutes as opposed to 20 mins for *Myco. tuberculosis* and 60 minutes for *Myco. avium-intracellulare* and *Myco. terrae* under clean conditions. These results were repeatable and elsewhere investigators in France reported similar findings (Cremieux, personal communication). There was no adequate explanation for these results. It could be that the glutaraldehyde might mask the mycobacteria either inducing cell death or a form of dormancy or stasis as can occur with bacterial spores (Gorman *et al.*, 1983).

In carrier tests *Myco. tuberculosis* was destroyed in 1 minute using 2% glutaraldehyde, under both clean and dirty conditions. Also, a >5 $\log_{10}$ reduction in *Myco. terrae* and *Myco. avium-intracellulare* was observed in 1 minute and 10 minutes respectively under clean conditions. However, in these carrier tests, the rate of kill decreased in the presence of an organic load, but still remained more rapid than in a clean suspension test. *Myco. avium-intracellulare* required 20 minutes and *Myco. terrae* 4 minutes to achieve a >5 $\log_{10}$ reduction.

Glutaraldehyde is a fixative and, as such, it might be expected to be less effective against organisms dried onto surfaces. However, contrary to this belief, it has been reported to be highly effective in stripping biofilms and destroying the microorganisms within it (Eager *et al.*, 1986; Russell, 1994). Biofilms present an even greater challenge to

disinfectants than the carrier tests described in this study. In a biofilm, microoganisms are not only dried onto the surface, but are attached to the surface and actively growing. Microorganisms in a biofilm are known to have a higher resistance to disinfectants than planktonic organisms (Schülze-Röbbecke & Fischer, 1989). Eager *et al.* (1986) reported that glutaraldehyde can penetrate a biofilm and exert an inhibitory effect on microorganisms. Glutaraldehyde also appears to increase the speed of the natural detachment of microorganisms from the biofilm. This supports the results obtained in carrier tests.

In comparison with many other disinfectants tested in this study, 2% glutaraldehyde is much slower in achieving its mycobactericidal effect although it can obtain a >5 $\log_{10}$ reduction in a realistic contact time. However, its spectrum of activity combined with its equipment compatibility means it remains the agent of choice.

Gigasept, a succinedialdehyde and formaldehyde mixture at 10% was less effective than glutaraldehyde. *Myco. chelonae* NCTC 946 proved most sensitive but even this organism required a10 minute contact time to achieve a >5 $\log_{10}$ reduction in clean and dirty suspension tests and 20 minutes in clean and dirty carrier tests. As expected, the glutaraldehyde-resistant strains of *Myco. chelonae*, Harefield and Epping, were similarly resistant to Gigasept and required 20 minutes and 60 minutes respectively in suspension tests and 60 and >60 minutes in carrier tests. *Myco. avium-intracellulare* and *Myco. terrae* were again found to be more resistant than *Myco. tuberculosis*, and *Myco. fortuitum* slightly more sensitive in suspension tests. In carrier tests, *Myco. terrae*, *Myco. fortuitum* and *Myco. tuberculosis* all required 60 minutes under clean and dirty conditions and only a 2.12 - 2.52

$\log_{10}$ reduction in *Myco. avium-intracellulare* was achieved in 60 minutes. Gigasept at 10% is clearly inferior as a mycobactericidal agent than 2% glutaraldehyde.

Chlorine releasing agents

Early reports presented chlorine as a poor mycobactericidal agent (Crowshaw, 1971). However, more recent studies have shown that they are in fact rapidly mycobactericidal (Dychdala, 1991). Two different agents were tested in this study; sodium dichloro-isocyanurate (NaDCC) and chlorine dioxide. NaDCC is available in powder or tablet form and is very stable until dissolved. It is also inexpensive, rapidly effective and slightly less damaging than sodium hypochlorite (bleach) which was formerly used. However, like all other chlorine releasing agents, the use of NaDCC in hospitals is usually limited to environmental surfaces eg laboratory benches and spillages, due to its corrosiveness. They are also adversely affected by organic matter (but less so than the hypochlorites) (Coates, 1988) and once a solution has been prepared is relatively unstable and should be used within 24 hours. Sodium dichloro-isocyanurate at 10,000ppm av Cl was rapidly active against all mycobacteria tested in this study in suspension and carrier tests in the presence and absence of serum, with the one exception ie. *Myco. avium-intracellulare.*

The lower concentration of 1,000 ppm av Cl, usually used on pre-cleaned surfaces, was rapidly effective against the type strain of *Myco. chelonae* in clean and dirty suspension and carrier tests, achieving a >5 $\log_{10}$ reduction in 1 minute. The much increased glutaraldehyde resistance in the washer disinfector isolates of *Myco. chelonae* was

accompanied by only a slight increase in resistance to chlorine. *Myco. avium-intracellulare* was more resistant to chlorine than *Myco. terrae* which in turn was slightly more resistant than *Myco. tuberculosis*. With NaDCC the carrier tests proved more stringent than the suspension tests. Results appeared very good in clean suspension tests but the addition of an organic load and the drying of the organisms onto glass surfaces led to a decrease in activity. *Myco. tuberculosis* was effectively destroyed in 1 and 4 minutes in clean and dirty suspension tests respectively and 10 and 20 minutes in clean and dirty carrier tests respectively. A 60 minute contact time was required to achieve a >5 $\log_{10}$ reduction in *Myco. avium-intracellulare* in suspension tests and clean carrier tests. The carrier tests with *Myco. avium-intracellulare* in the presence of 10% serum presented too difficult a challenge for 1,000ppm av Cl NaDCC in 60 minutes. *Myco. terrae* required 4 and 10 minutes in clean and dirty suspension tests and 20 and 60 minutes in clean and dirty carrier tests respectively. Hardie (1986) recommended that NaDCC could be considered as a glutaraldehyde alternative where protection from corrosion and repeated use were not required.

Chlorine dioxide in the form of "Tristel" (1,100 ppm av Cl) is a new addition to the UK disinfectant market. It is being sold primarily as an instrument disinfectant but is also used for environmental surfaces. Its microbicidal activity is recognized but there are still concerns regarding its corrosiveness and user safety. It was almost as effective as 10,000 ppm av Cl NaDCC against the mycobacteria tested. In suspension, under clean and dirty conditions, chlorine dioxide was effective in achieving a >5 $\log_{10}$ reduction in all test mycobacteria in 1 minute, including *Myco. tuberculosis* and *Myco. avium-intracellulare*. In

carrier tests, similar results were achieved in under 5 minutes for all test organisms except *Myco. terrae* under dirty conditions which required 10 minutes.

Chlorine has long been the preferred disinfectant for water treatment. As a result of our studies, 10,000ppm av Cl in the form of NaDCC is recommended to "purge" automated endoscope washer disinfectors which have become contaminated particularly with biofilm, *Myco. chelonae* and other environmental mycobacteria. Routine sessional disinfection with 1,000ppm av Cl has also been recommended but in both instances it is necessary to assess compatability with processor components.

Virkon

Virkon was ineffective as a mycobactericidal agent. In suspension tests 1% Virkon achieved a >5 $\log_{10}$ reduction of the type strain of *Myco. chelonae* NCTC 946 in 20 minutes in clean conditions and 60 minutes in dirty conditions. However, in all other tests 1% and 3% Virkon achieved, at best, only a $\log_{10}$ 3.41 reduction in 60 minutes. Virkon, could not therefore be recommended as a tuberculocidal or mycobactericidal agent. This is in agreement with earlier work published by Holton *et al.* (1994) and Broadley *et al.* (1993).

Peracetic acid

The microbicidal activity of peracetic acid has long been recognized. However, as with other strong oxidizing agents, it could not be recommended for use as an instrument disinfectant due to its

corrosiveness and instability. A new product, NuCidex, is now available in the UK which claims to have suitably overcome these problems and is being marketed as a glutaraldehyde alternative. This product has been shown to corrode brass and copper components but damage to other metals is relatively superficial (Johnson & Johnson Medical Ltd., Coronation Road, Ascot, Berks., 1995). Endoscopes themselves do not generally contain accessible copper or brass components, however most automated endoscope washer disinfectors do have brass and copper pipe fittings. These components are affected by most non-aldehyde disinfectants and are therefore being replaced in the newer endoscope washer disinfectors with stainless steel or plastic. Preliminary endoscope compatability test have been successful and any damage has been described as superficial or cosmetic not functional. In all tests ie. suspension and carrier tests, with/without 10% serum a >5 $\log_{10}$ reduction was achieved in 5 minutes or less.

Alcohol

Alcohol, in the form of industrial methylated spirits (IMS) at 70%, proved to be a highly effective mycobactericidal agent. Larson & Morton (1991) advise against the use of alcohol for an instrument disinfectant in the US as ethylene oxide and glutaraldehyde are readily available and more suitable. The major disadvantages with alcohol are its flammability and its weakening effect on the optic cements used in endoscope manufacture. Also, alcohol is a fixative and has been shown in studies with viruses to be unreliable in the presence of organic soils (Tyler & Ayliffe, 1987). In this study, it has been shown to be effective in 1 minute against all the test mycobacteria in suspension tests with/without 10% serum with one exception, i.e.

Myco. avium-intracellulare. The test organisms required 4 minutes exposure to 70% alcohol under both clean and dirty conditions in suspension to achieve a >5 $\log_{10}$ reduction. In the carrier tests, only *Myco. fortuitum* was effectively destroyed in 1 minute in clean and dirty conditions, all others required 4 minutes. *Myco. avium-intracellulare,* although destroyed in 4 minutes in clean conditions required 10 minutes in the presence of 10% serum.

4.3 GLUTARALDEHYDE-RESISTANT *MYCOBACTERIUM CHELONAE*

The presence of *Myco. chelonae* in both natural and treated waters, including tap water, is well recognized. Nye *et al.* (1990) were the first to indicate that tap water was the initial source of contamination of bronchoalveolar lavage specimens, although it had been the suspected source in a number of earlier incidences (Pappas *et al.*, 1983). Due to the concern of contamination of endoscopes and endoscope washer disinfectors from tap water it is currently recommended that sterile or bacteria-free water be used to rinse bronchoscopes and all other invasive surgical instruments, whether they are processed automatically or manually (Ayliffe *et al.*, 1993; Cooke *et al.*, 1993; MDA, 1996). To further minimize the risk of instrument contamination during rinsing, current UK guidelines advise that endoscope washer disinfectors be disinfected preferably during each cycle and at least at the start of each endoscopy session (MDA,1996). As 2% activated alkaline glutaraldehyde is currently used in the UK and elsewhere to disinfect both washer disinfectors and endoscopes, it is believed that continual exposure to this agent may have selected strains and thereby encouraged the proliferation of *Myco. chelonae* with

decreasing susceptibility to 2% glutaraldehyde (Van Klingeren & Pullen, 1993; Griffiths *et al.*, 1997)

Carson *et al.* (1978) assessed several strains of environmental mycobacteria including *Myco. chelonae* for growth in commercially distilled water and their resistance to disinfectants, including glutaraldehyde, formaldehyde and a chlorine-releasing agent. They found that 2% glutaraldehyde effectively destroyed the reference strain of *Myco. chelonae* (ATCC 14472) in 2 minutes but 12 clinical isolates were more resistant with some surviving for over 60 minutes. These clinical strains had not been subjected to the selective pressure of the disinfectant. Carson and colleagues were unable to conclude whether the greater resistance of the clinical strains to aldehyde disinfectants reflected their cultural history or whether they were variants. It could have been that they were resistant or that the reference strain was very sensitive due to its maintenance and subculture for prolonged periods. In this study the results were similar to those obtained by Carson *et al.* (1978). The type strain of *Myco. chelonae*, i.e. NCTC 946 was sensitive to all disinfectants tested and was destroyed by a 1 minute exposure to 2% alkaline glutaraldehyde. By contrast, the two endoscope washer disinfector isolates were unaffected by exposure to 2% glutaraldehyde for 60 minutes and also exhibited a slight increase in resistance to the chorine releasing agent at 1,000ppm av. Cl (NaDCC), peracetic acid and Virkon. The washer disinfector isolates also presented higher minimum inhibitory concentrations and minimum mycobactericidal concentrations to glutaraldehyde. These resistance patterns and MICs of the machine isolates remained unchanged even after repeated subculture. This suggests they are stable mutants/variants. With this in mind, tests were carried out to compare some of the phenotypic

characteristics of the three strains to assess if there were any apparent differences between the type strain and the two washer disinfector isolates.

Using the BATH method of Rosenberg *et al.* (1980), the Harefield and Epping washer disinfector strains of *Myco. chelonae* appeared more hydrophobic than the NCTC strain and also exhibited an increased resistance to heat and clear soluble phenolic ie. 1-2% Stericol. The observed increased hydrophobicity suggested a change in the cell wall leading to reduced affinity to glutaraldehyde, phenolics, chlorine and heat. Was it due simply to a reduction in permeability?

Glutaraldehyde uptake studies showed that both washer disinfector isolates took up increasing amounts of glutaraldehyde over a 60 minute period which indicated that there may be a quite specific change in the cell wall architecture, diverting the glutaraldehyde and preventing it reaching its target and exhibiting its effect. As the cell wall lipids of mycobacteria play a major role in their intrinsic resistance to antibiotics and chemical agents, both the extractable short chain fatty acids and the larger mycolic acids were analyzed using gas liquid chromatography and high performance liquid chromatography respectively. There was no apparent difference in the profiles which indicates there is no difference in the amount of mycolic acids or short chain fatty acids present in the three test strains. This would suggest that the mycolic acids and short chain fatty acids are not involved in the mechanism of increased resistance to glutaraldehyde unless their conformations have been significantly altered.

No further tests were carried out and additional work is necessary to find out what changes occur which lead to this reduced affinity, particularly for glutaraldehyde. One possibility is the cell wall proteins and peptidoglycan which are known to interact with glutaraldehyde (Russell & Hopwood, 1976; Scott & Gorman, 1991; Russell, 1994). Also, it may be beneficial to analyze the arabinogalactan as the washer disinfector isolates exhibited higher MICs to ethambutol than the NCTC strain and arabinogalactan has been identified as the target for this antibiotic (Takayama & Kilburn, 1989). No differences in the MICs of isoniazid were observed supporting the view that altered mycolic acids are not responsible for the resistance. However, a more sensitive method may show small differences.

4.4 SUMMARY

It is important to establish the mycobactericidal activity of disinfectants from 2 standpoints: 1) to prevent the transmission of *Myco. tuberculosis* and other pathogenic or problematic bacteria on instruments, equipment and contaminated surfaces; and 2) to establish which disinfectants are acceptable for intermediate level disinfection if this becomes part of international acceptance criteria. The work presented in this thesis has established a basis for evaluating the performance of disinfectants against mycobacteria. Carrier tests provided more stringent conditions for all disinfectants tested with the exception of glutaraldehyde. Suspension tests in the absence of any interfering substances, should be used only for initial screening of new formulations to determine their potential as mycobactericidal agents. If successful, suspension and carrier tests in the presence and absence of interfering substances, e.g. organic soil,

standard hard water etc. should then be performed to further confirm disinfectant efficacy and suitability for surface disinfection.

Myco. terrae is similar in its susceptibility to disinfectants to *Myco. tuberculosis* and could therefore be recommended as a surrogate for tuberculocidal tests. It is also a more rapid grower and is classified as a category 1 pathogen (ACDP, 1995). It is therefore much safer and easier to work with. However, a surrogate should be used only in screening or initial tests of a new product. It is preferred that confirmation of mycobactericidal activity is determined using clinically relevant strains, i.e. *Myco. tuberculosis* and perhaps *Myco. avium-intracellulare*. *Myco. avium-intracellulare* is the most resistant of the test organisms included in this study.

There appears to be no single neutralization system which could be used for all disinfectants. It is therefore necessary to assess the suitability of neutralization systems before commencing disinfectant tests.

Chlorine dioxide (1,100ppm av. ClO_2), peracetic acid (3,500ppm), alcohol at 70% and higher concentrations of NaDCC (10,000ppm av. Cl) are rapidly mycobactericidal. However, glutaraldehyde remains the disinfectant of choice for endoscopes. Instrument compatability, user friendliness and cost all have to be taken into consideration when choosing a disinfectant. The chlorine-releasing agents, although suitable as environmental disinfectants are too corrosive for instruments. Alcohol, is sometimes used to rinse decontaminated endoscopes if the quality of the rinse water cannot be assured. It is also used as a disinfectant for clean surfaces (e.g. table tops, electrical

equipment, patient thermometers etc.) and is included in many hand rubs. However it cannot be used in large quantities for disinfection due to its flammability.

Favourable results of tests with glutaraldehyde in the presence of protein as an organic load should be interpreted with caution. They are not a recommendation to use glutaraldehyde on endoscopes and other equipment without prior cleaning. They should be used only to initiate further studies.

The two washer disinfector isolates of *Myco. chelonae* constantly subjected to glutaraldehyde, were more resistant to 2% alkaline glutaraldehyde, 1,000ppm NaDCC, clear soluble phenolics, and heat, than the unexposed type strain NCTC 946. No difference in the short chain fatty acids and mycolic acids of the three strains of *Myco. chelonae* were observed, which suggests that the cell wall lipids are not involved in the mechanism of increased resistance to glutaraldehyde. The washer disinfector isolates of *Myco. chelonae* appeared to have higher MICs to ethambutol than the type strain but MICs to isoniazid were identical. The increased resistance to ethambutol may point to involvement of arabinogalactan in the mechanism of resistance to glutaraldehyde. Cell wall proteins are also worth consideration in any further studies.

It is hoped that the results and conclusions of this study may be presented as a useful contribution to the CEN TC216 committee for the standardization of disinfectant testing in Europe.

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APPENDIX B: Standard test method

Preparation of test suspensions

One colony of each test organism was taken from 7H11 plates and inoculated into 100ml Middlebrook 7H9 broth. The broths were incubated at 30°C for 14-15 days (*Myco. chelonae*) or 37°C for 14-15 days (*Myco. fortuitum*), 17 days (*Myco. terrae*) or 21 days (*Myco. tuberculosis* and *MAI*). The suspensions were sonicated in an ultrasonics waterbath at 50-60Hz for 10 minutes every second day and inverted several times to minimize clumping. On completion of the incubation period, the suspensions were mixed with 10% glycerol as a preservative and 1ml aliquots were decanted into 1.5ml microcentrifuge tubes. These were then frozen at -70°C until required. Immediately prior to testing, one of the suspensions stored at -70°C was removed from the freezer, thawed at room temperature, centrifuged, washed twice in sterile distilled water and a loopful spread onto a Middlebrook 7H11 agar plate. After the appropriate incubation, 7-10 loopfuls of growth were harvested, added to glass beads, moistened with sterile water and shaken for five minutes. Ten ml of sterile distilled water was added, agitated and the suspension left to settle for 30 minutes. The supernatant was removed to a second sterile bottle and left to settle for a further 2 hours. The supernatant from this suspension was sonicated for 10 minutes to disperse the organisms and this was used as the challenge in disinfectant tests. Films were prepared of these challenge test suspensions and stained by Ziehl Neelsen (ZN) to check for homogeneity.

Organic Material

For tests under dirty conditions, defibrinated horse serum (Tissue Culture Services Ltd) was added to the initial test suspension to give a final concentration of 10% to simulate an organic load.

Neutralization/Recovery

Prior to testing each disinfectant, neutralization tests were carried out to determine the most suitable recovery system. To mimic test conditions 100µl of sterile distilled water was added to 900µl of the disinfectant at the highest concentration, mixed and left for 1 minute. Ten µl of the mixture was then added to 990µl of Ringers solution containing 0.5% Tween 80. Tenµl of an undiluted test suspension of *Myco. tuberculosis* was added to this mixture (Neat) and serially diluted to 10^{-5} in Ringers solution only. One hundred µl of the neat and subsequent dilutions were spread onto 7H11 agar in duplicate, using sterile spreaders. The plates were incubated at 37^{0}C for up to 28 days. (in plastic bags to prevent drying out due to prolonged incubation) and colony forming units enumerated. The undiluted test suspension was used as the initial count. The test was repeated using water instead of the disinfectant as the control.

If the system failed to neutralize the disinfectant, another neutralizer in combination with dilution was tested, until an effective system was found e.g. a combination of dilution in Ringers and 0.5% sodium thiosulphate

A combination of dilution and 0.5% Tween 80 was used to neutralize all disinfectants with the exception of chlorine dioxide and peracetic acid. A combination of dilution, 0.025% catalase and 5% sodium thiosulphate was used for the peracetic acid compound and a combination of dilution, 0.5% Tween 80 and 0.5% sodium thiosulphate for chlorine dioxide.

Suspension Test

One hundred µl of the test suspension was added to 900µl of freshly prepared disinfectant in microcentrifuge tubes and vortex mixed for 20 seconds. The disinfectant/test suspension mixture was held at room temperature (20°C) and sampled at 1, 4, 10, 20 and 60 minute intervals. After the required contact time, 10µl were removed and added to 990µl of Ringers/Tween 80 or other suitable neutralization/ recovery system. This was then serially diluted to 10^{-3}. One hundred µl of the neat and subsequent dilutions were spread onto Middlebrook 7H11 agar, in duplicate, using sterile hockey stick spreaders. Plates were incubated at 30°C for up to 1 week (*Myco. chelonae*) or at 37°C for 1 week (*Myco. fortuitum*) 2 weeks (*Myco terrae*) or up to 6 weeks (*Myco. tuberculosis, MAI*) and colony forming units enumerated.

Carrier Test

Ten µl of the test suspension was placed on the base of a sterile glass cup (capacity 600µl), supported in a 24 well cell culture plate, and left to dry at 25°C for 90 minutes. Care was taken to ensure the culture

was only placed on the base of each cup. This inoculum was overlaid with 60µl of freshly prepared disinfectant and left at room temperature for contact times of 1, 4, 10, 20 and 60 minutes. After the required contact time the glass cup was removed, using sterile forceps, and placed aseptically into 2940µl of neutralization/recovery medium, in a sterile universal. Using a sterile loop the base of the cup was gently scraped to loosen any of the organism which may have been fixed by the disinfectant. The universal was then vortexed for 20 seconds and serially diluted to 10^{-3}. One hundred µl of the neat and subsequent dilutions were spread onto Middlebrook 7H11 agar, in duplicate, and incubated as previously described.

Controls

Controls were carried out in duplicate at 1 and 60 minute intervals using 900µl (suspension test) and 60µl (carrier test) of sterile distilled water instead of the disinfectant. The mean of the two counts obtained in the controls was used as the initial challenge.