Evaluation of Alternative Methods for Antimicrobial Susceptibility Testing of Fastidious Organisms

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____**Signed**

PONG WAI LEUNG ROCKY

Table of Contents

List of Abbreviations

AD	Agar Depth
AST	Antimicrobial Susceptibility Test
$BaCl_2$	Barium Chloride
BSAC	British Society of Antimicrobial Chemotherapy
BLNAR	Beta-lactamase Negative Ampicillin Resistant
BLPACR	β-Lactamase Positive Ampicillin Resistant
CAT	Chloramphenicol Acetyltransferase
CDC	Centre for Disease Control
CFU	Colony Forming Unit
CMRNG	Chromosome Mediated Resistant *Neisseria gonorrhoeae*
Conc.	Concentration
CSF	Cerebral Spinal Fluid
DF	Deposition Factor
$DiBAC_4(3)$	Bis-(1, 3-dibutylbarbitaric acid) Trimethine Oxonol
$DiOC_5(3)$	3, 3' – Dipentylozocarbocyanine Iodide
DNA	Deoxyribose Nucleic Acid
DST	Diagnostic Sensitivity Agar
EB	Ethidium Bromide
EDTA	Ethyl-Diamine-Tetra-acetic Acid

EMA	Ethidium Monoazide
FCM	Flow Cytometry
FDA	Food and Drug Administration
FS	Forward Scatter
GISP	Gonococcal Isolate Surveillance Project
HPCV	Half Peak CV
HTM	Haemophilus Test Medium
ICS	International Collaborative Study
ID	Inoculum Density
IEP	Immunoelectrophoresis
IN	Incubation Time
IST	Isosensitest agar
LHB	Lysed Horse Blood
H_2SO_4	Sulphuric Acid
MBC	Minimium Bactericidal Concentration
MDR	Multidrug Resistant Strain
MH	Mueller Hinton
MIC	Minimal Inhibitory Concentration
mM	milli Molarity
NAD	Nicotinamide Adenine Dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical Density
PBP	Penicillin Binding Protein

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PMT1	Photomultiplier 1
PMT2	Photomultiplier 2
PMT3	Photomultiplier 3
PPNG	Penicillinase-producing *N. gonorrhoeae*
RA	Radial Advance
Rh123	Rhodamine 123
RNA	Ribose Nucleic Acid
SAD	Standard Agar Dilution
SGE	Spiral Gradient Endpoint Method
SS	Side Scatter
ST	Standing Time
TMP-SMX	Trimethoprim-Sulfamethoxazole
TRNG	Tetracycline-Resistant *N. gonorrhoeae*
WHO	World Health Organization

List of Tables

List of Tables

List of Tables

List of Tables

List of Tables

List of Figures

List of Figures

List of Figures

List of Figures

List of Figures

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Abstract

Fastidious organisms such as *Haemophilus influenzae* and *Streptococcus pneumoniae* continue to cause considerable morbidity and mortality. In the past antimicrobial susceptibility testing was not needed as susceptibility patterns were predictable. However, considerable resistance has now emerged, and susceptibility testing is required to ensure successful therapy.

MICs of fastidious organisms are sometimes needed, when there is no standard diffusion method, or the organism has an MIC close to the susceptibility category breakpoint, or when there is unsuccessful therapy. Conventional dilution methods are intensive, time-consuming, and technically demanding. In addition, they lack precision, especially at high MIC values, since two-fold dilutions of antimicrobial concentrations are performed. Few automated systems for MIC determination of fastidious organisms are available with the exception of the Vitek 2, which only provides a panel for susceptibility testing of *S. pneumoniae*, after incubation for approximately 8.5 hours. The E test is a robust method and is simple to perform. However, it is quite expensive, especially if several drugs need to be tested, and its use can be effected by storage. There is still no economical and rapid method for MIC determination of fastidious organisms.

Spiral Gradient Endpoint test (SGE) utilizes a spiral plater to deposit antibiotic on the agar surface in a decreasing concentration gradient, providing an antimicrobial concentration gradient on the agar for determination of MIC. It is especially suitable for fastidious organisms since agar with different supplements can be used, and it can be

incubated in different atmospheres. Some preliminary studies on the use of SGE for susceptibility testing have been performed and the results were good. However, these studies did not use the SGE method for fastidious organisms.

In this study, SGE was evaluated for its reproducibility, and the effects of varying the SGE test parameters were determined. A comparison study of the SGE test with the conventional dilution method for susceptibility testing of 4 species of fastidious organisms was conducted. A cost analysis was also performed as shown in Appendix ix.

It was found that both the intrabatch and interbatch reproducibility of the SGE method were good, with a range of coefficients of variation (CV) of 6.22 to 14.56% for intrabatch and 11.06 to 18.56% for interbatch reproducibility, which were much lower than those of the standard MIC methods. It was also found that the parameters that would affect the SGE MIC were: incubation time, inoculum density, and antibiotic concentration. Using the optimal conditions obtained, SGE tests were performed on four fastidious organisms, and results were compared with standard methods. There was a high percentage of agreement between the two methods for all organisms. Cost beneficial analysis was also in favour of SGE test, which was found to be cheaper amongst E test and Vitek 2.

Early reporting of susceptibility test results can lead to reductions in mortality, morbidity, and shortening of hospital stay. However, all routinely used methods require overnight testing before results can be obtained. The flow cytometer is able to detect viable and non-viable bacterial cells after staining with fluorescent dyes. Its use for AST has been suggested but methods, especially for fastidious organisms have not been developed or

evaluated. This study has developed an accurate and precise method for AST of two fastidious organisms, *H. influenzae* and S. *pneumoniae*. This FCM-AST was rapid and could provide results within 6 hours. It also had the advantage of being able to analyse non-synchronous heterogeneic cultures, although it was found to be more expensive than more conventional AST methods (Appendix ix).

The kinetics of ampicillin and tetracycline were studied and a FCM-AST was evaluated for testing of *H. influenzae* isolates. Penicillin and erythromycin kinetics were investigated for *S. pneumoniae*, and FCM-AST was performed on the clinical isolates of the organisms against these antibiotics. The organisms responded well to the 1x MIC of antibiotics. Breakpoint antibiotic concentrations were then used for FCM-AST. The results of the AST were found to agree well with those of standard methods.

In an era of increasing antibiotic resistance, rapid, accurate, and economical methods for AST are essential. This study has successfully evaluated a simple, economic method, the SGE, and a rapid, though more expensive, method FCM-AST, for the accurate susceptibility testing of fastidious organisms.

Conference Presentations and Publications

Pong WLR, O' Donoghue MM, Boost MV (2000) Evaluation of the spiral gradient endpoint method for the determination of MIC of antimicrobial agents for fastidious organisms, 7th Western Pacific Congress of Chemotheraphy and Infectious Diseases. 11-14th Dec. 2000. Hong Kong.

PongWLR and O'Donoghue (2003) Optimisation of flow cytometry method for the antimicrobial susceptibility testing of *Haemophilus influenzae*. The Sixth Chinese Laboratory Medicine Conference 14-17th Nov. Hong Kong

Pong WLR, O' Donoghue, MM. (2005) Optimisation of the flow cytometer for use in antimicrobial susceptibility testing of *H. influenzae*. 10th Annual General Meeting and scientific meeting of HK Society of Flow Cytometry. 26th Feb. 2005. Hong Kong.

Pong WLR, M O'Donoghue, M Boost (2005) Susceptibility testing of *Haemophilus influenzae* using flow cytometry. 5th International Symposium on Antimicrobial Agents and Resistance. 27-29th April, 2005. Soeul, Korea.

Pong WLR, Boost MV and O' Donoghue MM, (1998). Comparision of the Spiral gradient endpoint technique with the E-test and standard agar dilution method for the susceptibility testing of *Streptococcus pneumoniae*. British Journal of Biomedical Science. 55:21.

Chapter 1

Antimicrobial susceptibility test – an overview

1. Introduction

Antimicrobial susceptibility tests (AST) to evaluate the effects of antimicrobial agents on microorganisms were first developed in the 1920s (Poupard *et al*, 1994) although knowledge of antibiotics had been observed as far back as the nineteenth century (Roberts, 1874). The need for AST became evident soon after antibiotics were made commercially available and resistant bacterial strains emerged during World War II (Poupard *et al*, 1994). Two methods, the quantitative dilution and the qualitative diffusion methods, were developed. Modifications to antimicrobial susceptibility testing methods are due to improvements in technology and represent responses to user's requirements and published research.

Antimicrobial susceptibility tests are performed on clinical isolates of specimens of bacteria determined as the probable cause of the patient's infection, both to determine suitable drugs for therapy and to monitor trends in resistance patterns. Some organisms, such as *Streptococcus pneumoniae,* which were previously susceptible to antibiotics are now often resistant (Appelbaum, 1992; Baquero, 1995; Karlowsky *et al*, 2003; Canton *et al*, 2003; Felmingham, 2004). Other important factors must be considered when determining whether AST is warranted. Usually, susceptibility tests are not performed on commensals that are normal inhabitants of non-sterile sites. However, species usually regarded as normal flora may be responsible for an infection in an immunocompromised patient and in this situation may require susceptibility testing.

The mechanisms of bacterial antimicrobial resistance are complex and, though some are intrinsic, many are the result of a variety of adaptations by the organism in response to the presence of drug. Some resistance mechanisms are a result of genetic mutation or are encoded by a chromosomal DNA, and can be transferred to other bacteria by transformation or transduction. Resistance genes may also be carried on plasmids that can pass from one organism to another by conjugation. Some resistant genes are carried on transposons that can move between chromosomes or between chromosomes and plasmids (Neu, 1992). Presence of a gene for resistance does not always render an organism resistant as expression of the gene is required for resistance to occur. Expresson may be affected by both intrinsic factors such as presence of other genes and relative fitness of strains expressing resistance, and extrinsic factors including the presence of the relavent antibiotic.

As most treatment for bacterial infection commences before the results of susceptibility tests are available, the need for antimicrobial susceptibility testing to be performed on all isolates has been challenged. However, susceptibility tests can provide information on suitable alternative drugs if the organism is resistant or if the patient is intolerant to a particular agent. This can reduce the risk of treatment failure due to use of an inappropriate antibiotic, and allows change from a broad spectrum to a narrow spectrum antibiotic, which can reduce the risk of inducing resistance in commensal organisms. AST results may also enable the use

of a cheaper antibiotic, reducing costs to the health care system. Routine testing can also generate information on local resistance rates and patterns, and therefore guide empirical therapy. Surveillance of antibiotic-resistant organisms in different parts of the hospital is an important component of efforts to prevent and control the spread of resistant strains.

2. Antimicrobial Susceptibility Tests

A) Selecting antimicrobial agents for testing and reporting

There are more than 20 classes, of antimicrobial agents some with many representatives, commercially available for the treatment of bacterial infection, consequently the laboratory cannot carry out tests on all drugs. For some agents a routine laboratory testing method is not available. If a commercial susceptibility test panel is used, there may be reduced flexibility in matching test batteries to the drugs of interest. Moreover, there is also a limit on the maximum number of drugs that can be economically tested by any susceptibility method. The laboratory has to select and report on the most appropriate antimicrobial agents for the bacterial species isolated, based on the site of infection, and the clinical condition of the patient.

For some antibiotic groups, it is sufficient to test one representative of the whole family, since they exhibit similar if not identical activities *in vitro*. In other groups, where differences of activities and spectrum exist, the laboratory has to

compromise by testing agents that can represent a subclass of the group. The choice of antimicrobial agents to test and report are affected by several factors. First, the antibiotics tested will depend on the characteristics of the patients served by the hospital. Second, the agents tested should closely reflect the antibiotic policy of the hospital. It is important that the antimicrobial agents are included in the institution's formulary. Finally, the species of bacteria to be tested and the possibility of encountering organisms resistant to the first choice agent, also strongly influence the choice of agents for testing and reporting (Turnidge and Jorgensen, 1999).

Guidelines that list the antimicrobial agents appropriate for testing various groups of aerobic, anaerobic and fastidious organisms have been published by the Clinical Laboratory Standard Institute, formerly National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2000, 2003a, 2003b, 2004a, 2004b; CLSI, 2005 a,b,c,d). These indicate the most appropriate drugs for each organism group and for infections at particular anatomical sites, and designate agents that are suitable representatives of antibiotic groups. Table 1 shows the agents suggested as most appropriate for testing against fastidious organisms (NCCLS, 2004). The list is a guideline only, and other variables may need to be considered in determining the agents to be tested in the laboratory. Other European organizations including the BSAC, SFM and DIN, also produce similar guidelines which can vary from those of CLSI (Doern, 1995; Andrews, 2001). The final decision of agents to test and report should be made after discussion between the

infectious disease physicians, pharmacists, the institutional formulary the microbiologist and infection control committee.

The identity of an isolate is often not known at the time the susceptibility test is performed, therefore some drugs may be inappropriate for reporting. The final reporting decision should be made once the isolates identity is known. Several strategies are used for reporting of susceptibility test results depending on local policy, needs, and resources. The primary objective is to use the most efficacious, least toxic, most cost-effective and most clinically appropriate agents, and refrain from broad spectrum agents that are unnecessary. CLSI provides guidance for developing a cascade reporting protocol (NCCLS,2003a,b;2004a,b; CLSI, 2005b), which tends to report narrower spectrum and less potent drugs. Selective reporting should help improve the clinical relevance of test reports, and help minimize the selection of multi-resistant nosocomial strains by overuse of broad spectrum agents (CLSI 2005b).

B) Conventional susceptibility test methods

The antimicrobial susceptibility of bacteria can be assessed in a variety of ways and factors considered when choosing an AST method are: a) ease of performance of test; b) cost; c) the nature of bacterial species under examination, and d) the degree of precision required. There are two major approaches to antimicrobial susceptibility testing: the diffusion method and the dilution method (CLSI,2005b; NCCLS, 2003; Andrews, 2004).

i) Disk diffusion test

In this method a 'dense but not confluent' suspension of the tested organism is inoculated onto the surface of an agar plate, and paper discs which act as the reservoir of each antibiotic to be test is applied. After incubation, there is a zone of inhibition of growth and the diameter of the zone is measured, and compared to published data to determine whether the strain is susceptible or resistant to the drug in question. This is a qualitative method and remains the most widely used routine method in clinical laboratories. The interpretative criteria of this method is based on regression lines plotted by comparing the log of inhibition zone diameter of at least 300 organisms of different sensitivity against the minimal inhibitory concentrations (MICs) of the organisms to the antimicrobial agent. Developments to this method have concentrated on the standardization of variations of test parameters and the criteria for interpretation of zone diameter into categories of susceptibility (Bauer *et al*, 1966). There are two versions of the disk diffusion test: the comparative method and the standardized method.

The most widely used standardized method is the CLSI method (NCCLS, 2003a which is a development of the Kirby-Bauer approach (Bauer *et al*, 1966). Other standard methods were developed as results of the International collaborative study (ICS) sponsored by the WHO (Ericsson *et al.*, 1971; WHO Expert Committee for Biological Standardization, 1977) and by the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2004). The approach is to

control variation by standardizing all details of the tests, with the aim to achieving reproducibility between different clinical laboratories. The method however, suffers from the inherent weakness that batches of agar medium may differ considerably in their performance (Amsterdam, 1996). Addition of cation supplements helps to reduce effects due to different levels of Ca^{2+} and Mg^{2+} between batches by providing excess of these ions (Kenny *et al*, 1980).

Comparative methods, such as the Stokes's method, were developed in the United Kingdom (Stokes *et al*, 1993), in which, the inhibition zone obtained with the test organism is compared directly with that obtained with a fully sensitive control strain on the same agar plate. Technical variations affect the test and control strains equally and are assumed to cancel each other out.as the control strain and the test strains are inoculated on the same plate, whereas in the other comparative methods the control strain may be put on separate plates. However, with the comparative method, the genus of the control strain may differ from that of the test strain, which is important when there is variation in the antimicrobial content of the disc recommended, and for interpretation of inhibition zone of intermediate susceptibility (Brown, 1990). Moreover, the comparative approach has not been systematically developed to take account of highly active newer agents or particular resistance mechanisms, and as a result *ad hoc* changes to the comparative criteria for interpretation, and to the range of control organism has been made (Brown, 1990). For these reasons, the value for the Stroke's method for AST is increasingly limited and is rarely used nowadays.

The disk diffusion test is simple to perform and, if performed carefully, very reproducible (Amsterdam, 1996). The cost is low and it does not require special equipment. Moreover, it allows flexibility of choice of antimicrobial agents for testing and provides qualitative results easily interpreted by physicians. However, the test should be applied only to bacterial species that have been completely evaluated and standardized (Turnidge and Jorgensen, 1999). Slow growing, anaerobic or fastidious bacteria should not be tested (Woods *et al*, 1995). Moreover, it provides only a qualitative result of resistance.

ii) Dilution test

A dilution test determines the minimum inhibitory concentration (MIC) of an antimicrobial agent, i.e., the lowest concentration of drug which inhibits growth of an organism. Generally, serial twofold - dilutions, representing concentrations of the antimicrobial attainable *in vivo* following standard dosages are tested. The concentration range tested varies with the drug, organism, and site of infection. Moreover, the tested range must include concentrations that allow categorisation of organisms into susceptibility categories and the reference ranges for quality control strains. The antimicrobial agent stock solutions are prepared and stored as specified in CLSI standard for dilution test (NCCLS,2004b) and the sensitivity testing method provided by the working party of the BSAC (Andrews, 2004). Cation-adjusted Mueller-Hinton agar/broth is the recommended medium for testing commonly encountered aerobic and facultatively anaerobic bacteria by

theCLSI, whereas the BSAC recommends Isosensitest agar/broth (Oxoid) and Diagnostic Sensitivity Test Agar (DST). The interpretative criteria of susceptibility categories of each organism are provided in the CLSI test document (CLSI, 2005b).

Some fastidious organisms may grow too slowly, may require special nutrients or atmospheres, or simply may not have been adequately tested to demonstrate they can be tested by the standard methods. The media recommended may not support their growth for susceptibility tests, consequently, routine methods must be modified for testing fastidious bacteria that require supplementary nutrients, modified incubation conditions or both.

Dilution tests include both macro (tube), or micro (microtitration tray) broth methods, and the agar dilution method.

a. Agar dilution test

In the agar dilution method, specific volumes of antimicrobial solutions are dispensed into pre-measured volumes of molten and cooled agar, which is subsequently poured into petri dishes. The agar is allowed to solidify, and then a standard number of tested bacteria (10^4CFU/Spot) for aerobic bacteria or (10^5 CFU/Spot for anaerobic bacteria are applied to the surface of the plates as spots, using a multipoint inoculator. Following incubation, the MIC is read as the

lowest concentration of antibiotic that inhibits visible growth. Since the initial WHO report of an international collaborative study on the agar dilution susceptibility testing method in 1971 (Ericsson, 1971), developments has concentrated on the standardization of method procedures. Full descriptions of methods have been published in several countries including the U.K. and USA (Philips *et al*, 1991; CLSI 2004b; Andrews 2004; CLSI 2005b).

The advantages of agar dilution method are: up to 36 isolates can be tested on one 10cm plate and some check on purity and identification is possible. It measures the population segment resistant to the amount of drug present and heterogeneity of antimicrobial sensitivity may be recognized. However, this method cannot be easily used to determine bactericidal activity.

b. Broth macrodilution test

Broth macrodilution tests are performed in test tubes with twofold serial dilution of antimicrobial agent. A standardized suspension of test bacteria is added to each dilution to obtain a final concentration of 5×10^5 CFU/mL. After incubation, the MIC is determined as the lowest concentration that visually inhibits growth (NCCLS, 2004b).

Although standard methods have been published for this method (NCCLS, 2004b; CLSI 2005b; Andrews, 2004), there has not been agreement on details as for the agar dilution method. The major advantages of the broth dilution test are its

suitability for testing a single isolate for drugs not routinely tested, and for testing fastidious organisms requiring special growth media. Moreover, the broth dilution method is more suitable for automation. This method is also used when minimum bactericidal concentration (MBC) needs to be determined. However, contamination of the culture is not easily recognized, and the method is impractical for multiple isolates or when several antimicrobial agents must be tested on an isolate.

c. Broth microdilution test

Broth dilution susceptibility tests performed in multi-well microdilution trays are known as the microdilution broth method. The trays, containing between 80 and 100 wells, are filled with small volumes of twofold dilution concentrations of antimicrobial agent in broth. The trays may be prepared in house by mechanical semiautomated or automated equipment (Gavan and Butler, 1973; Gavan and Barry, 1980) and are also available as either frozen or lypholized commercial test panels, customized for the user. Commercial panels need to be rehydrated by adding diluent and inoculum to each well. Before testing, the inoculum suspension is prepared and standardized as described by the CLSI (CLSI, 2005b; NCCLS 2003, 2004b,). An intermediate dilution of this suspension is prepared in water, and a multi-pronged inoculator or other type of inoculating device is used to inoculate the wells to obtain a final concentration of 1 to 5 x 10^5 CFU/ml. After overnight incubation at 35°C-37°C, the tray is placed on a tray reading device to allow close visual examination of each well. MIC is determined as the

lowest concentration of wells that show no growth. The procedure for the microdilution broth test is given in CLSI approved standard M7-A6 (NCCLS, 2004b) and M100-S15 (CLSI, 2005b).

Microbroth dilution method provides a reliable standardized reference method for susceptibility testing. The major advantage is that it is amenable to automation. Most commercial automated systems available are based on this technique (Amsterdam, 1996). Moreover, if the resources are available, local production of plates allows the use of a tailored panel of antibiotics, allowing changes to be made easily. Reading and inoculating procedures allow convenient simultaneous testing of several antimicrobial agents on the same isolate. The microdilution broth method also allows either visual examination or use of automatic or semiautomatic instruments to read the endpoint. However, microdilution method requires the preparation of microdilution trays and not all laboratories have the facilities for the preparation of dilutions. Moreover, it sometimes produces an MIC endpoint that is not clear cut, and growth in the wells may demonstrate trailing or skipped wells (Jorgensen *et al*, 1999). It is also difficult to appreciate and detect contamination. This MIC method is unable to produce a penicillin MIC that is consistently within the resistant range for *Staphylococci* that are low producers of β-lactamase. Staphyloccocal isolates for which the MIC falls between 0.06 μg/ml to 0.12 μg/ml should be tested for the presence of β-lactamase before the results are reported (NCCLS, 2004b).

d. Advantages and disadvantages of dilution methods

Bacteria multiply *in vivo* on solid surfaces where they adhere, and the ultrastructure of the bacteria grown on agar is more closely related to *in vivo* conditions than that of the same organism grown in broth (Acar and Goldstein, 1996). This may influence the results of susceptibility tests of antibiotics when they are performed on agar or broth culture media. MICs obtained by agar or broth dilutions are well within one $\log_2$dilution difference for many combinations of antimicrobials and organisms, as long as the bacteria are susceptible (Amsterdam, 1996). However problems exist for bacteria with acquired mechanisms of resistance, especially if they produce inactivating enzymes. The MICs are three to five dilutions higher in broth than on agar media and the organisms appear more resistant in broth (Acar and Goldstein, 1996). For this type of organism, the amount of inoculum also has significance effect in broth. The effect of inoculum density has been found in several bacterial species and is wide spread among the β - lactams when their activity is direct against β - lactamase producing bacteria (Amsterdam, 1996).Therefore, MICs obtained by agar dilution method may be lower and need interpretation (Amsterdam, 1996). When comparing MICs between laboratories, or when new methods for susceptibility testing are compared to reference dilution method, this must be taken into account. When new testing methods are performed on agar media, the agar dilution test should be used as the reference 'gold standard.'

Both broth or agar dilution methods may be used to provide 'gold standard' MIC data except for pneumococci, (CLSI only recommended broth dilution as a standard method) that may be used as a reference for evaluating the accuracy of other testing systems. When compared with disk diffusion, dilution methods are time consuming, labour intensive, and more expensive. However dilution methods allow the standard medium used to test non-fastidious microorganisms to be readily supplemented or even replaced with another medium to allow accurate testing of various fastidious bacteria that cannot be tested by disk diffusion method. Moreover, any drug available in powder form can be used for dilution methods. Dilution methods are used when a more accurate estimate of susceptibility than that provided by disk tests is needed. An MIC determination is desirable when the isolate is from a patient with septicemia, meningitis, endocarditis, or other life-threatening infection and when the disk diffusion test gives unclear susceptibility results. When there is an unexpected failure of treatment or there is an unusual resistance pattern, quantitative MIC results are also required.

There has been a gradual shift by laboratories using quantitative susceptibility tests to methods that can provide MIC results (Craig, 1993). MIC testing is of greater benefit since studies have shown that treatment failures within the susceptible category are associated with higher MICs than with therapeutic successes, and the dose of drug required for a given response in animal models is linearly related to the MIC (Craig, 1993). MICs values of antibiotics together with

drug pharmacokinetic and pharmacodynamic (PK/PD) features, such as serum concentration over time and area under the concentration- time curve are better predictors of bacterial eradication and clinical success, and can determine dosage regimens (Craig, 2000; Andes and Craig, 2002; Craig, 2003) Quantitative MIC results may enable more individualization of the therapeutic regimen than provided by qualitative category susceptibility tests such as the disk diffusion test. Dilution tests methods are usually selected as the benchmark for susceptibility testing, but there is no absolute agreement when different reference methods, such as the agar, broth and microdilution versions, are compared. Moreover, conventional dilution methods have the disadvantages of large increments at higher concentrations, and there is a high standard allowable error (±100%) (Wexler *et al*,1990). More precise and reproducible methods for quantitative susceptibility testing are obviously needed, but laboratories face the dilemma of comparing potentially superior susceptibility methods with established tests.

C) Other susceptibility testing methods

i) Breakpoint method

In the breakpoint method, test strains are inoculated onto the surface of agar plates or into broth medium containing different concentrations of antibiotics chosen to separate bacterial strains as sensitive, intermediate or resistant categories. Usually, two appropriate drug concentrations are selected and each interpretative category

can be determined. When growth occurs at both concentrations, it is a resistant strain, whilst growth only at lower concentration indicates an intermediate result. When there is no growth at both concentrations, it is interpreted as susceptible. This method employs a standard MIC technique, so requires the use of appropriately adjusted and supplemented Mueller-Hinton broth/agar or Iso-Sensitest agar/broth. Standard inoculation, incubation and interpretative methods recommended for standard dilution methods must be followed.

The agar dilution based breakpoint method has gained acceptance in U.K. as an alternative to disc diffusion (Faiers *et al*, 1992). The major advantages of the breakpoint method are the ease of data handling, which allows its amenability to automated inoculation and reading. Moreover this method generally has clear cut end-points and removes interpretative difficulties. The method is suitable for laboratories with high workloads, since large numbers of isolates can be tested using multi-point inoculation techniques. However, it relies on an all-or-none effect, it cannot discriminate the finer degrees of susceptibility. It is difficult to define control strains, so it is more difficult to control effectively. Furthermore, the method intrinsically has a low reproducibility for organisms with MICs close to a breakpoint.

ii) Automated methods

Due to the progress in the development of robotics, electronics and microprocessors since the 1980's, clinical laboratories have increasingly used

commercial overnight or short incubation instruments based on automated broth microdilution AST methods for the determination of susceptibility of isolates. By the early 1990s automated or mechanized systems were used in approximately 60% of clinical laboratories in the U.S.A. (Jones and Edson, 1991), and were increasingly commercially available being used elsewhere (Brown, 1994).

Automated susceptibility testing methods range in choice from simple to highly complex. The least automated of the instrument-based systems interprets growth endpoints of broth microdilution panels when they are placed into an automated reader device. Other systems incubate broth microdilution trays or special cards in an incubator and perform serial interpretation of growth pattern by a reader device. The instruments having the highest degree of automation accomplish these tasks mentioned above by incorporating simple internal robotics to move the trays during the incubation and reading sequences. The automated methods differ in the optical method used for examining the growth endpoint. Many use turbidimetric detection of growth in the broth medium by the use of a photometer. Others use fluorimetric detection of fluorescent indicators or of hydrolysis of fluorogenic growth substrates incorporated in special liquid medium (Bascomb *et al*, 1991; Godsey *et al*, 1991; Schernbra *et al*, 1993; Nolte *et al*, 1998). All instruments depend heavily on microcomputer processing functions, which include use of a microcomputer to generate final reports and to help in data storage and retrieval. Some instrument systems incorporate software that can provide "expert" automated reviews of test results for errors or can analyze

susceptibility test result to determine likely mechanisms of resistance (Ferraro and Jorgensen, 2004). Most instruments also offer identification of gram positive or gram negative bacteria as well as antimicrobial susceptibility testing and, in some cases, can assemble combined identification and susceptibility reports.

Broth microdilution test panels are often provided in freeze dried or frozen antimicrobial panels and are usually offered with a mechanized device to simplify hydration and inoculation of trays. Agar dilution tests can also be mechanized through the use of a Steers replicater device or a Cathra Replicator (AutoMed, Inc, Arden falls, Minn). An AutoMed Cathra AutoReader can be used to read the agar dilution plates through feeds, indexes, or by video image capture analysis (Ferraro and Jorgensen, 2004).

A variety of commercial systems are available including Vitek (bioMerieux), AutoSCAN WalkAway W/A (Baxter Diagnostics), Sensititre (Radiometer), ALADIN (Analytab Products), AutoSceptor (Becton Dickinson), ATB Expression (BioMerieux), and Cobas Micro (Roche). Only two of these instruments, Baxter MicroSCAN AutoSCAN WalkAway (Baxter Diagnostic West Sacramento, CA) and Vitek System (BioMerieux Vitek, Hazelwood, MO), are capable of generating rapid (4 to 8 hours) susceptibility test results and are approved by the Food and Drug Authority (FDA) (Ferraro and Jorgensen, 2004) and dominate the USA market, though other rapid systems are in use elsewhere

such as Becton Dickinson Phoenix system, Akis (Trek Inc.) and the mini API (BioMerieux).

a. Baxter MicroSCAN AutoSCAN WalkAway system

The WalkAway system developed in the 1980s uses conventional 96-well standard-size microdilution trays coupled with fluorogenic or standard substrates to detect growth. The instrument consists of a computer controlled microprocessor and a large self-contained incubator-reader unit, that holds standard microdilution trays and interprets biochemical and/or susceptibility results. The microdilution trays are read either fluorometrically (short incubation susceptibility test) by a fluorometer or photometrically (conventional overnight susceptibility test) by a photometer. Recently an updated version of the same instrument, renamed the MicroScan rapid/S plus system, that reads results photometrically after a short incubation period (Ferraro and Jorgensen, 2004). The system consists of a carousel that rotates the towers containing the panels, a bar code scanner, spectrophotometer/fluorometer, and mechanisms to move panels according to computer-controlled robotic steps and position trays to add reagent or read results. There is a database system capable of reproducing cumulative patient reports, epidemiologic reports and antibiograms. Special combination trays are available that can provide organism identification and susceptibility test results. Final endpoints can be read when the value of a growth indicator exceeds

a preset level (Godsey, 1991). The initial reports on performance of MicroScan turbidimetric appear quite promising (Balou *et al*, 2001)

b. Vitek system

The Vitek system (bioMerieux Vitek, Hazelwood MO) is an automated method for performing same-day identification and AST on non-fastidious bacteria, modified in the 1970s for clinical laboratory use. Subsequent modifications have expanded its capabilities to provide both qualitative and quantitative AST of almost all antimicrobial agents.

The Vitek system is modular, consisting of a filling-sealer unit, an incubator/reader module that incorporates a carousel to hold the test cards, a robotic system to manipulate the cards, a photometer for measurement of optical density and biochemical reaction color change, and a computer module with video display and printer. The essence of this system is the small thin plastic reagent card that contains 30 microcuvettes, which are available with a variety of predetermined configurations of antimicrobial agents and reagents for identification (Stager and Davis, 1992). Although the fixed configuration of the system imposes some limitations on the selection of antimicrobial agents, a combination of two cards, called Flex cards, can be tested together and the results from both cards can be merged to one report. The system can hold 30 to 240 reagent cards. Growth endpoint, which is the MIC, is determined

turbidometrically at hourly intervals and growth is compared to the baseline and expressed as a ratio. Normalized linear regression of this growth is used to calculate growth curves of best fit leading to computer algorithm-derived MIC values (Ferraro and Jorgensen, 2004).

Recently a more automated version of the Vitek system (Vitek 2) has been developed and marketed. The new system eliminates the need for filler-sealer module and the associated hand labelling and manipulation of cards. The Vitek 2 cards are labeled by barcode and use of a computer chip-containing 'smart-carrier' that holds and identifies the cards. Thus the Vitek 2 system reduces the technical time required (Ferraro and Jorgensen, 2004). A further advantage of the Vitek 2 system is its ability to test *S. pneumoniae* in addition to non-fastidious organisms (Goessen *et al*, 2000; Jorgensen *et al*, 2000).

c. Advantages and disadvantages of automated systems

The major advantage of using an automated system is the reproducibility of results obtained, since procedures for inoculum preparation, duration of incubation, and assessment of endpoint are highly standardized. Moreover, many of the drug-organism combinations have been approved by regulatory authorities. The results generated should have high precision (Peterson *et al*, 1986; Visser *et al*, 1992; Fekete *et al*, 1994; Berke *et al*, 1996). Another advantage of instrument based systems is derived from the potential to establish a link between the computer and the laboratory information system interface. Programmed software

can detect impossible or rare resistant patterns and flag these on the data terminal or laboratory report for senior staff review (Ferraro and Jorgensen, 2004). This precludes technologist or clerical errors on individual laboratory reports. The data management system of the instruments can provide clear reports and can also allow periodic reviews and analysis trends in antibiotic susceptibility patterns of organisms encountered in a clinical laboratory. As the automated system can generate rapid susceptibility results, this can allow timely changes to antibiotic therapy (Trenholme *et al*, 1989), which may have a major impact on patient care and help reduce hospitalization costs if both advanced computer programmes and change of prescription practice are introduced (Barangfranger, 2001). Another advantage of some of the instrument-based susceptibility test systems is the utilization of an expert software system for automated review and verification of the data generated (Courvalin, 1996; Sanders *et al*, 2000; Sanders *et al*, 2001).

Although, the automated approach to AST seems to be more efficient by use of robotics in performing these tests, workload gains in labor saving have been reported to be minimal (College of American Pathologists, 1992). In addition, the initial capital investment for purchase of hardware and costs in running the systems are relatively high. The recurrent or disposable cost may be more expensive than consumables needed for other AST methods. Maintenance costs of automated systems are also high. Fees for service contracts and change of broken parts may add to operational costs (Ferraro and Jorgensen 2004). Laboratories using these systems must purchase antimicrobial test panels preset

by the manufactures and these offer a limited choice. Therefore, the flexibility to change test panels is limited and the user may need to use a combination of panels to complete the range of drugs to be tested, further increasing the running cost. Furthermore, the flexibility to begin testing a new antibiotic is limited.

For short incubation systems, CLSI does not provide standards or quality control for automated test procedures. Some quality control organisms provided by the manufacturers fail to produce QC endpoints that are on scale (Kellogg, 1984; 1985), and their flexibility and precision are questionable. Furthermore, the range of drug concentration available for test of a given antimicrobial agent is narrow, so MICs out of range are frequently encountered and susceptibility test results can only be expressed semi-quantitatively. Moreover, a short incubation period of 4 to 6 hours is inadequate for expression of inducible β-lactamase mechanism (Visser *et al*, 1992; Schadow *et al*, 1993). The detection of plasmid mediated extended-spectrum β-lactamase is even worse for short incubation systems when compared with conventional methods (Katsanis *et al*, 1994).

Probably the major disadvantage of automated systems for susceptibility testing is their inability to test some clinically significant bacteria, such as certain fastidious organisms, anaerobes, and specific non-fermentative gram negative bacilli. An alternative MIC method is still needed for testing these groups of organisms. Some commercial microdilution systems offer panels for the susceptibility testing of *S. pneumoniae*. However there have been repeated incidents of inaccurate

reporting of susceptibility to penicillin for pneumococcal strains that are intermediate or highly resistant (Shanholtzen and Peterson, 1986; Krisher and Lincott, 1994; Kiska *et al*, 1995). These led the FDA to prohibit automated testing of *S. pneumoniae* with penicillin (FDA, 1994). Restrictions were also applied to automated testing of *Enterococcus* with vancomycin and ampicillin (FDA, 1994). In the past few years, one of the automated systems, the Vitek 2, and several semi-automated systems: PASCO (Difco, Wheatridge, Colo), MicroScan (Dade MicroScan Inc., Calif), MicroTech (Aurora, Colo) and Sensititre (AcuMed, Ohio) were found to produce MICs that are comparable to the dilution reference method (Gathrie *et al*, 1999; Goessen *et al*, 2000; Jorgensen *et al*, 2000; Mahammed and Tenover, 2000) and have been cleared by FDA for susceptibility testing of *S. pneumoniae* (Hindler and Swenson, 2004).

iii) E Test

The E Test (AB Biodisk, Solna, Sweden) is a concentration gradient method for direct quantification of antimicrobial susceptibility of micro-organisms. The E test is an inert thin plastic carrier strip with a predefined concentration of dried and stabilized drug on one side, and a continuous MIC interpretative scale on the other. To determine an MIC with the E test, the surface of an agar plate is swab-inoculated with an adjusted bacterial suspension in the same manner as for disc diffusion. One or more E test strips for the antimicrobial agents to be tested are then placed on the inoculated agar surface. After overnight incubation, the effect of the antimicrobial agent in a gradient on the test bacterial inoculum gives rise to

an elliptical inhibitory zone. The interphase of the growth ellipse margin with the E test strip gradient indicates the MIC of the drug for the organism. Fig 1 shows the E test principle. The choice of media, preparation of inoculum, incubation atmosphere, and incubation temperature used for E test are the same as those used for disc diffusion test.

E test is a robust, simple technique which combines the simplicity and flexibility of the disk diffusion test and is minimally affected by laboratory variation. Moreover, it is versatile and can be used with different *in vitro* parameters conducive to resistance detection e.g. inducible resistance mechanisms, high and low level resistance, subtle changes in susceptibility, and can detect resistant subpopulations. The major advantage of the E test is its ability to be used for testing anaerobic bacteria or fastidious organisms such as *S. pneumoniae, Haemophilus influenzae and Neisseria gonorrhoeae.* This is because the E test strips can be placed on enriched media or in special incubation conditions without affecting the accuracy of the result.

There have been numerous evaluations of E test performance in comparison with reference method results using commonly isolated bacteria (Baker *et al*, 1991; Brown and Brown, 1991; Ngui-Yen, 1992), in which E tests MICs demonstrated excellent agreement with the reference methods, and were found to have very good reproducibility. Evaluation of E-test AST for some fastidious organisms,

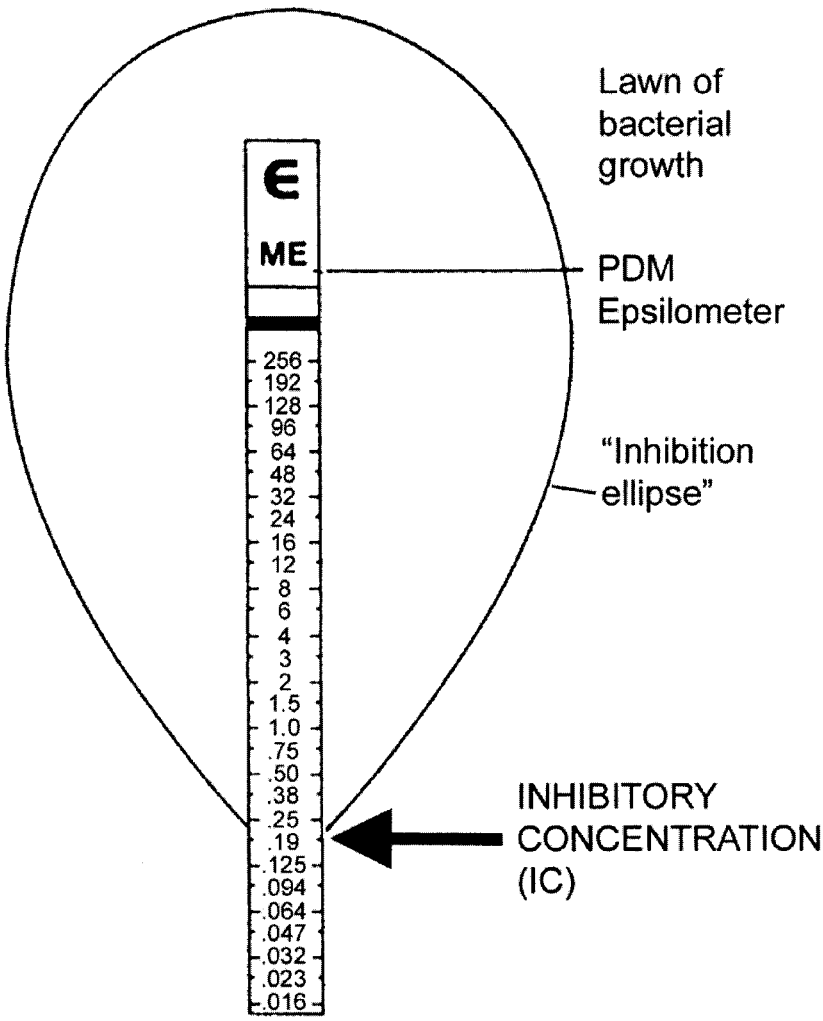


Figure 1: Principle of E test

(Reprint from Ericsson *et al.* A novel technique for direct quantification of antimicrobial susceptibility of microorganism. Poster session 10LICACC. 1988)

revealed that although there was a small amount of disagreement observed between methods, the E test has a high level of agreement with results of reference tests (Sanchez *et al*, 1992; Hughes *et al* 1993; Jorgensen *et al*, 1994a; Jorgensen *et al*, 1994b; Macias *et al*, 1994). E test is FDA approved for determination of MIC for some of the drugs for *S. pneumoniae,* and *N. gonorrhoeae* (Jorgensen *et al*, 1991; Bledenbach and Jones, 1996; Hindler and Swenson, 2004).

Since no more than six E test strips can be placed on the surface of 150mm diameter petri dish, and the E test strips are relatively expensive, this test is not an economical method for testing a large number of drugs on each isolate. E test also produces some minor discrepancies, when compared with reference methods (Jorgensen *et al*, 1994b; Macias *et al*, 1994; Skulnick *et al*, 1995). The difference in results reported by previous studies indicates an inherent disadvantage of the test: since the methodology is very simple, there are few parameters that can be standardized or controlled by the user. The thickness of the agar plates or the wetness of the plates may affect growth and therefore the result, but these factors have not been thoroughly evaluated. The performance of the tests cannot be totally monitored and depends absolutely on the quality of the E test strips. This can make evaluation of E test results difficult. There is also a storage problem associated with the test strips, which should be kept at -20^0C, but extreme care must be taken to avoid any moisture (AB Biodisk, 1992). There may be condensation of water when the strips are removed from the refrigerator.

iv) Direct detection of the expression of a resistance mechanisms

The major advantage of detection of resistance in the absence of growth is rapid reporting of the results within a short time, which may have benefits to patients (Barenfanger, 2001: Barenfanger et al, 2001). Detection of resistance mechanisms has been restricted in most laboratories to detection of antimicrobial-inactivating enzymes. Although Courvalin (1972) proposed a wider approach to characterization of resistance phenotypes by conventional testing of a range of antimicrobial agents to deduce the resistance mechanism, detection of antimicrobial-inactivating enzymes in the clinical laboratory is limited to tests for β-lactamase and chloramphenicol acetyltransferase (CAT).

β-lactamase production is a significant mechanism contributing to β-lactam resistance in *H. influenzae, N. gonorrhoeae, Moraxella catarrhalis, Staphylococcus sp., Enterococcus sp* and some anaerobes. Simple β-lactamase tests are performed in the clinical laboratory to identify β-lactamase production in these organisms. A positive reaction means that β-lactam agents may be ineffective to treat infection caused by these organisms, and a negative reaction is inconclusive. Members of the families *Enterobacteriacae* and *Pseudomonadaceae*, which produce a variety of β-lactamases, may have various susceptibilities to β-lactam agents. Direct β-lactamase testing cannot predict resistance to all β-lactam agents in these species and should not be used.

The most commonly employed β-lactamase test utilizes the chromogenic cephalosporin, nitrocefin. Nitrocefin has been incorporated into a widely used commercial product, the simple to use cefinase disk (Becton Dickinson Microbiology systems, Cockeysville, Md.) (O' Callaghan *et al*, 1972). Other β-lactamase detection methods, such as the acidometric and iodometric methods (Leitch and Boonlayangoor, 1992), are based on the detection of penicilloic acid, which results from the action of β-lactamase on penicillin. Tests involving difference in response to third generation cephalosporins alone and together with clavulanic acid are used in the detection of extended spectrum beta lactamases (Livermore and Woodford, 2004)

A rapid CAT test is useful when chloramphenicol is used to treat serious infection caused by *H. influenzae*. In addition, the CAT test has been used for *S. pneumoniae* and *Salmonella sp*. It can be performed either by a tube test or commercially available disks (Remel, Lenexa, KS) (Azemun *et al*, 1981). A positive test confirms chloramphenicol resistance, but a negative test does not exclude that the isolate may be chloramphenicol resistant by a non-enzymatic mechanism, which requires a conventional susceptibility test for confirmation. Currently, third-generation cephalosporins are used for treating serious *H. influenzae* infections, the CAT test is primarily used in research settings or to confirm equivocal results obtained with conventional susceptibility tests.

v) Genotypic detection of resistance mechanism

Genotypic detection of resistance genes in bacterial isolates is effected either by DNA probes or polymerase chain reaction (PCR) and is based on the assumption that resistance gene carriage equals resistance. However, there are subsets of strains which acquire resistance by either chromosomal mutation or by novel genes that cannot be detected by the designed probes or amplification techniques. Such resistance must still be detected by conventional susceptibility methods.

The detection of resistance genes by genetic methods can be used to directly detect resistance genes in the clinical specimen. Antimicrobial therapy can be guided much earlier before the reporting of the conventional antimicrobial susceptibility test. This has been shown for rifampcin resistance in *M. tuberculosis.* Moreover genetic methods are useful for interpreting traditional MIC results that are close to the breakpoint for resistance. Detection of resistance genes can be used as the 'gold standard' for resistance, when a new susceptibility test procedure is being evaluated using isolates with borderline MIC results (Bignardi *et al*, 1996). Genetic based tests are also a useful technique to determine the epidemiological spread of resistance in a hospital or community.

Many nucleic acid probes have been developed for the detection of various types of resistance. However, these are mainly used as research tools and few tests are as yet commercially available for routine use. The sensitivity of DNA probes can be greatly increased by gene amplification techniques such as PCR. Nevertheless,

primer sets used should be thoroughly evaluated for specificity, self-complementarily, and dimer formation before being used (CLSI, 2005a).

The major advantage of molecular methods is the rapid reporting of results. Moreover, the method is effective against non-growing and even dead bacteria. It can also improve safety in the laboratory since it reduces the handling of dangerous bacteria. However, simultaneous DNA probes for identification may be necessary since the identification of the organism is usually required, and this method is more feasible when the presence of wide range of organisms is possible. PCR may give inaccurate resistance predictions as sometimes, more than one gene may confer resistance; the detected gene may not be expressed; or the gene probe cannot detect resistance due to random mutation. In summary, molecular method can augment but not replace the traditional AST methods. Further commercial development of molecular methods is required before they can be widely used.

vi) Spiral Gradient Endpoint (SGE) Method

The use of a spiral plater has become a well established method for the enumeration of bacteria and has been particularly applied to determination of bacterial counts in foods and water. The use of the spiral gradient endpoint (SGE) method for determination of MICs has been suggested by several workers (Hill and Schalkowsky, 1990; Paton *et al*, 1990). This is another concentration

gradient method, utilizing the spiral plating method to deposit a liquid suspension in a spiral pattern on the surface of a prepoured agar plate. For its application to AST, a small volume of a stock concentration of antibiotic is dispensed in an Archimedes spiral onto a rotating agar plate (Fig 2). A variable cam is used to dispense the antibiotic solution at a slower rate the further away the dispensing arm moves from the starting point. This creates a radial concentration gradient of antimicrobial agent in the agar, decreasing from near the centre of the plate outward thereby eliminating the need to make a large number of serial dilutions for MIC determination. Test strains can be inoculated on to surface of the agar by swabbing along radial lines across the concentration gradient. After incubation, visible growth along the radial streak ceases where the concentration of the antimicrobial agent has reached the bactericidal or/and bacteriostatic concentration (Schalkowsky, 1985). The concentration of the antimicrobial agent at this growth endpoint can be calculated after measurement of the radial distance of the growth transition endpoint to the commencement of the antibiotic deposition (13mm from center). The concentration of antimicrobial agent at the growth endpoint serves to compute the MIC of the drug/strain interaction. The distance between the growth endpoint and commencement of antibiotic deposition (13mm from centre) is known as the radial advance (RA) (Fig 3). The volume of antibiotic stock solution deposited by the spiral plater at any location on the

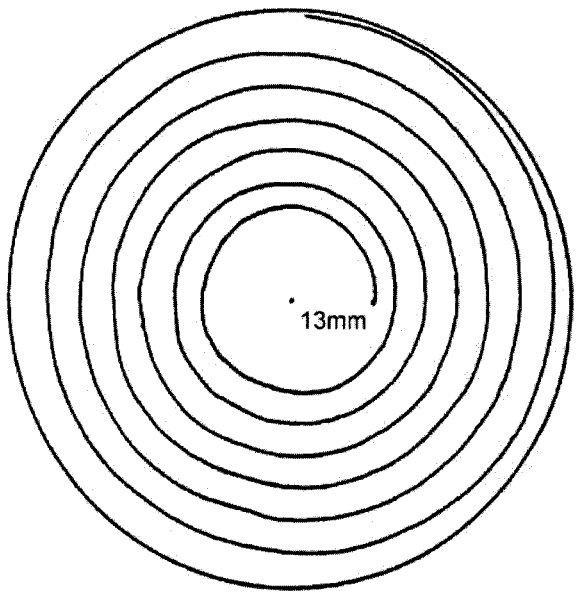


Figure 2: Spiral deposition of antibiotics onto agar plate

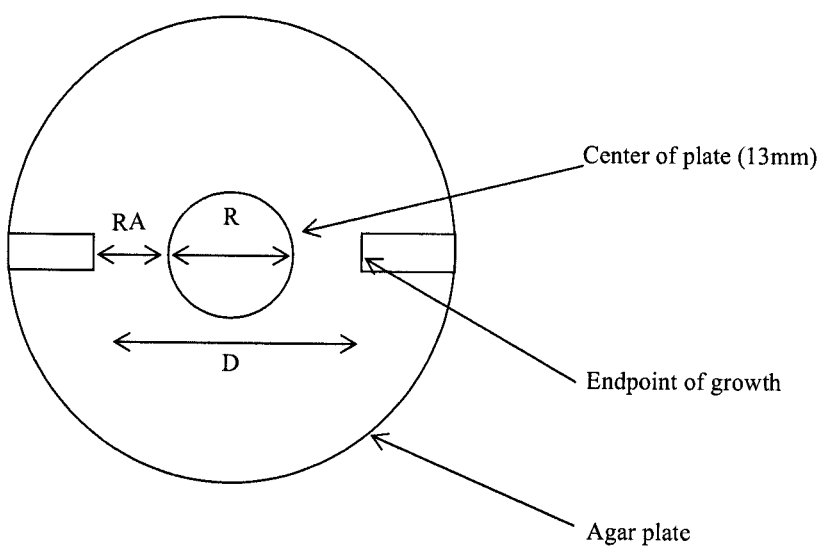


Figure 3: Diagram of MIC measurement by SGE method

surface of the plate is known. This quantity is referred to as the Deposition Factor (DF) and can be calculated using an equation. The formula used to calculate the average concentration of antibiotic at any radial advance of the plate is:

$$\text{Average concentration} = \frac{\text{DF}(\mu l/mm^2) * \text{Stock concentration (mg/L)}}{\text{depth of agar (mm)}}$$

The DF values are expressed as a function of the Radial Advance (RA) in millimeters, allowing the antimicrobial concentration at any point on the plate to be calculated. Tables are available from the manufacturer of the spiral plater, which have pre-calculated deposition factors for a given RA. However, it should be noted that the DF varies for different models of spiral plater.

Fifteen cm diameter pre-poured agar plates are used to provide a concentration range on one SGE plate equivalent to up to 8 twofold dilution plates in the SAD method and a wide range of concentrations of antibiotic (300:1) can be deposited on each plate. The stock concentration of antibiotics should be selected to cover the range of MIC expected. A concentration range that includes the breakpoint concentration as well as the known MIC of at least one of the two quality control strains is required. This range is sometimes provided by using plates with two different SGE concentration ranges - a high range plate and a low range plate with concentration overlapping in the midrange.

The SGE test is not a diffusion method and does not rely on drug diffusion to create the drug concentration gradient. It relies upon the precisely known

volumetric deposition rates, and the known concentration of antibiotic in the stock solution, to determine the amount of antibiotic solution at any location on the plate. However, diffusion will alter the deposited gradient in prolonged incubation. This is of particular importance with anaerobes which require two days of incubation (NCCLS, 2004a). Relevant diffusion rates were initially studied by Schalkowsky *et al* (1985) in the course of the development of SGE by using spectrophotometric readings of agar plugs taken at different RA following deposition of a methotrexate gradient. It was found that at RA locations, concentration of antimicrobial in the agar, as computed from deposition factor table, were somewhat lower than actual. The correction for the diffusion in the computation of the drug concentration was incorporated into a revised methodology (Hill and Schalkowsky, 1990) and was found to be effective for antimicrobial agents spanning a wide range of molecular weights. The major determinants of diffusion rate are molecular weight of the drug and time of incubation. A computer program is required to calculate the required stock concentrations for various antimicrobial agents according to the range of test concentrations desired and to calculate the endpoint MIC of an antimicrobial agent for each strain. Nevertheless, diffusion has a more pronounced effect when testing slow growing anaerobes. Evaluation utilizing non-fastidious aerobic test strains showed good correlation to results from parallel tests with SAD method even without correction for diffusion (Weckback and Staneck, 1987; Paton *et al*, 1990). Omitting additional calculations makes the original SGE method more convenient to use the to perform AST for aerobic bacteria or fastidious organisms.

Studies have shown that there is excellent agreement between the MIC obtained by the SGE method compared with those obtained by the SAD method for non-fastidious organisms (Hill and Schalkowsky, 1990; James, 1990; Paton *et al*, 1990; Wexler *et al*, 1991). Several studies have been performed on AST of anaerobic bacteria (Hill and Schalkousky, 1990; Hill 1991; James *et al*, 1991; Wexler *et al*, 1991, 1996) and the revised SGE method was found to have high reproducibility and accuracy with correlation coefficients of greater than 0.9 when compared with reference methods. There are few published studies on the AST of aerobic bacteria (James, 1990; Paton, 1990; James, 1991). In those studies using the original SGE method, the correlation coefficient between the SAD method and SGE method was found to be 0.74 to 0.97 ($P<0.05$). Agreement between the two methods was good, with percentage agreement ranging from 46% to 88% within a twofold dilution, and from 88% to 100% within 2 twofold dilutions. Although the SGE method has been shown to be a reproducible and accurate method for MIC determination of aerobic and anaerobic organisms (Hill and Schalkowsky, 1990; James, 1990; Paton *et al*, 1990; Hill, 1991; James *et* al, 1991; Wexler *et al*, 1991; 1996), little work has been performed on its use for AST of fastidious organisms.

The major advantages of SGE method are decreased labour and materials, and more accurate mechanical dilution compared with SAD method. The cost of consumables is low when compared to other MIC methods such as the E test, although there is a large initial investment for purchase of the spiral plater. The SGE measures a similar type of endpoint to the SAD method but with increased

sensitivity since a continuous scale of MICs is obtained. The overall usefulness of the SGE method would be increased if it can be shown to produce precise MICs for the AST of fastidious bacteria on enriched media.

When compared with the SAD method, it is easier to visualize the growth endpoint of a radial streak on a single plate than in a series of twofold dilution plates. One or more drugs and bacterial strains can be tested economically and at short notice. Another potentially interesting facet of the SGE test is that it allows a more detailed analysis of bacteria-drug interactions, that includes a sensitive evaluation of the characteristics of the growth inhibition zone of the streak and of the nature of the bacterial population present within these zones, where confluent growth along the radial streak changes to diminished growth and finally ceases, with or without outlier colonies. In serious infections such as meningitis, pathogens are promptly eradicated only if the antibiotic concentration in CSF reaches the minimal bactericidal concentration (MBC) - the lowest concentration of a drug required to kill a pathogen, by 8 to 10 fold. SGE method provides a sensitive method to measure the MBC when the growth endpoint is carefully examined. Studies have shown that the SGE method gave very high reproducibility for MBC determination (James, 1990) and the method has been used for study of penicillin tolerance in Streptococcus species in patients with endocarditis (James, 1990; James *et al*, 1991)

Breakpoints in disc diffusion tests are obtained from "scattergram" plots of the discrete twofold reference MIC values against the corresponding inhibition zone diameters. The scatter observable in such plots is due to the incremental nature of the twofold MIC of the reference SAD or broth dilution method and to the high variability in their measurement. However, zone diameters in disk diffusion tests are derived from a continuous scale. This can lead to unacceptable low correlation coefficients for a regression analysis. The calibration of zone diameter can be done in terms of the corresponding MIC value in parallel with SGE test, which is read on a continuous scale. Considerable reduction of scatter should result and acceptable correlation is likely to be obtained. SGE method could improve calibration of zone diameter of disk susceptibility testing if it is shown to be a precise and reproducible method.

The standard allowable error for the SAD method is considered to be one twofold dilution (NCCLS, 1985). When the MIC value of a particular isolate is near the breakpoint, the isolate termed susceptible on one occasion may be retested and termed resistant due the to the inherent error of the test. This clustering around the breakpoint is a characteristic of organism-drug interaction and is present to some degree, in every technique. The greater precision of the SGE test to determine MICs due to the continuous concentration gradient reduces the number of strain within this population. (Wexler *et al*, 1991)

Shifting of endpoint on SGE plate towards the outside of the plate and vice versa is dependent on the strength of the stock concentration of the antimicrobial used to produce the gradient. The ability to shift endpoint location of the SGE method provides a reliable basis for testing the efficacy of drug combinations, circumventing the fundamental deficiency of the checkerboard method due to its reliance on twofold dilutions and its use of computer null reference, which cannot be accurately measured in the test (Kouick and Yu, 1991). The synergistic effect of combination therapy may be needed to treat serious infections. With the current lack of new antibiotics, drug combination might be the only way in short run. In the treatment of meningitis due to *S. pneumoniae*, combined drug therapy with vancomycin and an extended spectrum cephalosporin to obtain a synergistic effect on multiply-resistant strains has been suggested (Klugman *et al*, 1995). Other workers have suggested vancomycin and rifampcin (Pallares *et al*, 1998) or vancomycin and gentamicin (Cottagnowd *et al*, 2003). Because most enterococci are tolerant to the bactericidal activity of β-lactam and glycopeptide antibiotics, bactericidal synergy between one of these antibiotics and aminoglycoside is needed to treat serious enterococcal infection (Moellering, 1981). In serious *Pseudomonas aeruginosa* infection, combination therapy is significantly better than monotherapy in improving outcome (Kouick and Yu, 1991). If SGE method can be successfully used for the AST of these organisms, the method can be readily adapted for testing drug combination efficacy for treatment of multiple-resistant strains.

vii) Flow Cytometry

(a) Introduction

Flow cytometry (FCM) is a technique in which measurement of physical characteristics of cells is made as the cells pass in a fluid stream through a measuring point surrounded by an array of detectors (Shapiro, 2003). Figure 4a shows a diagram of a typical flow cytometer.

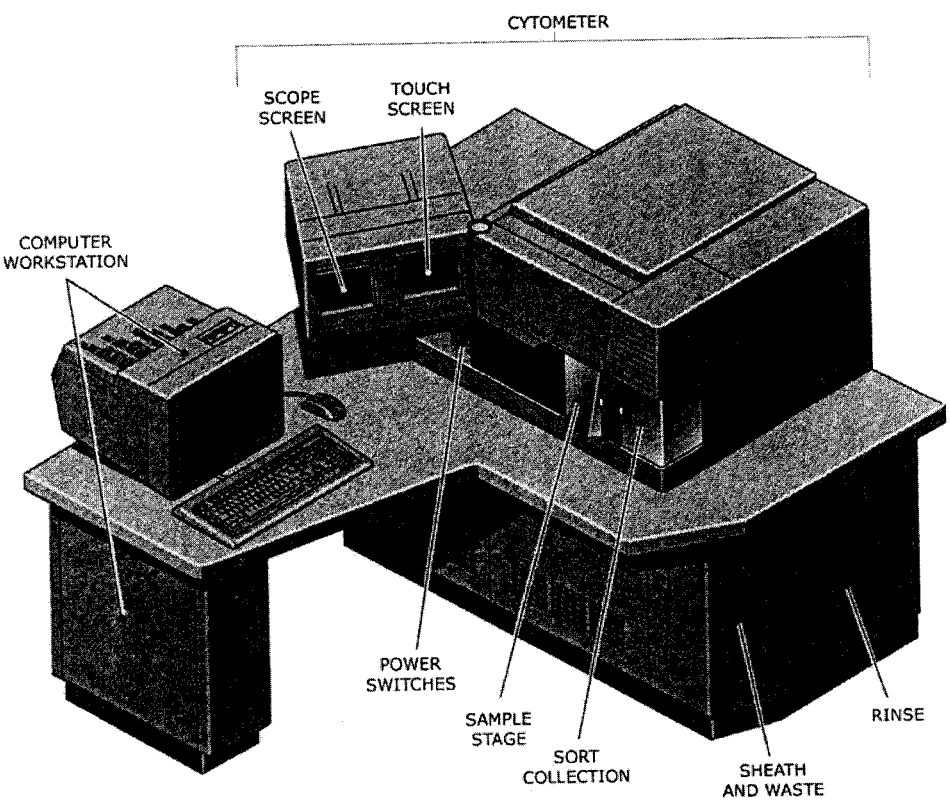


Figure 4a: Diagram of an EPICS Elite flow cytometer.

In a typical FCM, the streams of cell particles intersect with a beam of light from an arc lamp or a laser lamp. When the light beam meets the cell particles, some of the light is scattered out, and this scattered light is collected over a range of angles by photomultipliers (PMTs) or diode detectors positioned around the measuring point. Such measurements of each cell or particle are made separately and the results represent cumulative individual cytometric characteristics. The important feature of FCMs is their ability to measure multiple cellular parameters through the arrangement of PMTs and optical filters. Figure 4b show the diagram of optical filter setting of a Coulter EPICS Elite FCM.

(b) Historical Development of FCM

The ancestor of the modern FCM was developed by Gucker and colleagues in 1947 (Gucker *et al*, 1947) as an instrument for the analysis of dust aerosol particles for the determination of the efficiency of gas mask filters. This apparatus was used in World War II by the US Army in experiments for the detections of bacteria and spores. In the mid 1950's, particle counters based on the Coulter orifice principle were developed. The difference in electrical conductivity between the cells and the medium in which they were suspended, is measured by changes in electrical impedance produced as they pass through an orifice, were developed. However, the first real FCM was built by Kamentsky and coworkers (1965), who described a two parameter FCM that measured absorption and back-scattered illumination of unstained cells and this was used to determine cell nucleic acid content and size.

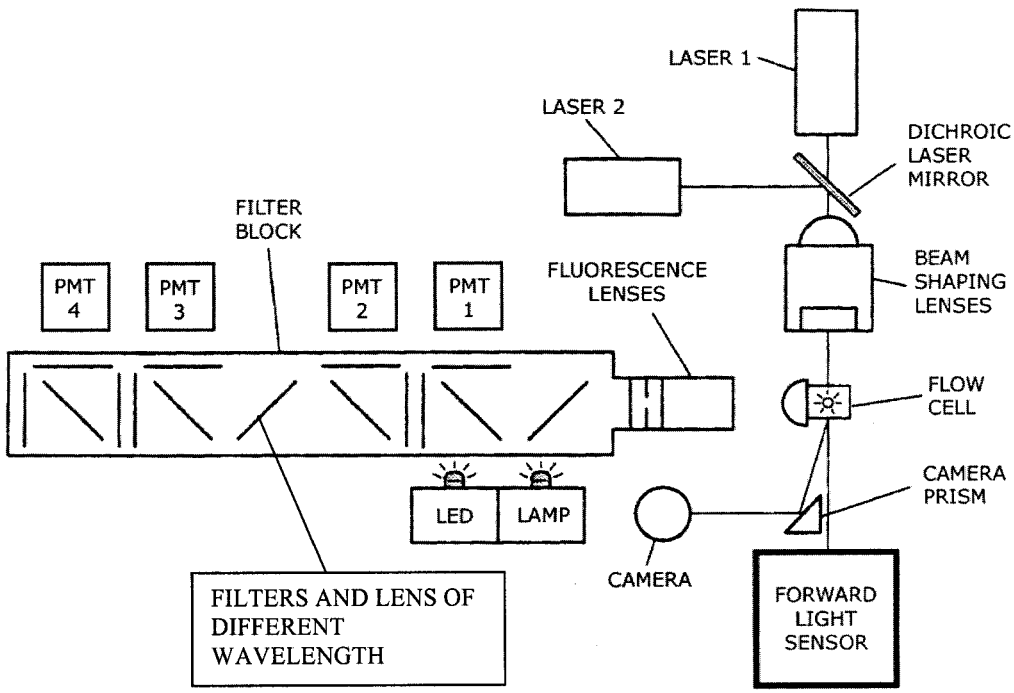


Figure 4b. Optical settings of a Coulter EPICS ELITE flow cytometer.

Later in the same year, the first flow sorter was described by Fulwyler (1965).

During the 1970's, applications of FCM to research into mammalian cells advanced rapidly, but few instruments were developed for microbiological studies (Alvarez-Barrientos *et al*, 2000). The subsequent applications of FCM to microbiology were due to optical improvement in FCMs and newly developed fluorochromes. The development by Steen and co-workers of a sensitive arc lamp-based instrument with a redesigned flow chamber designed specially for work with bacteria was an important step in increasing the use of FCM techniques in microbiology (Steen and Lindmo, 1979). The high signal-to-noise ratio of the new flow chamber was ideal for measuring parameters on microorganisms (Steen and Boye, 1981; Steen *et al*, 1982; Boye *et al*, 1983; Skarstad *et al*, 1983; Steen *et al* 1990; Allman *et al*, 1992; Brailsford and Getty, 1993).

Although the earlier laser lamp FCMs were not sufficiently sensitive to measure smaller bacteria, developments in technology have resulted in instruments such as EPICS Elite FCM (Beckman Coulter Ltd.) that can be successfully utilised for microbiology studies (Sander *et al*, 1990; Gant *et al*, 1993).

(b) Flow Cytometry Susceptibility Test

FCM has proved to be very useful for studying the physiological effects of antibiotics on bacteria due to their effects on different metabolic parameters: including cell size, amount of DNA, membrane potential and membrane integrity.

Moreover, FCM can provide a reliable method for AST, providing information on the effect of bactericidal or bacteriostatic antimicrobial agents (Steen *et al*, 1982; Martinez *et al*, 1982; Gant *et al*, 1993, Durrdie *et al*, 1995; Pore, 1994; Walberg *et al*, 1997).

It was in the early 1980's that the first FCM studies on the effects of antibiotics in bacteria were performed (Hutter and Oldiges, 1980; Steen and Boye, 1981; Steen *et al*, 1982; Steen *et al*, 1986). FCM use for AST of bacteria was first reported by Steen *et al* (1982) demonstrating two-parameter histogram (light scatter/fluorescence) when bacteria were stained with mithramycin. Martinez and coworkers demonstrated the filamentation of *E. coli* when treated with β-lactam antibiotics (Martinez *et al*, 1982), while Boye and Lobner-Olesen (1990) demonstrated the inhibition of *E. coli* growth within minutes following antibiotic treatment. Gant *et al* (1993) showed that a modern laser-based FCM was sufficiently sensitive to detect changes in bacterial morphology on entry into the growth cycle after exposure to bactericidal antibiotics.

Using FCM and fluorescein diacetate (FDA), susceptibility testing of *Mycobacterium tuberculosis* has been successfully performed (Norden *et al*, 1995). In contrast to the delay in obtaining data in current antimycobacterial drug AST, results were available within 24 hours. Other Mycobacterium species treated with different concentration of ciprofloxacin, kanamycin and other drugs were easily differentiated from untreated cells after 6 to 24 hours of incubation

(Bownds *et al*, 1996). Moore *et al* (1999) have developed a safer method using paraformaldehyde to inactivate the TB cells. However the time needed to obtain the AST results was increased to 72 hours.

In addition to the increasing sensitivity of the instruments, another factor in the evolution of FCM AST was the identification of fluorescent indicators. Two major categories of indicators have been identified for the study of antimicrobial effects: DNA or RNA labelling dyes and protein binding or metabolic probes, which include membrane potential sensitive probes. The choice of these probes is related to their excitation and emission properties and the mode of action of the antimicrobial agents.

Fluorescent RNA or DNA intercalating dyes are generally used to measure membrane integrity. Propidium iodide (PI), ethidium bromide (EB) and ethidium monoazide (EMA) are all examples, which, at low concentration do not normally cross intact membranes and intercalate into DNA or RNA (Shapiro, 2003). Ethidium bromide may slowly enter intact cells at high concentration (Shapiro, 2003) and can stain microbial nucleic acid after cell cycle development and indirectly, indicate viability (Martinez *et al*, 1982). PI is more appropriate than ethidium bromide for AST (Pore, 1994) as it cannot permeate and is excluded by viable cells, making the measurement of loss of viability produced by antimicrobial agents or other compounds possible. Other new nucleic acid

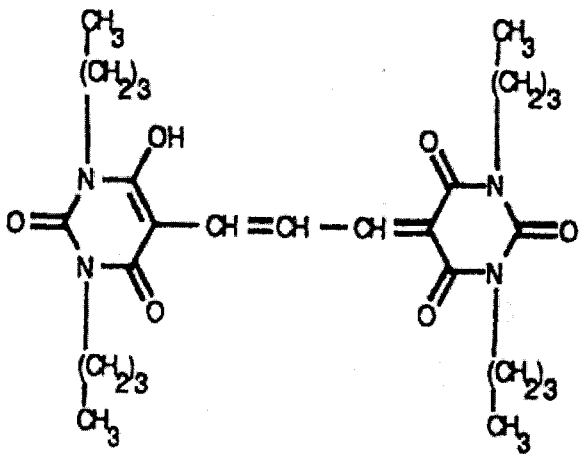
staining fluorochomes have been used for FCM including SYTO-13 and SYTO-17, which label both DNA and RNA (Haugland, 1996).

Other compounds used include fluorogenic substrates, which are lipophilic, non-toxic, uncharged and nonfluorescent (Shapiro, 2003). The fluorogenic substrates are taken up by viable cells, and are hydrolysed by non-specific esterases to polar fluorescent products that are retained by cells with intact membranes. These dyes include fluorescein diacetate (FDA). Dead cells with leaking membranes rapidly leak the dye; quantitative assays of this leakage can be used to determine cytotoxicity induced by antimicrobial agents (Bercovier, 1987). However, there is some evidence of incomplete hydrolysis of the esters and that compartmentalization of the dye in intracellular organelles may occur, resulting in a complex and uninterpretable signal from the cells. Furthermore, the reaction products from ester hydrolysis may be cytotoxic (Shapiro, 2003).

In bacteria, the cellular apparatus for energy metabolism is on the cytoplasmic inner membrane. The potential across this membrane is dependent on energy metabolism and is decreased following removal of the energy source or when the membrane is perturbed by physical or chemical agents such as antibiotics. Cell death and/or membrane damage brings about a collapse of the electric membrane potential can be effectively measured by FCM using membrane potential-sensitive probes (Pore, 1994). Some of these probes belong to the carbocyanide group, which includes 3,3'-dipentyloxocarbocyanine iodine ($DiOC_5$) (Ordonez

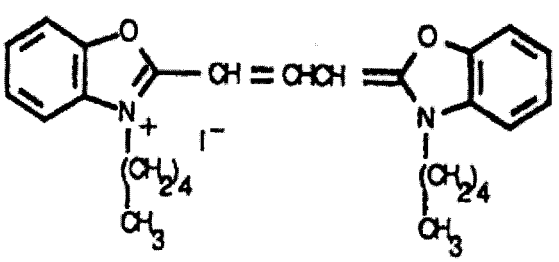
and Wehman, 1993). Mason *et al* (1995) has assessed various membrane potential dyes for their ability to discriminate between viable and non-viable bacteria.

Rhodamine 123 (Rh123) and oxonol (bis-1,3 dibutybarbituric acid trimethine oxonol – $DiBAC_4(3)$) are other membrane potential sensitive probes that are frequently used to assess drug effects by FCM (Jepras *et al*, 1995; Comas and Vives-Rego, 1997; Suller *et al*, 1997). The structure of these membrane potential dyes is shown in Figure 4c. Viable bacteria accumulate Rh123, which is a lipophilic cation, but non-viable bacteria do not. The extent to which bacterial cells take up Rh123 quantitatively reflects the extent of their viability. However, Rh123 is not readily taken up by gram negative organisms (Matsuyama, 1984; Kaprelyants and Kell, 1992), which require pre-treatment with EDTA to improve the uptake of Rh123, and allow the detection of antimicrobial agent effects on the membrane potential (Alvarez-Barrientos *et al*, 2000). However, after pre-treatment, the result may depend on the strain's susceptibility to either the EDTA, or the antibiotic, or both. There is a similar problem if the bacteria require washing before staining. Washing lengthens a so-called rapid method and there is a possibility of loss of bacterial cells, making standardization and commercial exploitation more difficult. Moreover, active cells can actually pump such dyes as Rh123 out (Jernaes and Steen, 1994). Additionally, some dyes are less tightly bound by energized membranes and more active cells may be stained less brightly.



bis (1,3-dibutyl-barbituric acid) trimethine oxonol

DiBAC$_4$(3)



3,3′-dipentyloxa-carbocyanine iodide

DiOC$_5$(3)

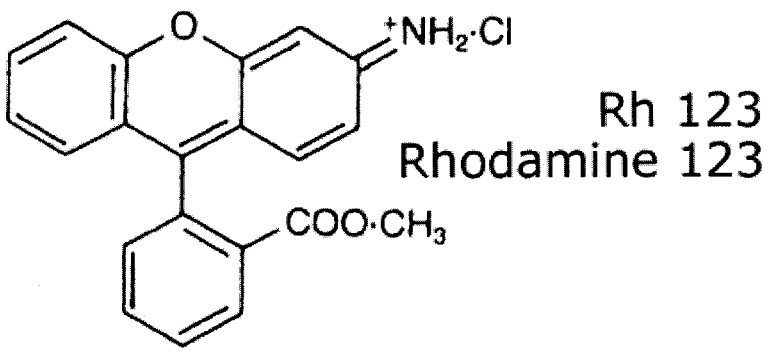


Figure 4c: Structure of i) $DiBAC_4(3)$ and ii) $DiOC_5(3)$ and iii) Rh123

The lipophilic Oxonal dyes are anionic and have the opposite effect of Rh123 (Ordonez and Wehman, 1993). The bis-(1,3-dibutyl-barbituric acid) trimethine oxonal ($DiBAC_4(3)$) has a high degree of voltage sensitivity (Braeuner *et al*, 1984) and enters depolarized cells where they bind lipid-rich intracellular macromolecules. Therefore, cells emitting fluorescence have a lower membrane potential and the greater the decrease in membrane potential, the higher the intensity of fluorescence (Kaprelyants and Kell, 1992; Davey *et al*, 1993; Diaper and Edwards, 1994; Lopez-Amoros, 1995; Mason *et al*, 1995).

Oxonol has been used to study the susceptibility of different genera of bacteria to different classes of antimicrobial agent. Suller *et al* (1997) studied the effect of penicillin, methicillin and vancomycin on bacterial cells. Mason *et al* (1984) used this dye to study membrane damage caused by gentamicin and ciprofloxacin. Jepras *et al* (1995) has also used the dye to study the susceptibility of *E. coli* on azithromycin, cefuroxime and ciprofloxacin. The major advantage of oxonol is versatility. It can be used directly in liquid culture of bacteria without the requirement of any pretreatment steps. Thus any perturbation in bacterial cells and interference with the effect of antibiotics is avoided. Moreover, unlike cationic dyes, oxonol is non-toxic to bacterial cells (Diaper *et al*, 1992).

(d) Advantages of FCM as a susceptibility test

An increasing number of clinical microbiology laboratories have installed automated systems for AST, which provide results in 2 to 7 hours instead of

overnight as with traditional culture methods. Although this increases laboratory costs, studies have shown that rapid identification and AST significantly lower mortality rates and shorten hospital stay thereby reducing hospital costs (Doern *et al*, 1994; Barangfanger and Short, 2001; Barangfanger, 2001). A reduction in the time taken for AST results from days to hours enables physicians to choose more quickly appropriate, narrower spectrum, and less costly antibiotics. The successful use of rapid mycobacterial AST (Moore, 1999) would lead to improvements in the care of patients with mycobacterium infection now that resistance to first line drugs is increasing. However, there are few automated ASTs for fastidious organisms, such as the *S. pneumoniae* panel of Vitek 2, which can provide results rapidly. Nevertheless, the initial investment for FCM is high. FCM doesn't rely on growth inhibition, but on rapid detection of antibiotic effects on bacterial cells measured by light scattered on fluorescent probes. Only a brief incubation time (several hours) is required before results are reported. The justification for developing FCM techniques for rapid susceptibility testing is strong, especially for fastidious organisms.

Standardized AST results are reported on the basis of zone size of growth inhibition or MIC. Unless the initial amount of bacterial inoculum is quantified, only the MIC, which indicates the bacteriostatic effect, is usually tested. The MBC, which may be more relevant for clinical decisions and indicates the bactericidal effect, is difficult to perform and rarely determined. FCM is a reliable

approach for AST, offering results in terms of bactericidal or bacteriostatic effect (Martinez *et al*, 1982; Gant *et al*, 1993; Pore, 1994; Durdie *et al*, 1995).

Moreover, neither MIC nor MBC determination take account of heterogeneity within the bacterial suspension and only produce average values which overlook effects related to cell cycle and physiological status (Kell *et al*, 1991). FCM could be useful in revealing numbers of resistant cells in heterogenous populations such as MRSA and VRSA. The exponential growth culture expresses a mixture of dissimilar morphological, physiological and reproductive properties. A major advantage of FCM as an AST is that it is possible with the use of selective fluorescent probes, to analyze the morphological and physiological properties of individual cells in non-synchronous culture (Kell *et al*, 1991).

Studies performed using FCM methods for assessing viability have shown this to be a very useful method for analysis of the interactions between bacteria and antimicrobial drugs. The effect of new or old antimicrobial agents on metabolic parameters such as membrane potential, cell size and amount of DNA, can easily be studied and provides another fruitful area for FCM methods (Steen and Boye, 1981; Pore, 1994).

Few studies have addressed the applicability of FCM to AST (Steen *et al*, 1982), or compared it with conventional AST method. Studies that have been performed using FCM for AST have concentrated on non-fastidious organisms (Steen *et al*,

1982; Martinez *et al*, 1982; Gant *et al*, 1993; Pore, 1994; Durdie, 1995; Walberg *et al*, 1997; Braga *et al*, 2003) and there are no published studies of FCM for AST of fastidious organisms such as *H. influenzae, S. pneumoniae, N. gonorrhoeae,* although work has been published on Mycobacteria (Moore et al, 1999). Development of FCM –AST method would be useful as there are still very few automated, rapid methods for susceptibility testing of fastidious organisms.

D) Standardization of Susceptibility Tests

Technical variations can have many effects on the results of AST (Brown and Blowers, 1978; Amsterdam, 1996; Andrews, 2004). Several variables must be strictly controlled when performing *in vitro* AST in order to obtain accurate and reproducible results. The NCCLS publishes standards for these and other tests on a continuing basis (NCCLS, 2000, 2003, 2004a, b). Some important facets of AST that have been standardized are discussed below.

i) Test Medium

The agar media used for SAD must meet the same requirements as the medium for disk diffusion test. The media should support growth of the test organism, enable reading of endpoint after incubation, and not contain inhibitors of antimicrobial activity. The NCCLS has suggested MH broth and agar for testing aerobic and facultative anaerobic bacterial isolates (NCCLS, 2003a, 2004a, b). A range of different supplements are necessary to support the growth of fastidious organisms. Defibrinated sheep blood (NCCLS, 2003a, 2004a) is commonly added for fastidious organisms such as viridans Streptococci. For *Haemophilus*

species, the CLSI has recommended Haemophilus test medium (HTM), which consists of MH agar plus NAD, yeast extract and haemin (Jorgensen *et al*, 1987). This medium must be prepared carefully to avoid batch to batch error. For *Neisseria* species, several supplements including lysed blood and chocolated blood and a complex defined supplement have been suggested (NCCLS, 2003a, 2004b). The working party of the BSAC recommended IST agar/broth (Oxoid) or DST (DST) Agar for susceptibility testing of rapidly growing, non-fastidious organisms (Andrews, 2004). Saponin-lysed horse blood (5%) is suggested as a supplement for nutritionally demanding species, and for the testing of sulphonamides or trimethoprim, with other supplements for some species. The suggested MH broth and agar most closely approximate the criteria for reproducible media. However, it should be noted that all the suggested media are not chemically defined. The CLSI has proposed a standard for evaluating MH agar (NCCLS, 1987), which attempts to minimize the variation among lots of MH agar. There is no global consensus on the media used and several media may be acceptable for AST.

ii) Inoculum

Inoculum preparation is the most important step in AST. The inoculum is usually prepared from a broth culture that has been incubated for 4 to 6 h, when growth is in the logarithmic phase. Inocula may also be prepared directly by inoculating fresh colonies grown overnight on an agar plate into saline or broth, though this may be result in a higher MICs. This direct inoculum suspension

preparation can be performed for bacteria that do not grow easily in broth (fastidious bacteria such as *S. pneumoniae, H. influenzae, N. gonorrhoeae*). When preparing the suspension, 4 to 5 colonies with similar appearance should be sampled to minimize variation in the bacterial population (NCCLS, 2003a, 2004a, b).

The density of bacteria tested must be standardized regardless of the method used. An inoculum that is too light may result in a decrease in MIC, and one that is too heavy may yield a significant increase. The effect of inoculum density is more important for the broth dilution test than for disk diffusion and agar dilution methods. The inoculum effect may be explained by the selection of resistant mutants or to drug breakdown by drug targeted inactivating enzymes (Chapman and Steigbigel, 1983). In the disk diffusion test, the inoculum is standardized by adjusting the density of the inoculum to equal the turbidity of a barium sulfate 0.5 McFarland turbidity standard (NCCLS, 2003). For the broth macrodilution method, a standardized suspension of tested bacteria is added to each dilution to obtain a final concentration of 5×10^5 CFU/mL. However, inocula of 10^4 CFU/spot, equivalent to 5×10^6 CFU/mL are used for SAD method. The working party of the BSAC also recommended that for testing sulphonamides, the inoculum must be reduced to 10^3 CFU/spot (Andrews, 2004). Additionally for testing antibiotics that are unstable to β-lactamase, a high inoculum gives more clinically relevant and reproducible results, so an inoculum of 10^6 CFU/mL is used for SAD of Staphylococci (Andrews, 2004).

For the broth microdilution method, the recommended inoculum is system dependent and varies from 2 x 10^4 to 1 x 10^6 CFU/mL (Amsterdam, 1996). The inoculum required for E test of aerobes and anaerobes is an emulsification of well isolated colonies in a suitable suspension to achieve a concentration of 1.5 x 10^8 CFU/mL as described in the CLSI standard (NCCLS, 2003a, 2004b; Bolmstrom *et al*, 1988). The preliminary user guide of the SGE method (Schalkowsky, 1985) recommended the test culture to be deposited on the agar surface at a concentration of about 10^8 CFU/mL. This recommended bacterial concentration derives from the fact that this surface density of depositing bacteria is about the same as that produced when following the recommendation of the CLSI in performing SAD. Paton *et al* (1990) assessed the effect of variation of the inoculum size in SGE test for non-fastidious aerobic organisms and recommended an overnight broth of 10^9 CFU/mL.

It is imperative that the inoculum suspension be properly standardized to the appropriate turbidity standard. The most widely used method of inoculum standardization involves McFarland turbidity standards, which are prepared by adding specific volumes of 1% sulfuric acid and 1.175% barium chloride to obtain a barium sulfate solution with a very specific optical density. The most commonly used is McFarland 0.5 standard which is prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 0.36N H_2SO_4, providing a turbidity equivalent to a bacterial suspension containing 1.5 x 10^8 CFU/mL. Further adjustment to the

inoculum depends on the type of test employed. Alternatively a nephelometer or spectrophotometer may be used for turbidity adjustment. The equivalent of the 0.5 McFarland standard, using a 1 cm path, with an absorbance at 625 nm wavelength should be 0.08 to 0.10 (Woods and Washington, 1995).

iii) Inoculation Conditions

NCCLS suggest 35°C as the mean incubation temperature and all AST should be carried out at this temperature (NCCLS, 2003a, b, 2004a, b). However, the Working Party of the BSAC recommends an incubation temperature within the range 35 - 37°C (Andrews, 2004). Lower temperatures should be avoided since the rate of growth for most bacteria would be decreased, and in the disk diffusion tests, antimicrobial agents would diffuse more slowly and inhibition zone become larger (Acar and Goldstein, 1996).

In disk diffusion tests, the antimicrobial disks should be applied to the agar plate no longer than 15 minutes before incubation (Woods and Washington, 1995). More than 15 minutes delay leads to excess pre-diffusion of antimicrobial agent and decrease in inhibition zone (Woods and Washington, 1995). Influence of pre-diffusion in E test was evaluated by Bolmstrom *et al* (1988). The E tests strips were applied onto the agar surface and allowed to pre-diffuse at 22°C for 0 to 6 h before inoculation. It was found that identical MICs were obtained throughout the varying pre-diffusion period, showing rapid stabilization of antibiotic concentration gradients in the agar in E test and that the gradient was maintained

for at least 6 hours after application of the strips (Bolmstrom *et al*, 1988). In the manufacturer's reference data of the SGE method (Schalkowsky *et al*, 1985), it was suggested the antimicrobial agent was deposited one day or at least several hours prior to application of the bacterial culture in order to maximize the useable test region on the plate and to stabilize the concentration gradient due to vertical stabilization. Paton *et al* (1990) assessed the effects of varying time of inoculation of organisms after antibiotic deposition in the SGE method. The result, based on testing of non-fastidious organism on DST agar, indicated that the time of inoculation had little effect from 1 h up to 24 h. Hill and Schalkowsky (1990) studied the effect of differences in the interval between deposition of the antibiotics and the inoculation of strains in the susceptibility of gram negative anaerobes, and found that the diffusion interval could be set at any time span from 0.75 h to 8 h without significantly affecting the MIC (Schalkowsky *et al*, 1985). The effect of varying time of inoculation after deposition of antibiotic has not been investigated for AST by SGE for fastidious bacteria.

For susceptibility testing of non-fastidious bacteria, agar or broth should be incubated in an ambient incubator. A CO_2 incubator should not be used since the carbonic acid formed on the agar surface or in the broth can cause a decrease in pH, affecting the activity of antimicrobial agents such as aminoglycosides, macrolides, and tetracycline (Ericsson and Sherris, 1971). However, incubation in an atmosphere of increased CO_2 for 18 to 24 hours is recommended for testing

some fastidious bacteria (NCCLS, 2004a, b; Andrews 2004), but effects on interpretation of results is not routinely considered.

iv) Agar Depth

In the disk diffusion test, agar plates must have a depth of 3 to 5 mm (NCCLS, 2003). Thinner plates will result in larger zones of inhibition, thicker plates having the opposite effect. For E test, a plate with an agar depth of 4.0 ± 0.5 mm should be used (Bolmstrom *et al*, 1989). In the SGE method, the deposition factor (DF) at any radial advanced (RA) (refer figure 3), divided by the agar depth (H) defines the average amount of stock solution, dispersed per unit volume in the column of agar below the point of measurement (Schalkowsky, 1985). The DF/H ratio multiplied by the concentration of antibiotic in the stock solution gives the concentration of antibiotic at any point in the agar. For the same stock solution, the average concentration of antibiotic at any point in the agar is inversely proportion to the agar depth of the plate. Therefore, with thicker plates, the radial advance (RA) would be expected to decrease, whilst with thinner plate, the RA would increase. Paton *et al* (1990) has studied the effect of varying the volume of agar in a 10 cm plate (16, 20 and 30 ml) for AST of non-fastidious bacteria. It was found that a reduction in calculated MICs was obtained with increasing volume of agar although they were within a two-fold dilution step.

v) Antimicrobial Agents

Reference antimicrobial agents for use in dilution tests should be obtained from the manufacturer. A uniform code list based on generic names for labelling antimicrobial disks has been published (WHO, 1982). When preparing antibiotic stock solutions, it is necessary that the powdered material is properly dissolved. As some antimicrobials do not dissolve in water, some non-aqueous solvents are commonly used (NCCLS, 2004b). The amount of powder weighed must be adjusted for the activity of each lot (NCCLS, 2004b).

Most antimicrobial agents are temperature-labile and should be handled according to the manufacturer's recommendation. Many antibiotics are more stable at temperatures below –20°C. Stock solution should be stored at –20°C or preferably at –70°C. Moreove,r once the stock solutions are thawed, they must never be refrozen (NCCLS, 2004b). Antibiotic powders, disks or solutions should not be stored in a frost-free freezer as there are continual heating and cooling cycles (NCCLS, 2004b).

Cartridges of antibiotic disks should be stored at –20°C or lower in a container with desiccants. A working supply of disks can be stored at 2°C to 8°C for up to a week (NCCLS, 2003a). Unopened cartridges of disks should be allowed to equilibrate to room temperature before opening. For E test strips, all unopened packages should be stored at –20°C until the given expiration date (Bolmstrom, 1989). E test strips should be protected at all times from moisture, heat and direct strong light. After removal from the freezer, the package should be allowed to

equilibrate to room temperature for 20 minutes and moisture condensing on the outer surface must be allowed to evaporate before opening (AB Biodisk, 1992).

vi) Reading Endpoint

In the disk diffusion test, plates are examined from the agar side while resting on or held 2 to 3 inches above a black non-reflecting surface, and the plates should be illuminated with reflecting light (Barry *et al*, 1979). Faint growth of tiny colonies at the zone edge is ignored, and any colonies within an otherwise clear zone should be retested to determine if this represents a small resistant subpopulation (Acar and Goldstein, 1996). In disk diffusion testing of enterococci with vancomycin, and staphylococci with methicillin, transmitted light is used for examination. Any evidence of growth within a zone of inhibition is considered significant and the isolate should be reported as resistant (NCCLS, 2003a).

In SAD tests, the use of a magnifying glass to detect the endpoint is not recommended (Acar and Goldstein, 1996). Broth dilution method, especially broth microdilution method may not produce a clear cut MIC endpoint, due to growth in the tubes or wells demonstrating trailing or skipped wells. Trailing appears as small buttons of growth that persists through several dilutions in broth dilution tests or a haze in the SAD test. This commonly occurs with sulfonamides, trimethoprim, and trimethoprim/sulfamethoxazale. In this case, the endpoint is read as 80% reduction in growth or a button 2 mm or less in diameter is disregarded in broth microdilution method (Ferguson and Weissfield, 1984;

NCCLS, 2000). If there is a single agent with skipped wells, the test should be repeated. However, when multiple drugs exhibit skipped wells, this may represent a mixed culture or a resistant subpopulation, and the test should be repeated (NCCLS, 2004b).

In E test, the MIC of the isolate corresponds to the point of intersection between the zone edge of the culture ellipse and the E test strips. In the presence of a dip-effect, the MIC should be the intersection on the strip after extrapolation. When there are microcolonies within original inhibition zone, the intersection of the strip with the most resistant microcolonies should be read as the final MIC. For SGE test, problems with reading are more explicit because of the greater detail shown by the continuous gradient in the growth transition region. While sharp transitions are common with aerobes, growth tailing, extending over a range of one or more twofold dilutions, are frequent with anaerobes. Tailing may also be encountered for susceptibility to biostatic drugs. In the absence of tailing transition from growth to no growth, MIC can readily be obtained after measurement of corresponding RA before growth has appeared (Schalkowsky, 1994). A twofold MIC value, corresponding to that obtained from dilution test can readily be obtained by taking the adjacent twofold value above the minimum activity concentration – the point where abrupt change of confluent growth occurs (Schalkowsky, 1994). For organisms and antimicrobial agent with growth trailing, the radius where the nature of growth changes (tail beginning radius) and the radius at which growth ends (tail ending radius) are both measured and should be

used for calculation of the corresponding MIC concentration. Since, the drug concentration at which the time kill has a zero slope is at a drug concentration greater than the end of the tail, the MIC should be based on the highest twofold concentration showing growth trailing. This is also applied for the testing of fastidious organisms. There is an option for the computer program provided with the revised SGE method to use the tail beginning concentrations as endpoints for analysis (Wexler *et al*, 1996), and these endpoints are used when they are consistent with the CLSI recommendations (CLSI, 2005a) for reading substantial reduction or barely visible hazes.

E) Modified AST Methods For Fastidious Organisms

As stated above, some clinically significant bacteria have characteristics that preclude their being tested by standard methods. In the past, many of these organisms did not require susceptibility testing since they were known to be consistently susceptible to some antimicrobial agents. However, more recently resistant strains have emerged and have produced challenges for the clinician because previously employed empirical therapy has become inadequate. AST for these fastidious organisms is required to ensure successful therapy.

i) *Haemophilus influenzae*

a) Resistance pattern and mechanism of resistance

β-lactamase mediated ampicillin resistance in *H. influenzae,* first reported in 1974 (Khan *et al*, 1974; Tomeh *et al*, 1974), has become increasingly more prevalent

(Beekmann *et al*, 2005). Currently, 5% to 40% of *H. influenzae* isolates produce TEM-1 or ROB-1-type β-lactamase and, as a result, are resistant to ampicillin and amoxicillin (Sahm *et al*, 2000; Biedenhach *et al*, 2001; Hoban *et al*, 2001; Beekmann *et al*, 2005). The prevalence of β-lactamase-mediated ampicillin resistance among non-type b strains continued to increase and reached levels of 36.4% by the mid 1990's (Doern *et al*, 1997). Rates of β-lactamase mediated ampicillin resistance are two to four-fold higher among encapsulated type b strains. However, infections due to encapsulated type b *Haemophilus* strains began to fall after introducing a protein-conjugated capsular antigen vaccine (Black *et al*, 1992). In 2004, after extensive vaccination against *H. influenza*e type b, ampicillin and cotrimoxazole resistance occurred at a frequency of 24.2% and 48.4% respectively (Campos *et al*, 2004). However, in countries not using the vaccine, such as India, *H. influenza*e type b infections are still an important cause of morbidity and mortality in children with 97% of *H. influenzae* infections caused by type b. Until recently these infections were caused by strains resistant to at least one drug, and 32% were resistant to three drugs. (Invasive Bacterial Infections Surveillance Group, 2002). In addition, serogroup a infections are now being reported (Adderson *et al*, 2001). In Hong Kong, where Hib vaccination is not used, 23% of *H. influenzae* were reported to be β-lactamase-producing (Felmingham, 2004).

In 1980, isolates of *H. influenzae,* resistant to ampicillin and amoxicillin by mechanisms other than production of β-lactamase, were described (Bell and

Plowman, 1980; Markowitz, 1980). The mechanism of resistance in this case was caused by elaboration of altered penicillin-binding proteins, (PBP) (Mendelman *et al*, 1984; Clairoux *et al*, 1992). These strains, referred to as β-lactamase negative but ampicillin intermediate or resistant (BLNAR), Although remaining uncommon in Hong Kong, represented 21.3% of isolates in Japan in 2002 (Hasegawa *et al*, 2004) and 30% in France (Dabernat *et al* 2003). These isolates are less susceptible to narrow spectrum cephalosporins and amoxicillin-clavulanic acid than ampicillin susceptible or ampicillin resistant β-lactamase producing isolates (Jorgensen, 1988). Screening by cefixime or cefpodoxime may be useful, with strains having an MIC of 0.25 ug/ml likely to be BLNAR. Recently, Barry *et al* (2001) concluded there should be a universal definition, and identification method for BLNAR strains.

Cephalosporin resistance in *H. influenzae* is uncommon except for narrow spectrum cephalosporins such as cefaclor, cephalothin, cefazolin and cephalexin. In large scale surveillance studies, the combined percentages of strains intermediate and resistant to cefaclor and cefprozil were 16.3% to 29.8% (Doern *et al*, 1997). Four to six percent of strains were resistant to cefuroxime (Doern *et al*, 1999; Felmingham and Gruneberg, 2000; Sahm *et al*, 2000; Biedenbach *et al*, 2001). Cefixime and cefpodoxime were the most active cephalosporins and the frequencies of resistance were 0.1% and 0.3% respectively. Reports of strains which were β-lactamase positive, but were resistant to amoxicillin-clavulanate

(BLPACR) (Doern *et al*, 1997), were later refuted by Jacobs and Bajaksouzian (1997)

As a result of the production of the inactivating enzyme chloramphenicol acetyltransferase (CAT), resistance occurs to chloramphenicol (Doern *et al*, 1987). Resistance has also been reported to macrolides, tetracycline, trimethoprim/sulfamethoxazole and rifampin. In the U.S.A., 0.5-1.9% of strains were resistant to macrolides (Doern *et al*, 1997), and more recently, resistance to TMP-SMX was 10.0% to 15% and is still increasing (Biedenbach *et al*, 2001; Blondeau *et al*, 2001). Resistance to chloramphenicol, tetracycline, and rifampin was less than 2%. (Felmingham and Gruneberg, 2000; Biedenbach *et al*, 2001; Blondeau *et al*, 2001), and *H. influenzae* remains susceptible to levofloxacin (Felmingham, 2004). In Hong Kong, the percentage of quinolone resistance was about 0.9% among young children in 2000 (Ho *et al*, 2004).

b) Susceptibility test methods

In vitro test methods have been standardized for AST of *H. influenzae* and these involve slight modifications of the routine disk diffusion and broth dilution MIC procedures (NCCLS, 2003a, b;2004a, b). Before 1990, the CLSI recommended the use of Chocolate MH agar for disk dilution tests and MH broth supplemented with 3-5% lysed horse blood for dilution tests (NCCLS, 1985). This resulted in a test medium which was opaque and interpretation of results was difficult. In 1987, a simplified medium known as HTM (Haemophilus test medium) was developed (Jorgensen *et al.* 1987). Commercially available HTM is a better medium since it

is optically clear, stable, and reproducible from lot to lot. The broth version of the medium is cation adjusted and contains 0.12 IU of thymidine phosphorylase per ml. This medium was adopted by the CLSI for broth dilution and disk diffusion susceptibility testing of *H. influenzae* (NCCLS, 2003b, 2004b). The Working Party of the BSAC suggested the use of ISA agar supplemented with 5% v/v of lysed horse blood and 20mg/L NAD as the optimum medium for AST of *Haemophilus* species (Andrews, 2004).

In a recent study (Reynolds *et al*, 2003), 936 *H. influenzae* strains were tested by the BSAC agar dilution test (Andrews, 2001, 2002) and the CLSI broth microdilution method (NCCLS, 2004b). The MIC results were compared between the two methods. Most of the organism-agent combinations showed excellent MIC agreement (>90%) within one double dilution except cefaclor and trimethoprim with only in 57.6 and 63.9% agreement respectively. Difference in breakpoints largely explained the major discrepancies with cefaclor and cefuroxime, which occurred at a rate of 95.6 and 18.5% respectively.

A β-lactamase test should be performed on all isolates as a rapid and reliable means of assessing the susceptibility to ampicillin and amoxicillin. A positive β-lactamase test result implies resistance to ampicillin, but a negative result does not indicate the isolate is susceptible (Hindler and Swenson, 2004). For disk diffusion tests of *H. influenzae*, colonies grown for 16 to 24 hours are directly suspended in MH broth to obtain a turbidity of McFarland 0.5 standard (NCCLS,

2003). Since the number of bacteria in the inoculum is critical for a reliable result, a spectrophotometric device should be used for inoculum standardization. The HTM test plates are inoculated as for standard disk diffusion and incubated in 5% to 7% CO_2 at 35°C for 16 to 18 hours (NCCLS, 2003b). There is a zone-interpretative table specific for *H. influenzae* provided by CLSI (CLSI, 2005b).

The standard NCCLS MIC testing method is the microdilution method in HTM broth (NCCLS, 2004b). The inoculum requirement is the same as for the disk test and incubation is at 35°C for 20 to 24 hours. However, the incubation atmosphere is in ambient air (NCCLS, 2004b). Guidelines for interpretation of MIC and QC results with the *H. influenzae* reference strain and suggested by NCCLS are shown in Table 2.

CLSI standard M7-A6 (NCCLS, 2004b) recommended that in SAD tests of *H. influenzae,* plates should be incubated 20-24 hours at 35°C in 5% CO_2. For bacteriostatic drugs such as SXT, the MIC is recorded as the lowest drug concentration that inhibits 80% of the test isolate growth. The SAD method using MH agar supplemented has been evaluated and the results compared to a reference microdilution procedure that utilized HTM broth (Giger *et al*, 1996). The MICs obtained by SAD were the same or 1 to 2 dilutions lower than MICs obtained with the reference HTM broth microdilution. Moreover, SAD on MH-LHB correctly identified all of the BLNAR isolates included in the study. The SAD showed good agreement with reference broth microdilution MICs except for

cefaclor and SXT. The poor agreement with SXT may be due to the difficulty of reading the 80% MIC endpoint on the agar surface (Giger *et al*, 1996).

Several commercial systems for quantitative AST of *H. influenzae* including the Vitek Haemophilus susceptibility cards, E test using HTM and Chocolate MH agar plate, MicroMedia and MicroScan have been developed and evaluated (Giger *et al*, 1996). All systems failed to detect one of the nine BLNAR strains, either because of growth failure in MicroScan and MicroMedia, or failure to categorize the strains as ampicillin resistant in the Vitek and E test (Giger *et al*, 1996). Moreover, none of the commercial systems could correctly identify all 6 amoxicillin/clavulanic acid resistant strains categorized by the standard microdilution method. It was also found that growth of most isolates was poor in MicroMedia and MicroScan and 4-5% of the isolates did not growth at all (Giger *et al*, 1996). The MicroScan and Vitek system did not correlate well with the reference method with very major errors of 11.9% and 8.1%, major error of 0%, and minor errors of 8.1% and 3.1% respectively for the susceptibility testing of ampicillin and cefuroxime. The MicroMedia method appears to be a better method for susceptibility testing of *H. influenzae* with very major errors of 1.7%, a major error rate of 0% and minor error rate of 4.8% for susceptibility testing of ampicillin and cefuroxime. However, there is limited data for the MicroMedia system since there is no panel for testing of ampicillin/clavulanic acid and SXT, and there is only a breakpoint dilution of cefaclor. Nevertheless, MicroTech and Sensititre have been cleared by the FDA for the use of commercial systems for AST *of H. influenzae.*

Jorgensen *et al* (1991) has evaluated the E test for AST of *H. influenzae* using both HTM and chocolate agar. E test MICs for a total of 10 antimicrobial agents were compared with standard broth microdilution MICs. E test MICs were easily interpreted for most of the drugs with either of the agar media (Jorgensen *et al*, 1991). The overall agreement between E test MICs measured on HTM agar and standard microdilution MICs was 89.8%. The highest agreement occurred with erythromycin tests (99%) and the lowest agreements with trimethoprim-sulfamethoxazole (67.7%) and ampicillin (76.5%) test. The overall agreement with standard dilution method was 81.4% (Jorgensen et al, 1991). Giger *et al* (1996) reported E test HTM and E test Choc-MH MICs agreed most closely with HTM broth microdilution test when compared with the other commercial systems. For the 8 drugs being tested, the overall agreement for E test HTM and E test Choc-MH were 79.2% and 79% respectively (Giger *et al*, 1996). E test HTM agreed most closely with the reference method with the exception of amoxicillin/clavulanic acid and appears to be an alternative to the standard microdilution method for AST of *H. influenzae*.

ii) Neisseria gonorrhoeae

a) Resistance pattern and mechanism of resistance

N. gonorrhoeae has developed resistance to most agents that have been recommended for therapy. Penicillin was the therapeutic choice for *N.*

gonorrhoeae infection until penicillin resistance began to increase significantly in the mid 1970s. Antimicrobial resistance in *N. gonorrhoeae* may be mediated by R-factor plasmid or multiple chromosomal mutations. Plasmid mediated resistance to penicillin is due to the production of a plasmid-associated TEM-1 type β-lactamase (Elwell *et al*, 1977) and the strains are called penicillinase-producing *N. gonorrhoeae* (PPNG). PPNG are now disseminated worldwide and plasmids of different sizes have been reported (Embden *et al*, 1985; Gouly *et al*, 1986; Brett, 1989), but all are related to the original plasmid and encode for TEM-1 β-lactamase. PPNG express high levels of resistance to penicillin, ampicillin and amoxicillin, but remain susceptible to most other beta-lactams such as cephalosporins, imipenem, and β-lactamase inhibitor combinations (Doern, 1995).

Chromosomal resistance to penicillin is low-level and due to the additive effects of mutations at multiple loci encoding for PBP-2. The result of mutation is a change in a single amino acid in PBP-2, leading to decreased affinity for penicillin (Spratt, 1988). Chromosomal-mediated resistance to penicillin may also be caused by diminished outer membrane permeability (Dougherty *et al*, 1980). These isolates are termed chromosomally-mediated resistant *N. gonorrhoeae* (CMRNG). CMRNG have reduced susceptibility to cephalosporins, such as cefotaxime, ceftriaxone and cefuroxame, and resistance appears to be conferred by mutations in the same loci (Ison *et al*, 1990). CMRNG also express a low-level resistance to tetracycline, erythromycin, aminoglycosides, and TMP-SMX. The Gonococcal Antimicrobial Surveillance Project (GASP) showed that 15% of

isolates were PPNG and 15.4% were CMRNG in 1993 in USA. PPNG has decreased to 2.1% in 1999; and CMRNG to 19.2% in Canada in 1999 (Ng *et al*, 2003). In China, PNPG has increased from 2% to 21.8% between 1996 to 2001 (Zheng *et al*, 2003). For management of sexually transmitted diseases, the WHO changed their recommendation for first line therapy from penicillin to spectinomycin, ceftriaxone or ciprofloxacin unless the gonococcal isolates were proven to be susceptible to penicillin (WHO/UNAIDS 1999)

In 1985, high level plasmid-mediated tetracycline resistance in *N. gonorrhoeae* (TRNG) was first reported (Morse *et al*, 1986). It is due to the acquisition of the *tet M* determinant by the conjugative plasmid. The plasmid can move between other genera and gonococci, and is self-mobilisable, thus TRNG are spread more quickly than the PPNG because *tetM* may be found in other flora found in the urinary test. The overall proportion of isolates with penicillin or tetracycline resistance in 1999 was 28.1% in USA (CDC, 2000). Selection of resistance may also occur by the use of tetracycline for Chlamydial infection. Other types of resistance in *N. gonorrhoeae* occur infrequently in USA including resistance to fluoroquinolone, extended-spectrum cepholosporins, and spectinomycin (Birley *et al*, 1994; CDC, 1995; Tapsall *et al*, 1995). However, fluoroquinolone resistance has become widespread throughout Asia (Tanaka, 2000). In a recent study of 115 isolates in sex workers in the Philippines, the MICs of ciprofloxacin for 49% of isolates were $\geqq$4 ug/ml, with 63%, having MICs $\geqq$1 ug/ml (De Los Reyes *et al*, 2001). Although decreased susceptibility to ceftriaxone (MIC 0.06 μg/mL) has

been reported in both China and Australia (Zheng, 2003; AGSP, 2003) it remains the drug of choice in Hong Kong and resistance has not been reported (Ling *et al*, 2003).

c) Susceptibility test method

PPNG can be reliably detected by β-lactamase tests such as the chromogenic nitrocefin test (O' Callaghan *et al*, 1972; Livermore, 2001) but CMRNG cannot be detected by this method. Consequently, conventional susceptibility methods must be employed to detect other CMRNG strains. A standardized laboratory method to determine the susceptibility of *N. gonorrhoeae* was established and has been recommended by NCCLS based upon a multicenter study to standardize SAD and disc diffusion tests (Jones, 1989; NCCLS, 2003a,; King, 2001). SAD is preferred to broth dilution as the reference method to determine MICs for *N. gonorrhoeae* since gonococci tend to lyse in broth medium (Hindler and Swenson, 2004). The NCCLS recommended test medium for both disc diffusion and agar dilution test is GC agar base with a defined supplement which is basically IsoVitaleX with a much lower concentration of cysteine. A lower concentration of cysteine is required to avoid inhibition of carbapenem and clavulanate activities (Jones, 1989). The NCCLS recommends the use of GC agar with 1% supplement for AST of *N. gonorrhoeae*, whereas the Working Party of the BSAC recommends the use of DST or IST agar supplemented with 5% LHB in addition to 1% IsoVitaleX (Andrews, 2001). The composition of the test medium and supplement does not affect the susceptibility results of antimicrobial agents such

as penicillin, spectinomycin, and cephalosporin. However, supplemented GC agar may give discrepant results compared with DST supplemented with LHB and IsoVitaleX for erythromycin and tetracycline (Dillon *et al*, 1987). Inocula for testing are prepared using the direct inoculum method, and tests are incubated for 20 to 24 h in an atmosphere of 5% to 7% CO_2 (NCCLS, 2003a, b; 2004b). For standardization between laboratories, the inoculum for SAD MIC test should be 10^4 CPU/Spot. The listing of antimicrobial agents for which standardization methods are applicable, interpretative criteria for results and QC range are shown in Table 3. The QC strain used for *N. gonorrhoeae* is ATCC 49226.

Chocolate MH agar can also be used for disk diffusion with *N. gonorrhoeae* in addition to supplemented GC agar. Interpretative criteria for disk diffusion results have been promulgated for penicillin, tetracycline, spectinomycin, and cefoxitin (Ison, 1996). Agar incorporation technique can be used to categorise isolates into susceptible or resistant using the breakpoint technique where only one or two concentrations of antimicrobial agent are used (Ison *et al*, 1991). However, the concentrations of antimicrobial agent used for breakpoint should be used in combination with the medium since sensitivity test agar generally give an endpoint one dilution lower than GC agar base (Ison, 1996). MICs can be determined with the E test with either Choc-MHA or GC agar base supplemented with 1% defined supplement as test medium. The E test has proven useful in measuring gonococcal susceptibility to a variety of antibiotics including penicillin, ciprofloxacin, chloramphenical, tetracycline, erythromycin, SXT, and other

cephalosporins. The total quantitative accuracy (± 1 $\log_2$ dilution step) obtained between the E test and the agar dilution results on GC agar were 87% to 98%. It was also found that the E test MICs were generally the same or one to twofold dilution lower than the MICs generated by the SAD method for penicillin and tetracycline (Coole *et al*, 1993; Yeung *et al*, 1993; Van Dyck. 1994; Daly *et al*, 1997). In addition, there were also some errors in susceptibility interpretative categories for the E test when compared with reference agar dilution methods.

Ceftriaxone is currently the drug of choice for treating uncomplicated gonorrhoea and, as high level resistance to this drug has not been reported, AST is not usually required for therapy except in cases when ceftriaxone cannot be used or suspected treatment failure. However, AST is extensively performed for surveillance. The worldwide emergence of plasmid-mediated and chromosomal resistance emphasises the importance of antimicrobial susceptibility surveillance of gonococcal isolates. A large number of consecutive isolates needs be tested to detect resistance. It is more informative to determine the pattern of susceptibility over time so that the likelihood of cure by empirical therapy can be predicted.

iii) Moraxella catarrhalis

a) Resistance pattern and mechanism of resistance

Most clinical isolates (>90%) of *M. catarrhalis* produce a beta-lactamase (Doern *et al*, 1999; Felmingham and Gruneberg, 2000; Sahm *et al*, 2000; Durbar, 2003;

Beekmann, 2005). The β-lactamase is chromosomal, constitutively produced in small amounts and is inhibited by sulbactam and clavulanate (Doern, 1986; Wallace *et al*, 1990). Up to 6 or 7 different β-lactamases, based on IEP patterns have been demonstrated (Labia, 1986). There are two principal types of β-lactamase produced by *M. catarrhalis*: the BRO-1 and BRO-2 (Wallace *et al*, 1990). The amount of BRO-1 enzyme produced is much greater than BRO-2 enzyme. BRO-1 type β-lactamase-producing strains thus have high MICs to β-lactam antimicrobial agents such as penicillin and ampicillin. However, BRO-2 enzyme producing strains typically have a low MIC indicating susceptibility to β-lactam agents (Doern, 1986). Patients infected with strains which produce BRO-2 type β-lactamase, have been reported to respond to therapy with penicillin, ampicillin or amoxicillin. However, BRO-1 producing *M. catarrhalis* strains are 10 times more prevalent that BRO-2 enzyme producing strains. A third enzyme BRO-3 has been described (Christensen *et al*, 1991). Resistance to alternative therapeutic drugs is rare (Felmingham, 2004), and tetracycline and macrolides are usually active against *M. catarrhalis*, but some resistant strains have been reported (Brown *et al*, 1989). Resistance to all antibiotics other than penicillin remains at less than 1% except for trimethoprim-sulfamethoxazole (1.5%) (Zhanel *et al*, 2003). First generation oral cephalosporins such as cephalexin, cephradine and cefadroxil should not be used for therapy of *M. catarrhalis* infection because they have a lower inherent potency against the bacteria, and they may also be hydrolysed by the enzyme produced by this species. However, *M. catarrhalis* is typically susceptible to other cephalosporins, amoxicillin-clavulanate, ampicillin-

sulbactam and imipenem (Doern *et al*, 1999; Felmingham and Gruneberg, 2000; Sahm *et al*, 2000; Biedenhach *et al*, 2001; Hoban *et al*, 2001; Beekmann, 2005).

b) Susceptibility test methods

AST of *M. catarrhalis* may be performed by disk diffusion and dilution procedures recommended by the CLSI for non-fastidious bacteria (NCCLS, 2003a, 2004b) since it grows readily on unsupplemented MH medium at 35^0 C to 37^0C in ambient atmosphere. The CLSI method has been reported as suitable for testing with *M. catarrhalis* for more than 21 antimicrobial agents including penicillin, ampicillin, amoxicillin-clavulanate, cephalothin, cefaclor, cefuroxime, erythromycin, tetracycline, chloramphenicol, and TMP-SMX (Traub and Leonhard, 1997). HTM and MH broth supplemented with LHB has been used for MIC determinations of *M. catarrhalis* by microdilution method (Doern and Vautour, 1992; Barry *et al*, 1993). It is convenient to use media other than that recommended by the CLSI for AST of *M. catarrhalis* since it is preferable to use a single medium for testing of all fastidious respiratory pathogens. This is justified as previous studies have documented essential equivalence between MIC results obtained with HTM versus MH broth when *M. catarrhalis* was tested (Barry *et al*, 1993a).

It is recommended that clinical isolates of *M. catarrhalis* be tested with the nitrocefin-based chromogenic cephalosporin β-lactamase assay (Doern, 1995; Livermore, 2001). Almost one third of β-lactamase producing strains have an MIC that is susceptible for penicillin and ampicillin according to the NCCLS

breakpoint definition (Doern and Tubert, 1987). There are a number of case reports of treatment failure with infection due to β-lactamase producing strains of *M. catarrhalis* that were treated with ampicillin, penicillin, or amoxicillin (Ninane *et al*, 1978; Motte *et al*, 1987; Bourgeois *et al,* 1993). Thus, all β-lactamase producing strains of *M. catarrhalis* should be considered resistant to penicillin, ampicillin and amoxicillin after screening by the nitrocefin based chromogenic method for β-lactamase regardless of the MICs (Walllace *et al*, 1990).

For surveillance purposes, it is justifiable to perform routine susceptibility testing of TMP-SMX combination for *M. catarrhalis*. However, the prevalence of strains resistant to erythromycin and tetracycline remains low, thus routine susceptibility testing of *M. catarrhalis* against these drugs is not recommended (Felmingham, 2004). Other antibiotics are either uniformly active or inactive against *M. catarrhalis,* and do not need to be tested (Durbar, 2003).

iv) ***Streptococcus pneumoniae***

a) Susceptibility pattern and mechanism of resistance

Streptococcus pneumoniae with reduced susceptibility to penicillin were first reported in 1967, and strains with high levels of resistance to penicillin and other antimicrobial agents have been recognized since 1977 (Klugman, 1990). Since these early reports, penicillin resistance in pneumococcal strains has been encountered with increasing frequency around the world (Baquero, 1995;

Karlowsky *et al*, 2003; Canton *et al*, 2003; Felmingham, 2004). 28.9% of Hong Kong strains showed reduced penicillin susceptibility in 1995 including 19.6% with frank penicillin resistance (Kam *et al*, 1995). These rates have continued to increase to more than 60% (Ip *et al*, 2001; Ho *et al*, 2001; Boost *et al* 2001). The rates for strains not susceptible to penicillin elsewhere in the world also exceed 50% (Whitney *et al*, 2000; Blondeau and Tilloston, 2001; Felmingham, 2004). The mechanism of resistance to penicillin is alteration of targets of penicillin action, that is, PBPs. This can cause increased resistance to other β-lactams (Caputo *et al*, 1993), and the activities of ampicillin, amoxicillin, the β-lactamase inhibitor combinations, and cephalosporins like cefotaxime, ceftriaxone and carbapenems against these strains are also diminished (Linares *et al*, 1992).MIC to amoxicillin may be lower than to penicillin, which has led to recommendations for the use of augmentin (amoxicillin/clavulanate) for treatment of community-acquired pneumococcal infections. When such β -lactam and β - lactamase inhibitor combinations are used, the active component for pneumococci resides solely in the β -lactam, the inhibitor being useful for empiric treatment of *Haemophilus* or *M. catarrhalis*. While cephalosporin resistance is more common in strains highly resistant to penicillin than in intermediately resistant strains, some strains intermediately-resistant to penicillin may be highly resistant to newer cephalosporins (Jorgensen, 1994). With the exception of three cephalosporins (ceftazidime, cefixime and ceftibuten) with limited intrinsic activities against *S. pneumoniae*, all of the beta-lactams described are uniformly active against penicillin-susceptible pneumococci.

Penicillin-resistant pneumococci are frequently resistant to non-β-lactam antibiotics as first reported in South Africa (Appelbaum *et al*, 1977). Such strains have elevated MIC values for trimethoprim-sulfamethoxazole and variable susceptibility for erythromycin, tetracycline, chloramphenicol and fluoroquinolones (McDougal *et al*, 1992; Farrell, 2004; Felmingham, 2004). However, other than in Hong Kong, Toronto, and Spain, quinolone resistance remains low in most other countries. Trimethoprim-sulfamethoxazole resistance has become more prevalent and now occurs at rates of up to 20-36% in the United States (Whitney *et al*, 2000; Hoban *et al*, 2001), though levels in Asia exceed 70% (Ho *et al,* 2001; Boost *et al*, 2001; Felmingham, 2004.) Erythromycin resistance, often linked to resistance to the tetracyclines and chloramphenicol, tends to occur with penicillin resistance in pneumococci. Erythromycin resistance, which may induce penicillin resistance, is also high with levels in Europe up to 60% (Felmingham, 2004) and around 25% in USA (Doern *et al*, 2001). Resistance levels to macrolides exceeding 80% to 90% have been reported in Asia (Blondeau and Tillotson, 2001). A CDC survey defined multidrug-resistant (MDR) strains as those strains resistant to at least three antimicrobial agents (Breiman *et al*, 1994) - a definition that applies to most penicillin-resistant strains of pneumococci. In the research conducted in Hong Kong (Kam *et al*, 1995; Ip *et al*, 2001; Boost *et al*, 2001; Chiu *et al*, 2001), at least 30% of pneumococcal isolates were resistant to three or more drugs. As penicillin resistance is associated with

resistance to tetracycline, erythromycin and sulphonamide, though not fluoroquinolone, sensitivity testing is essential.

Pneumococci, are always susceptible to vancomycin, although many strains will also respond to imipenem/cilistatin and meropenem as well (Jorgensen *et al*, 1990). Increasing prevalence of MDR pneumococci may lead to use of vancomycin as empirical therapy for meningitis, although ceftriaxone and carbapenem are much preferred,. This would greatly increase the selective pressure for emergence of vancomycin-resistant pneumococci, as seen with emergence of vancomycin resistance in enterococci (McGowan and Metchock, 1995).The identification of vancomycin tolerance in pneumococci, and its association with treatment failure is of concern.

b) Susceptibility test methods

Disk diffusion tests have been well standardized for testing *S. pneumoniae* (NCCLS, 2003a) by supplementing MH with 5% sheep blood (MHA-SB) and by incubating the plates for 20-24 hours at 35°C in 5-7% CO_2. The NCCLS recommended MHA-SB whereas the Working Party of the BSAC (Andrews, 2004) recommended DST or IST Agar supplemented with 5% LHB for the disk diffusion test of pneumococci. There is clearly no consensus with regard to medium or supplements to be used.

Penicillin resistance as defined by MICs is not reliably identified by disk susceptibility testing with a 10 U penicillin disk (Tarpay, 1978). The CLSI recommends the use of 1 ug oxacillin disk for the screening of penicillin resistance (Swenson, 1986; NCCLS, 2003a, 2004b). However, the screening method does not distinguish between penicillin resistance and relative resistance (Jacobs *et al*, 1978), a distinction which is important in both meningeal and non-meningeal foci of infection with *S. pneumoniae*. The latter infection may be adequately treated with high dose of penicillin therapy, but failure in treatment of meningitis caused by relative resistant pneumococci has been reported (Klugman, 1990; Friedland and McCraken, 1994). Strains displaying an inhibitory zone ≤ 19 mm may be either resistant or intermediately resistant to penicillin. An MIC must be determined in order to precisely categorize an isolate of *S. pneumoniae* as being intermediate or frankly resistant to penicillin, though some strains will have an MIC in the sensitive range.

Disk diffusion screening methods for cefotaxime and ceftriaxone resistance have been described (Tenover *et al*, 1992; Friedland *et al*, 1993). However, there are excessive interpretive errors (mostly minor errors) with cefepime, cefotaxime, ceftriaxone, imipenem, cephalothin and ceftizoxime (Friedland *et al*, 1993; Jorgensen *et al*, 1993). Friedland *et al* (1993) found that cefotaxime MICs were always within one twofold dilution of the ceftriaxone MICs. Tenover *et al* (1992) proposed the use of a 30 μg ceftizoxime disk as a surrogate disk for predicting cefotaxime and ceftriaxone susceptibility. However, regardless of the zone size

breakpoints used, a large number of highly ceftriaxone-susceptible organisms would be classified as definitely or possibly resistant pending MIC determination (Tenover and Swenson, 1993; Barry and Fuchs, 1996). There are still no definite screening strategies for disk susceptibility testing for resistance of ceftriaxone and cefotaxime for pneumococci, and MICs must be determined.

Susceptible and resistant strains are readily distinguished by the current CLSI method (NCCLS, 2003a, 2004b) in tests with erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. Screening vancomycin resistance by the disk diffusion test is also well standardized (NCCLS, 2003a). But the absence of resistant strains precludes defining any results category other than susceptible with zone size $\geq$ 17 mm.

Dilution methods for pneumococci are also well standardized for MIC determination (NCCLS, 2004b). MICs can be obtained by broth macrodilution in cation-supplemented MH broth supplemented with 3-5% LHB incubated for 20-24 hours at 37°C in ambient air (NCCLS, 2004b). MICs can also be determined by SAD with MH agar supplemented with 5% whole defibrinated sheep blood or horse blood. Both of the dilution methods are regarded as the reference "gold standard" for pneumococcal susceptibility testing (NCCLS, 2004b; Andrews, 2001). HTM was formerly proposed as an alternative medium for MIC testing of *S. pneumoniae*, but, as the mean MIC of penicillin G was found to be lower and a large proportion of pneumococcal isolates failed to generate turbid growth in

HTM compared to unsupplemented MH broth (Marshall *et al*, 1993), this recommendation was removed by the CLSI.

However, for broth dilution tests of pneumococci, many extra steps are required to prepare the medium since it is not commercially available. So, testing of pneumococci by broth dilution method is not convenient. The SAD methods are also very laborious, costly, and practical only if large numbers of isolates are tested. The recommended MIC breakpoint and QC range for *S. pneumoniae* ATCC 49619 for susceptibility testing of pneumococci is shown in Table 4 (NCCLS, 2003a, 2004b).

Commercial microdilution systems that provide panels for susceptibility testing of *S. pneumoniae* include: Sensititre trays (Radiometer Inc., Westland, OH), JustOne (Baxter Diagnostics), MicroScan Pos MIC Panal Type 6 (Baxter Diagnostics), Fastidious Antimicrobial Susceptibility panel (FAS) (Microtech Medical Systems), and the Fox Fastidious Panel (FOX) (Micro Media Systems). However, most may inaccurately indicate susceptibility to penicillin for pneumococcal strains that are intermediately or highly resistant, and problems with their use has repeatedly been documented (Shanholtzen and Peterson, 1986; Krisher and Lincott, 1994; Kiska *et al* 1995). Some of them have been cleared by FDA for susceptibility testing of *S. pneumoniae* (Hindler and Swenson, 2004). They include Pasco (Difo, Wheatridge, Colo), MicroScan (MicroScan Inc., West Sacramento, Calif), Sensititre (Trek Diagnostic Systems, Westlake, Ohio), and

Vitek 2 (BioMerieux, Inc., Hazelwood, MO). Some of them have been recently evaluated and found to give MICs which were comparable to reference method (Guthrie *et al*, 1999; Mohammed and Tenover, 2000; Jorgensen *et al*, 2000). However, none of these methods can issue a rapid result.

For susceptibility testing of pneumococci, E test is FDA approved for determination of pneumococcal MICs with penicillin, cefotaxime, erythromycin, tetracycline, and chloramphenicol. The quality control measures developed by the CLSI are also applicable for penicillin and cefotaxime. E test has shown excellent correlation with SAD and broth microdilution methods for penicillin G, cefotaxime, ceftriaxone, chloramphenicol, erythromycin, and tetracycline (Jacobs *et al*, 1992; Jorgensen *et al*, 1994; Macias *et al*, 1994). Evaluation of E test for detection of penicillin resistance among *S. pneumoniae* clinical isolates found that 90% to 100% isolates yielded MIC values within one $\log_2$ dilution for the E test and microdilution method. However, there were 9.5% to 17.6% minor interpretive errors involving intermediate resistant strains. Jorgensen *et al* (1994) found that the cefotaxime MICs agreed within one $\log_2$ dilution for 96.6% of isolates by the standard MIC method, and only 5.4% minor errors occurred. Moreover, the MICs generated by E tests were found to be the same or one $\log_2$ dilution lower than those determined by broth micro dilutions for both penicillin and cefotaxime. In another study by Skulnick *et al* (1995), the accuracy and reproducibility of E test were evaluated for penicillin, cefotaxime and ceftriaxone, and almost prefect agreement was shown for both intra and interobserver

assessment, and the reproducibility of E test was good, though some minor errors occurred. Most of the minor errors for penicillin and ceftriaxone were made due to shifting of one log_2 dilution higher by the E test. Comparison of studies is made difficult by differences in inoculum and agar base used. Vancomycin susceptibility testing of pneumococci by E test has also been evaluated (Hashemi *et al*, 1996), and MICs of vancomycin were found to be elevated in comparison with the levels obtained by broth and SAD methods.

Although, E test is claimed to represent the optimal MIC test method for pneumococci (Doern, 1995), some minor susceptibility errors still occurred in all of the studies (Jacobs *et al*, 1992; Jorgensen *et al*, 1994; Macias *et al*, 1994; Skulnick *et al*, 1995; Hashemi, 1996). Moreover, as E test strips have a high retail price, the E test does not represent an economical method for testing a large number of drugs on each isolate. Therefore, E test method may require further evaluation to determine whether it represents the best susceptibility testing method for pneumococci

F) Quality Control of Susceptibility Tests

In order to obtain accurate results to select appropriate antimicrobial agents, AST need to be accurate and reproducible. Emphasis has been focused on strict adherence to procedures (NCCLS, 2004b). Quality control (QC) has played a significant role to ensure the high level of performance of AST in most laboratories. Routine QC procedures involve performance testing of standard

reference strains that have well-characterized susceptibility characteristics. It is important to use strains that are representative of the types of clinical isolates that are tested. Several ATCC or NTCC reference strains are recommended by the CLSI or Working Party of the BSAC respectively (NCCLS, 2004b; Andrews, 2004). However, performance limits for control strains are not published for all methods, and the controls are of limited value if acceptable performance is unknown. Some studies of the reproducibility of broth dilution methods have been conducted showing broth dilution could achieve 94% reproducibility (Hacek *et al,* 1999).

Ideal QC strains for MIC tests should have MIC endpoints near the middle of the range of concentrations being tested for a particular antimicrobial agent, or at least no closer than two dilutions from the extremes of the test range included (Thornberry *et al*, 1977). Therefore, the established QC strains that have been used for disk diffusion testing may be different from QC strains for dilution testing. Moreover, control reference strains should remain genetically and phenotypically stable over many replications and in long term storage.

When a laboratory has documented proficiency in performing a specific AST, reference QC strains need only be tested once each week instead of daily and or when new lots of materials are put into use. Routine QC procedures provide a review of variables such as antimicrobial potency, test medium, incubation condition, standardization of inoculum, instrument function, and technical

proficiency. However, there are several test parameters that are not controlled by QC procedures. These include individual organism-antimicrobial test problems, contamination, sporadic instrument malfunction, subjective reading of equivocal endpoints, interpretation of results, and individual transcription or technical errors. Thus, control results with reference QC strains do not always guarantee that all test results will be consistently reproducible and accurate (Zoutman *et al,* 2002).

For broth dilution tests, additional QC procedures are necessary that are less critical for SAD and disk diffusion. A control tube, free of antimicrobial agents, should be included for each panel set to ensure there is adequate growth. This tube can also serve as a control of turbidity to aid reading endpoints. A purity plate for each tested isolates should be plated directly from the final inoculum suspension onto an appropriate agar medium to detect potential contamination. Moreover, the proficiency of the endpoint readers should be monitored periodically by comparing their results with those of a standard reader (NCCLS, 2004b; CDC 2002; Doern *et al*, 1999).

In addition, supplemental QC strains may be tested periodically to control specific organism-antimicrobial interaction. Another supplemental QC measurement is the use of antibiograms to verify results generated on an isolate. Certain species have "typical" susceptibility or resistance to a battery of drugs, and the identification of a specific isolate can be checked against its expected antibiogram. There are also external quality assessment schemes which provide an independent

assessment of the performance of an individual laboratory. These may highlight hidden problems and provide a useful educational role.

3. Interpretation of ASTs into Categories of Susceptibility

In almost all contexts, the laboratory provides information that designates organisms as sensitive, intermediate sensitive or resistant. Susceptible strains are likely to respond to treatment with normal dose of an antibiotic. Resistant strains would not respond to treatment. The intermediate category may act as a buffer zone to prevent major or very major errors in reporting. It is also a category to allow for high dose for a site where the antibiotic is concentrated (Jones and Dudley, 1997; Jorgensen *et al*, 1996; Jorgensen *et al*, 1994).

The categories of susceptibility are distinguished by definition of MIC breakpoint concentrations of the antimicrobial agents. Defining MIC breakpoint involves the interpretation of the quantitative MIC susceptibility test results and has three major components. They are the clinical considerations, microbiological considerations and pharmacological considerations.

Ideally, breakpoint MIC should be based on data relating clinical response to *in vitro* test results. Data should be collected and evaluated from prospective clinical investigations that reflect the *in vitro* response to treatment of patients with specific infections caused by various strains of species with known MICs.

Although guidelines for performing these types of experiments have been included in a NCCLS document (1986), such data are rarely available (Philips, 1986). Thus, both the influence of host factors and the different growth states of the isolates in the patients and in the laboratory are not taken into account. However, there is now greater awareness of the need for data relating clinical response to *in vitro* test results, and such data is now being collected during clinical trials (Houton, 2003; Nguyen *et al*, 2000; Mason *et al*, 2000; Cunha, 1997, Jones and Dudley, 1997).

Microbiological factors affecting choice of breakpoints relate to the distribution of susceptibilities of the organism, i.e. the relationship of the strain under test to that of other members of same species. The MIC breakpoint should fit within the limits of clusters of microorganisms having comparable susceptibilities. The selection of resistant strains with extrachromosomal determinants of resistance has led to the appearance of populations separate from wild type that were uniformly susceptible to the drugs. The resulting bimodal distribution of susceptibilities correlates well with clinical responsiveness. However, there are species that do not have clearly defined populations at any specific MIC. The development of resistance may be a stepwise process. There is a continuous distribution of MICs, and those lying in the middle cause an oscillation of MIC about the breakpoint which leads to variations in classification of susceptibility. In these cases, it may be necessary to shift breakpoints or to introduce two

breakpoint concentrations to minimize the problem (Morrisey *et al*, 2005; Klugman *et al*, 2004; Jorgensen 2004).

For pharmacokinetic and pharmacodynamic (PK/PD) considerations, the MIC upper limit for susceptibility must be lower than the level of antibiotic attainable in the blood or tissues with clinically accepted dosages and routes of administration. It also includes the decisions on the relevance of blood concentrations when the site of infection may be varied, concentration maintained for half the dosage interval, the relevant of protein binding and metabolism, and variation in pharmacokinetic factors in different individuals (Houton, 2003; Jones and Dudley, 1997). Time above MIC for free drugs concentrations is the important PK-PD parameter correlating with the efficacy of beta-lactam antibiotics. The 24 hours AUC-MIC (Area Under Curve) is probably the important PK-PD parameter correlating with efficacy of vancomycin and teicoplanin. Breakpoints are selected based upon the pharmacokinetics of the agent in humans, the ability to separate resistance and susceptible bacterial species and minimization of the major error of interpretation. (Amsterdam 1996, Craig, 2003)

4. Selection Criteria for a new AST Method

Clinical microbiology laboratories can choose from several methods for performance of their routine AST. These include the disk diffusion (Kirby-Bauer) test, SAD or broth dilution test, antibiotic gradient methods, or commercial

automated microdilution test. There are two antibiotic agar gradient methods which laboratories may consider for susceptibility testing. They are the E test and the spiral gradient endpoint method (SGE). These methods are especially useful for susceptibility of fastidious organisms because special enriched media or a special incubation atmosphere (increase CO_2) may be used. However, the SGE method has not been evaluated for the susceptibility testing of fastidious organisms.

If a new susceptibility testing method like SGE method is suitable for fastidious organisms, it is essential that the test system provides reproducible results and that the results generated can be comparable to results determined by an acknowledged "gold standard" reference method such as SAD or broth dilution method. Acceptable precision has been defined for MIC tests by the CLSI (1990) as in repeated tests more than 95% of MIC should fall within an expected range of 3 $\log_2$ dilution. This contention that the acceptable precision is a range of target value $\pm$ 1 dilution is now well established. In 1980, Thornsberry *et al* (1980) used the categories of very major, major and minor to describe errors of false susceptibility or false resistance or a response involving an intermediate result respectively in their study of evaluation of a new automated system. In their study, a reference antibiogram was established for each of their isolates on the basis of knowledge of the organisms' resistance mechanisms. They utilized a challenge collection of organisms with known resistance properties for evaluation of a new AST method. Thornsberry and Gavan (1980) also suggested that it is

essential for a new test method, that complete category agreement should be over 90% and that the total of very major error and major errors should be less than 5%. Sherris and Ryan (1982) additionally suggested that very major errors attributable to the new procedure should be less than 1.5% for all isolates tested. However, the characteristics of the test isolate population affect the susceptibility test error rates. Murray and colleagues (1987) confirmed that the calculation of very major error should be based only upon the number of resistant strains tested and major errors should be calculated only on the basis of susceptible strains, which cannot contribute errors of false susceptibility. So, in evaluation of a new AST method, it is important to examine a collection of strains which includes an adequate number of resistant strains. Jorgensen (1994) proposed that a new test method should provide > 90% agreement with MICs determined by the reference method and very major errors determined on a large sample of equal or greater than 35 known resistant strains should be less than or equal to 3%, and that the combination of major and minor errors attributed to the new test should be ≤ 7% when determined on a large number of known susceptible or random clinical isolates. Nevertheless, for antibiotics without an intermediate interpretive category, slight modification of these criteria is required. Thus, the legitimate measure should be whether the within 1 dilution agreement between the two procedures is > 90%. The criteria proposed for the acceptable accuracies of new AST methods should not be interpreted too rigidly. It is important to consider the degree of difficulty involved in detecting resistance in some organisms. It is also possible that a new test with a lower degree of correspondence with the reference method might in

fact be more useful for detecting resistance mechanisms. Moreover, if strain resistance to an antibiotic has not been recognised, the accuracy of a new susceptibility test method for that drug cannot be predicated with absolute accuracy.

When considering introduction of a new AST system, a microbiologist should be aware of other evaluations, which have been published in well-respected journals regarding the new system. A laboratory should perform a limited on-site evaluation using selected control and clinical isolates to validate claims made in the literature and by the manufacturer regarding the advantages and reproducibility of a new system. The reputation of the instrument manufacturer with respect to routine service and likely response to unanticipated problems should also be assessed (Nonhoff *et al,* 2005, Donay *et al*, 2004).

5. Conclusion

The disc diffusion test is simple, inexpensive, reliable, and allows drug selection flexibility. It is commonly used as a routine test method in many laboratories. However, dilution methods are used when a more accurate estimate of susceptibility than that provided by disc tests is needed. An MIC determination is desirable when the disc diffusion test gives unclear susceptibility results, when there is unexpected failure of treatment, or there is unusual resistance pattern.

Some clinically significant fastidious bacteria have characteristics that preclude their being tested by standard methods. Dilution methods offer flexibility that the standard medium used to test non-fastidious micro-organisms may be supplemented or even replaced with another medium to allow accurate testing of various fastidious bacteria. Moreover broth or agar dilution methods continue to provide "gold standard" MIC data that may be used as a reference for evaluating the accuracy of other testing systems. However, SAD methods are time consuming, labor intensive, technically demanding, and expensive. They also have the disadvantages of large increments at higher concentrations, and there is a high standard allowable error (±100%) (Wexler *et al*, 1990). There is increasing use of commercial instruments based on automated broth microdilution AST methods in clinical laboratories (Jones and Edson, 1991). But, these automated systems are unable to test all clinical significant groups of bacteria. AST of fastidious bacteria, anaerobes, and certain non-fermentative gram negative bacilli is often not available. An alternative MIC method must be available for testing these groups of organisms.

Molecular methods can only augment but not replace the traditional AST methods, and they require further commercial development before they can be widely used. E test offers another option for the AST of fastidious organisms, as it combines the simplicity and flexibility of the disk diffusion test. But, E test has a high retail price and entails storage problems.

Spiral gradient endpoint (SGE) method is a concentration gradient method, which may be used to produce precise MIC for the AST of fastidious bacteria in various enriched mediums and incubation atmospheres. The advantages of the SGE method are decreased labor and materials, and more accurate mechanical dilution. The SGE measures the same type of endpoint as the dilution methods but with increased sensitivity since a continuous scale of MIC is obtained. Evaluation of non-fastidious aerobic and anaerobic test strains showed good correlation to results from parallel tests with SAD method even without correlation for diffusion (Weckback & Staneck 1987; Hill & Schalkousky 1990; James 1990; Paton *et al*, 1990; Hill 1991; James *et al*, 1991; Wexler *et al*, 1991; Wexler *et al*, 1996). However, there has been no reported study of AST of fastidious organisms with SGE methods. Although, preliminary standard parameters have been provided by the preliminary user guide of SGE method (Bolmstrom *et al*, 1988), concerning the requirement of agar depth, inoculum density, and elapsing before culture deposition, technical variations of these parameters may affect the results of AST by SGE method. These variables must be strictly controlled in order to obtain more accurate and reproducible results. Therefore, further studies on optimisation of these variables on SGE test are required.

Results of studies of AST using FCM on non-fastidious organism have given promising results and this method may be useful for AST of fastidious organisms. It offers the possibility of rapid results and allows investigation of resistance mechanisms. The method may be developed as a qualitative method using a fixed

concentration, or it may be possible to extend this to a breakpoint method which would provide more useful data for the treatment of infectious diseases.

Table 1. Antimicrobial agents that should be tested by standard antimicrobial susceptibility testing and reported for fastidious organisms (NCCLS, 2000)

Haemophilus influenzae[a]	*Neisseria gonorrhoeae*[b]	*Streptococcus pneumoniae*	*Moraxella catarrhalis*
Primary agent:- Amoxicillin/clavulanic acid or Ampicillin/sulbactam Ampicillin Azithromycin or Clarithromycin Cefaclor, cefprozil or L aracarbef Cefixime or Cefpodoxime Ceftriaxone, Cefotaxime, Ceftrizoxime, or Cetazidime Cefuroxime Chloramphenicol Tetracycline Trimethoprim/ Sulfamethoxazole		Primary agents:- Cefotaxime or Ceftriaxone Chloramphenicol Erythromycin Ofloxacin Penicillin Tetracycline Trimethoprime/ Sulfamethoxazole Vancomycin	Primary agents:- Penicillin Ampicillin Cloxacillin Trimethoprim/ Sulfamethoxazole Tetracycline Erythromycin
Supplementary agents:- Aztreonam Cefonicid Ciprofloxacin, Lomefloxacin, or Ofloxacin Imipenem Rifampin	Supplementary agents:- Penicillin Cefixime, cefotaxime, Ceftriaxone or Ceftizoxime Cefmetazole Cefotetan Cefoxitin Ciprofloxacin or Ofloxacin Spectinomycin Tetracycline		

a. For isolates recovered from blood and CSF, only results of testing with ampicillin, chloramphenicol and a third-generation cephalosporim should be reported. Results of tests with oral agents should be reported only against isolates recovered from localized uncomplicated infection.

b. A β-lactamase test should be used to detect the most common form of penicillin resistance. It will also provide useful epidemiologic information.

Table 2. Recommended MIC interpretation and QC range for susceptibility testing of *H. influenzae* against clinically-relevant antimicrobial agents (NCCLS, 2000)

Agents	Breakpoint (ug/ml)				
				QC range (ug/ml)	
	Susceptible	Intermediate	Resistant	*H. influenzae*	
				ATCC 49247	ATCC 49766
Ampicillin	≤ 1	2	≥ 4	2.0 – 8.0	
Trimethoprim/ Sulfamethoxazole	≤ 0.5 / 9.5	----	≥ 4 / 76	0.03/0.57 0.25/4.75	
Amoxicillin/ Clavulanic Acid	≤ 4 / 2	----	≥ 8 / 4	2/1 – 16 /8	
Azithromycin or Clarithromycin	≤ 4	----	----	1.0 – 4.0	
Cefaclor	≤ 2	4	≥ 8	----	1 - 4
Cefuroxime	≤ 4	8	≥ 16	0.06 – 0.25	
Choramphenical	≤ 8	16	≥ 32	0.25 – 1.0	
Tetracycline	≤ 4	8	≥ 16	4.0 - 32	
Rifampin	≤ 4	8	≥ 16	0.25 – 1.0	
Cefonicid	≤ 4	8	≥ 16	----	0.06 – 0.25
Imipenem	≤ 4	----	----	0.12 – 1.0	

* There is no QC β-lactamase positive strain and an β-lactamase positive *E. coli* strain is used as control.

Table 3. Recommended MIC interpretation and QC range for susceptibility testing of *N. gonorrhoeae* against clinically-relevant antimicrobial agents (NCCLS 2000)

Agents	Breakpoint (mgL^{-1})			
	Susceptible	Intermediate	Resistance	QC range (mgL^{-1}) for *N. gonorrhoeae* ATCC 49226
Penicillin	≤ 0.06	1	≥ 2	0.25 – 1
Spectinomycin	≤ 32	64	≥ 128	8 – 32
Tetracycline	≤ 0.25	0.5 – 1	≥ 2	0.25 - 1
Ciprofloxacin	≤ 0.06	----	----	0.001 – 0.008
Ceftriaxone	≤ 0.25	----	----	0.004 - 0.015
Cefmetazole	≤ 2	4	≥ 8	0.5 - 2
Cefotetan	≤ 2	4	≥ 8	0.5 - 2
Cefoxitin	≤ 2	4	≥ 8	0.5 - 2
Cefuroxime	≤ 1	2	≥ 4	0.25 - 1

Table 4. Recommended MIC interpretation and QC range for susceptibility testing of *S. pneumoniae* against clinically-relevant antimicrobial agents (NCCLS, 2004)

Agents	Breakpoint (mgL^{-1})			QC range(ug/ml)
	Susceptible	Intermediate	Resistant	*S. pneumoniae* ATCC 49619
Penicillin G	≤ 0.06	0.1 - 1	≥ 2	0.25 – 1
Cefotaxime	≤ 0.5	1	≥ 2	0.06 – 0.25
Ceftriaxone	≤ 0.5	1	≥ 2	0.03 - 0.12
Cefepime	≤ 0.5	1	≥ 2	0.06 – 0.25
Cefuroxime (oral)	≤ 0.5	1	≥ 2	not available
Imripenem	≤ 0.12	0.25 – 0.5	≥ 1	0.03 – 0.12
Erythromycin	≤ 0.5	1 - 2	≥ 2	0.03 – 0.12
Chloramphenicol	≤ 4	----	≥ 8	2 – 8
Ofloxacin	≤ 2	4	≥ 8	1 – 4
Tetracycline	≤ 2	4	≥ 8	0.12 – 0.5
Trimethoprim / Sulfamethoxazole	≤ 0.5	1	≥ 4	0.12/2.3- 1/19
Rifampin	≤ 1	2	≥ 4	not available
Vancomycin	≤ 1	----	----	0.12 – 0.5

Chapter 2

Aims and Objectives

Two standard types of AST method are used routinely– quantitative dilution and qualitative diffusion tests. Both depend on growth of the organism and require overnight incubation. Moreover, neither method takes account of heterogeneity within the bacterial suspension and produces only an average value which overlooks effects related to cell cycle and physiological status (Jorgensen *et al* 1994). Until recently, fastidious organisms like *H. influenzae, S. pneumoniae, N. gonorrhoeae, and M. catarralis* did not require AST since they were consistently susceptible to antimicrobial agents commonly used for therapy. However, with the emergence of resistant strains empirical therapy can no longer be used and AST is required for successful therapy.

Standard methods cannot be used for fastidious bacteria as they may grow too slowly, may require supplemental nutrients or modified incubation condition, or simply it may not have been demonstrated that they can be tested accurately by standard methods.

Automated or mechanized susceptibility systems are used in about 60% of clinical laboratories in the U.S.A. and many European countries (Jones and Edson, 1991; Brown, 1994). The major advantages of automated systems are their high reproducibility and precision, since most of the procedures are highly standardized in these systems (Peterson *et al*, 1986; Visser *et al*, 1992; Fekete *et al*, 1994; Berke and Teeino, 1996). Their data management systems can mimic the microbiologist to detect rare resistance patterns and flag for senior staff review. In particular, automated systems can generate rapid susceptibility results. However, most automated systems do not offer testing of fastidious bacteria. Recently, BioMerieux (Vitek 2 Hazelwood, MO) introduced a testing panel for *S. pneumoniae* and this was approved by the FDA. However, this method requires more than 8 hours of incubation before reports can be issued (Jorgensen *et al*, 2000).

AST of fastidious organisms tends to depend on conventional standardized dilution and diffusion methods, the former being used when an accurate estimate of susceptibility is needed, such as isolates from a patient with septicemia, meningitis or other life – threatening infections, or if the disk diffusion test give unreliable results. Conventional dilution methods are

labour intensive and require much technical skill. They suffer from inaccuracy due to large increments at higher concentrations, and have a high standard allowable error (±100%) (Wexler *et al*, 1990). A more precise and reproducible method of quantitative susceptibility testing is obviously needed.

E test (PDM Epsilometer; AB Biodisk, Solna, Sweden) is a concentration gradient method for determination of MIC. It is useful for fastidious organisms (Sanchez *et al*, 1992; Hughes *et al*, 1993; Jorgenson *et al*, 1994; Macias *et al*, 1994; Skulnick *et al*, 1995) because E test strips can be placed on special enriched media or used in special incubation conditions. The MICs generated generally yield excellent agreement with all reference methods and have very good reproducibility. However, E test strips are relatively expensive and there are problems of storage, making E test less convenient and economical as an MIC method.

The spiral gradient endpoint (SGE) method is another concentration gradient method for susceptibility testing, utilizing a spiral plater to deposit a stock concentration of antimicrobial agent in a spiral pattern on the surface of a prepoured agar plate. Test strains of bacteria are streaked radially across the

concentration gradient. After incubation visible growth ceases where the concentration of the antimicrobial agent reaches the bactericidal or/and bacteriostatic concentration. The distance from the commencement of antibiotic deposition (13mm from center) to the endpoint of growth is measured and used to calculate the MIC value.

Since the SGE test is performed with prepoured agar plates, it is significantly less labour intensive and more convenient to use than dilution methods. Although SAD method can also use with prepoured plate, not all the antibiotics can remain in solution form in SAD prepoured plate left at room temperature or 4°C for a long time. However for SGE test, the antibiotics can be left in powder form until it is used. And most of the antibiotics can be left in powder form for a long time when properly stored (NCCLS, 2004b). Consumable costs are low compared with E test or Vitek 2 (refer to cost analysis; Appendix ix). SGE measures the same type of endpoint as the dilution methods but with increased sensitivity since a continuous scale of MICs is obtained. The agar used may be supplemented and incubated in different conditions, suggesting that the SGE test would be suitable for AST of most fastidious bacteria. Studies have shown excellent agreement between

SGE and standard methods for MIC determination of both aerobic and anaerobic organisms (Hill *et al* 1990; Paton and Bywater 1990; Hill, 1991; Wexler *et al* 1991; Wexler *et al* 1996;), but no results have been reported for AST of fastidious organisms using the SGE method.

Flow Cytometry (FCM) measures physical and morphological characteristics of cells in a fluid system at a measuring point where a stream of cell particles intersects with a beam of light from an arc lamp or a laser lamp, and is useful for studying the physiological effect of antimicrobial agents on bacteria (Steen *et al* 1981; Martinez *et al* 1982; Gant *et al* 1993; Pore 1994; Durrdie *et al* 1995; Walberg *et al* 1997). FCM-AST does not rely on growth inhibition but allows rapid detection of antimicrobial effects on individual cells as measured by fluorescent probes. Thousands of cells can be rapidly and accurately assessed, making results available after a few hours.

Rapid AST can significantly lower mortality rates and shorten hospital stays (Doern *et al,* 1994; Barangfranger, 2001; Barangfranger and Short, 2001). It allows physicians to prescribe more appropriate, narrower spectrum and less costly antibiotics. FCM can offer results in terms of bacteriostatic or

bactericidal effects (Martinez *et al* 1982; Bolmstrom *et al* 1988; Pore 1994; Durrdie *et al* 1995). Further work is needed to develop FCM techniques for rapid AST of fastidious organisms.

Specific Aims

1) To assess the reproducibility of the SGE method for AST of *S. pneumoniae, H. influenzae, N. gonorrhoeae* and *Moraxella catarrhalis.*

2) To assess the effects of varying time of inoculation after antibiotic deposition, depth of agar, and length of incubation time on accuracy of SGE for AST of fastidious organisms (*H. influenzae*).

3) To compare and correlate the MICs of clinical isolates of *S. pneumoniae, H. influenzae, N. gonorrhoeae* and *M. catarrhalis* by SGE and standard dilution methods.

4) To assess advantages, disadvantages and potential application of the SGE method for AST of fastidious organisms.

5) To optimize concentrations of Propidium Iodide and $DiBAC_4(3)$ and incubation time for FCM determination of MIC for a control strain of *H. influenzae.*

6) To determine the optimal media to give maximum signal for FCM studies on fixed control *H. influenzae* and *S. pneumoniae* cells.

7) To determine the effects of concentration and exposure time to antibiotics on *H. influenzae* (ampicillin and tetracycline) and *S. pneumoniae* (penicillin G and erythromycin) control strains.

8) To determine numbers of fluorescent events (cells labeled) and the mean fluorescent channel for the PI and $DiBAC_4(3)$ signal on *H. influenzae* and *S. pneumoniae* clinical isolates treated with antibiotics at susceptibility breakpoint concentrations and untreated controls.

9) To determine the susceptibility categories of clinical isolates of *H. influenzae* and *S. pneumoniae* by FCM-AST by using a derived formula and to compare results with standard dilution methods.

10) To assess the cost and benefit for performance of SAD, E test, Vitek 2, SGE and FCM-AST test.

Significance

Standardisation of SGE may provide a reliable and inexpensive alternative test for MIC values of fastidious organism. FCM methods for fastidious organisms may offer rapid and accurate AST. Standardisation of these methods is required. The availability of a standardized method can also allow the effects of antimicrobial agents on metabolic parameters to be easily studied.

Assumptions and Limitations

Due to time and financial constraints, it was not possible to perform optimization of SGE for all fastidious organisms. A control strain of *H. influenzae* was used throughout the optimization studies. It is assumed that it would be representative of all the fastidious organisms, since it requires agar supplementation is and incubated in CO_2 for growth. Others species of bacteria were assumed to behave similarly to the *H. influenzae* control strain under various condition of the SGE test.

For the FCM AST, the sizes of the fastidious organisms (*H. influenzae* and *S. pneumoniae*) were just above the detection limit of the Coulter EPICS Elite ESP flow cytometer (Coulter corporation, Miami, FL, USA) (particles size detection limit is 0.5 μ). To monitor the cell flow, a dot plot of log forward scatter (FS) against log side scatter (SS) was set with an analysis gated around the bacterial populations. It was assumed that all the organisms were detected by the machine. Slight modifications of the machine for the FCM AST were made, including installation of disposable self-bleeding in-line sheath filter cartridges (0.2 μm pore size) in parallel, close to the flow cell to minimize particles background noise; setting of all detector amplifiers on logarithmic gain mode, and setting of a minimum gate discriminator on side scatter criteria to exclude sub-cellular debris and optical 'noise'. *N. gonorrhoeae* was not included in the study since the organism would not grow well in liquid medium (Amsterdam, 1996) and the FCM-AST required the organism to growth in liquid medium and growth to log phrase before antibiotics were added.

Clinical isolates were collected on a consecutive basis from several district hospitals located in different areas of Hong Kong. For some species, numbers

of the resistant strains were low for some antibiotics so more resistant strains were added in order to cover all the susceptibility categories.

Chapter 3

Materials, Methods and Experimental Design

The thesis is written in the form of a series of monographs, with an introduction, materials and methods used and the study described individually and in detail in Chapters 4 to 8. Chapter 9 summarizes the results of the studies and in Chapter 10, the overall study is discussed and suggestions for further research are described.

All the Chapters of the thesis are designed to be read and understood independently. Although it has not been possible to avoid duplication, repetition has been minimized, while attempting to maintain clarity.

All bacterial strains involved in the studies were collected in various clinical laboratories of hospitals of Hong Kong with the approval of the senior staff. The strains were come from various sites including blood, sputum and other body sites. All the laboratory work was performed according to recommended microbiology laboratory safety standards in order to prevent laboratory infection. A special flow cell was used that all the samples and wastes were

delivered within an enclosed system. Flow cytometry should better be conducted in a negative pressure laboratory and operated by a well trained operator. Proper personal protective equipment must be worned. When possible, biological samples should be fixed usually with 1 % formaldehyde before run through the flow cytomter.

Statistical analysis was performed using SPSS 10.1 software and log fluorescent histograms of the flow cytometer were generated from the gated data by the Coulter Elite Software Version 4.5 Revisions B.

Techniques involved in the studies include; agar dilution test, broth micro dilution test, E test, standard plate count, SGE test, and flow cytometry. All tests were performed under standard conditions given by the CLSI (NCCLS 2003a, 2004b;CLSI, 2005b). Tests were standardized under conditions described in the materials and methods section of each chapter.

All sample collection, laboratory work, reagent preparation, equipment set up and testing was performed by the author personally. Nevertheless, there was

some assistance from Ms Eva Cheung and Ms Sindy Lai in minor work and their help is fully and gratefully acknowledged.

A cost analysis was also performed (see Appendix ix) to compare the price for 100 tests using SAD, E test, Vitek 2, SGE and FCM-AST.

Chapter 4

Optimization of the SGE test for susceptibility testing of fastidious organisms

Introduction

Since the emergence of resistance in strains of fastidious organisms, previously employed empirical therapy for these organisms has become inadequate. AST of these organisms is required to ensure a successful therapy.

Disk diffusion testing has an inherent flexibility and cost effectiveness, but a quantitative minimum inhibitory concentration (MIC) value of drugs may be required in some cases. MIC values are desirable when the isolates are from patients with life-threatening infection or if disk diffusion provides unclear susceptibility results.

Standard dilution methods for MIC determination are labour-intensive and time-consuming and also have the disadvantage of large increments at high concentrations (Wexler *et al,* 1990). Use of automated broth microdilution methods has increased, but there is little difference in the labour required in comparison to conventional methods, and the running costs are relatively high. In addition, these systems cannot perform susceptibility testing of

fastidious organisms, except the Vitek 2 system for *S. pneumoniae*, which requires overnight incubation.

E test is suitable for testing anaerobic bacteria or fastidious organisms. However, the strips are expensive, and storage of test strips is a problem as they are very sensitive to environmental change.

The Spiral Gradient Endpoint (SGE) method is an alternative technique for MIC determination utilizing a spiral plater with a variable cam to produce an antimicrobial drug concentration gradient on an agar plate. Bacteria are streaked in radial lines across this gradient and the drug concentration at the endpoint location where growth stops is known as the Radial Advance (RA). The RA, combined with parameters such as the agar depth, stock drug concentration, and deposition factors are used to calculate the MIC.

SGE method has previously been shown to be a reproducible and accurate test for MIC determination of aerobic and anaerobic organisms (Hill and Schalkowsky, 1990; Paton *et al*, 1990; James, 1990; Wexler *et al*, 1991; Hill, 1991; James *et al*, 1991; Wexler *et al*, 1996). However little information is available on susceptibility testing of fastidious organisms using this method.

SGE is not a diffusion method and does not rely on drug diffusion to create the drug concentration gradient. However, diffusion can alter the deposited gradient. This point is of particular importance with anaerobes which require two days of incubation for AST (NCCLS, 2003b). Early studies (Hill and

Schalkowsky, 1990) demonstrated the need for a standardized procedure, various technical improvements, and revised SGE formulae to correct for drug diffusion in calculation of stock concentrations and endpoint concentrations for testing anaerobic bacteria. There have been few studies of SGE susceptibility testing of aerobic bacteria (Paton *et al*, 1990; James, 1990; James *et al*, 1991). In those studies using the original SGE method, the correlation between the SGE and standard agar dilution methods (SAD) were good. It is more convenient to use the original SGE method for the AST of aerobic bacteria, since there is no need for purchase of license for use and, no need for calculations of stock solution concentrations and endpoint MICs.

This study aimed to optimize the laboratory conditions for SGE for AST of fastidious organisms. In order to determine the usefulness of SGE for fastidious bacteria AST, a preliminary study was performed to assess the intrabatch and interbatch reproducibility of SGE. Additional validation studies were performed under varying laboratory conditions using a control strain of *H. influenzae*.

Materials and Methods

Strains

A quality control strain, *H. influenzae* ATCC 49247, was used in these studies. It was recovered from lyophilized condition by plating out on chocolate agar. It was subsequently subcultured 3 times before the commencement of the

sensitivity testing. A wild type strain of each of the other species was used for reproducibility testing.

Antimicrobial Agents

Standard antimicrobial powders (Sigma Chemical Co., St. Louis, MO.) were used to prepare antibiotic solutions for reference dilution tests (SAD and broth microdilution test) and susceptibility testing using SGE. Agents tested were ampicillin for *H. influenzae* and penicillin G for *S. pneumoniae*, *N. gonorrhoeae* and *M. catarrhalis*. The concentration ranges for reference dilution tests were:

- ampicillin for *H. influenzae* from 0.06 ug/mL to 128 ug/mL;
- penicillin G. for *S. pneumoniae* from 0.06 IU/mL to 128 IU/mL;
- penicillin G. for *N. gonorrhoeae* from 0.06 IU/mL to 128 IU/mL;
- penicillin G. for *M. catarrhalis* from 0.06 IU/mL to 128 IU/mL .

Master stock solutions of the drug were prepared according to the recommendation of the CLSI (NCCLS, 2004b). The solvent and diluent for penicillin G was sterilized distilled water, whereas for the sodium salt of ampicillin, the solvent was phosphate buffer (0.01 mmol/L, pH 8) and the diluent was phosphate buffer at pH 6.0 (0.01 mmol/L). A master stock solution of penicillin G (10,000 IU/mL) was prepared and stored at -70°C, and used within one month as recommended by CLSI (NCCLS, 2004b). Ampicillin stock solution was freshly prepared on every occasion and used within 24 hours.

SGE Test

The SGE test was performed based on the existing preliminary user manual for the SGE method of susceptibility testing (Schalkowsky, 1985). The conditions were varied as described below in the reproducibility test. A spiral plater (WASP, Don Whiteley Scientific Ltd., Shipley, U.K.) was used to deposit the stock solution to appropriate agar plates. 15 cm agar plates with culture media recommended by the CLSI (NCCLS, 2004b) were used. The stock solution concentrations were selected to cover the range of MICs expected and included the MICs breakpoint concentrations as well as the known MICs of various control strains. Haemophilus Test medium (HTM) (Oxoid, UK) supplemented with 15 ug/mL of hematin and 15 ug/mL of NAD was used as medium for *H. influenzae*. HTM medium were homemade and were used within one week.50 mL agar + supplement were used in each plate to give an agar depth of 3.57 cm. GC Sensitivity Agar (Oxoid, UK) was used for *N. gonorrhoeae*. Mueller-Hinton (MH) agar (Oxoid, UK) supplemented with 2-5% lysed horse blood was used for *S. pneumoniae* and *M. catarrhalis*. The same batches of media were used throughout the experiments. The inoculum was prepared by making a direct MH broth suspension of isolated colonies selected from an 18 to 24 h chocolate agar plate. The density of the suspension was adjusted and verified by using a spectrophotometer with a 1 cm light path. After dispensing of antimicrobial stock solution by the spiral plater, plates were allowed to stand for 1 h to allow absorption of the antibiotic. Inoculums prepared by direct suspension method (Poupard et al, 1994) and adjusted to 0.5 McFarland Standard density (concentration around 10^8/mL were streaked radially from the periphery of each plate to a distance

of 13 mm from the centre of the plate. The organism would not be streaked from opposite direction i.e. from the center of the plate to the periphery, since this may lead to carry over of drugs from high concentration to low concentration. The use of a replicator for inoculation may generate a more consistent results. Plates were incubated for the appropriate period immediately after streaking. All plates were incubated in 5% CO_2. After incubation, the distance from the commencement of antibiotic deposition (13 mm from centre) to the endpoint of growth (RA) was measured. (see Fig 3). The MIC value was then calculated using the formula provided by the manufacturer (Schalkowsky, 1985).

For the reproducibility study, the stock concentration used for low range and high range plates, and the corresponding MIC range measured for each organism are shown in the Table 5.

Reproducibility study

In order to assess the validity and reproducibility of SGE, an intrabatch reproducibility study was performed on a single occasion using 48 replicates of a single isolate of *H. influenzae* with ampicillin, and 48 replicates of single isolates of *S. pneumoniae*, *N. gonorrhoeae* and *M. catarrhalis* with penicillin. SGE tests were performed on six plates, and on each plate the test was repeated eight times.

An interbatch reproducibility study was performed on separate occasions by SGE using single isolates of *H. influenzae* with ampicillin, and *S. pneumoniae, N. gonorrhoeae* and *M. catarrhalis* with penicillin on 6 separate occasions. On each occasion, SGE was repeated eight times on one plate, and the mean MIC of the plate was calculated. A control strain with known MIC for each organism was included in the test on each batch of testing.

Validation Studies

Validation studies were performed for variability under various laboratory conditions. The purpose was to counteract the decrease in MIC due to diffusion. One strain of *H. influenzae* (ATCC 49247) and ampicillin was tested 32 times by SGE and the mean MIC results generated by each condition were compared. Haemophilus Test Medium (HTM) (Oxoid, UK) supplemented with 15 μg/mL of haematin and 15 μg/mL of NAD was used as medium. All the plates were incubated in 5% CO_2 at 35°C

(A). *To assess the effect of varying the volume of agar in the 15cm plate.*

Plates were poured with 45mL, 50mL, 55mL. and 60mL of media. For each volume, the control strain *H. influenzae* was tested 32 times (4 plates, 8 replicates on each plate) for each agar volume. The plates were inoculated with a 10^8 CFU/ ml inoculum for 2 h after antibiotic deposition, incubated for 24 h, using a low range plate (1,000 ug/mL) and a high range plate (12,000

ug/mL). After comparing the MICs of using different volumes of agar, the optimal agar depth was used in assessing other parameters.

(B) *To assess the effect of varying time of inoculation of organisms after antibiotic application (ST): 1, 2, 4 & 8 hours.*

H. influenzae was tested 32 times (4 plates, 8 replicates on each plate) for each time of inoculation of organism after antibiotic deposition, using the optimum agar density determined in (A) with other conditions as in (A). The optimal ST was used for evaluation of other parameters.

C) *To assess the effect of varying the length of incubation before reading plates (In) : 18 hours, 20 hours and 24 hours.*

The control strain was tested 32 times as above for each incubation time and the result evaluated. The other parameter settings were the same as part (a) except the AD and ST settings were based on the results of (A) and (B). The optimal (In) was used for further parts of the evaluation.

(D) *To assess the effect of varying the Inoculum Density (ID): 10^6, 10^7, 10^8 and 10^9 CFT/mL.*

The control strain was also tested 32 times as described above for each (ID) The other parameters were dependent on the optimal results of (A), (B) and (C). The optimal settings for ID were used for further evaluation.

(E) *To assess the effect of varying the stock solution concentration (Conc.) : 500 ug/mL, 1,000 ug/Ml, 5,000 ug/mL and 10,000 ug/mL.*

Sensitivity testing of the control strain was repeated 32 times for each concentration and results compared. Other parameters were as determined by earlier evaluation steps.

Statistical Analysis

Statistical analysis was performed using the SPSS program for Windows (release 10.1). Differences in sample means for different values of optimizing parameters were tested for significance. Since it is a one-way analysis of variance for a quantitative dependent variable (the MICs) by a single factor independent variable (the value of a particular parameter), one-way ANOVA procedure was used to test the hypothesis that the means were equal. It was assumed that the sets of data (MICs after varying the tested parameter) constituted independent simple random samples from the several populations that were similar except for the difference due to varying the value of the parameter, and that the several populations of measurements were normally distributed with equal variances. The statistics were chosen to include descriptive tests and tests for homogeneity-of-variance. Homogeneity of variance was tested by calculation of the Levene statistic to test for the equality of group variance. The p value for the F test is set at 0.05. Post hoc test, S-N-K and Duncan, were used to perform multiple comparisons of pairs of means.

Results

Reproducibility of SGE Test

H. influenzae.

The results of the intrabatch reproducibility for *H. influenzae* are shown in Table 6. The overall standard deviation (SD) and coefficient of variation (CV) were 0.101 and 6.22% respectively. The average SD & CV on each plate were 0.0893 and 5.56% respectively. The results of the interbatch reproducibility are as shown in Table 7. The overall mean MIC was 1.817ug/mL. The average SD and CV on each plate were 0.114 and 6.29%. The overall SD and CV for the interbatch reproducibility were 0.201 and 11.06% respectively. For the conventional broth micro dilution method, the MIC values obtained are shown in Table 8. All the readings on each plate were the same as for the reference test. The mean MIC was 3.667ug/mL. The SD and CV of the reference test were 2.338 and 63.8% respectively.

S. pneumoniae.

The MIC readings for *S. pneumoniae* are shown in Table 9. The average MIC obtained was 0.689 Iu/mL on the single occasion. The average SD and CV on each plate were 0.0752 and 10.9% respectively. The overall SD and CV of the MIC values were 0.0832 and 12.1%. The results of the interbatch reproducibility of the SGE test are shown in Table 10. The overall mean MIC was 0.772 Iu/ML. The mean SD and CV on each plate were 0.0756 and

9.79% respectively. The overall SD and CV were 0.100 and 13.0%. The interbatch reproducibility of the broth micro dilution test for *S. pneumoniae* is as shown in Table 11. The mean MIC was 3.00 I/ML. The overall SD and CV for the reference method were 1.095 and 36.5% respectively.

N. gonorrhoeae.

The results of the intrabatch reproducibility of SGE test are shown in Table 12. The overall mean MIC for the reproducibility was 0.568 I/ML. The average SD and CV on each plate were 0.034 and 6.07% respectively. The overall SD was 0.041 and CV was 7.17%. The results of MIC readings of SGE test on 6 separate occasion for are shown in Table 13. The overall mean MIC was 0.554 I/ML. The average SD and CV on each plate for the interbatch reproducibility test were 0.037 and 6.76%. The overall SD and CV of the interbatch tests were 0.078 and 14.02%. Results of the SAD method on 6 separate occasions are shown in Table 14. The mean MIC determined by the SAD test was 0.75 I/ML. The SD and CV SAD method for on six separate occasions was 0.274 and 36.52% respectively.

M. catarrhalis.

M. catarrhalis showed a lot of clumping no matter how to prepared the suspensions when the density of suspensions were high. This lead to inconsistence in inoculation density and lead to a high CV and SD in the reproducibility tests. The intrabatch reproducibility and interbatch

reproducibility of the SGE test for MIC determination of *M. catarrhalis* strain are shown in Table 15 and 16. The mean MIC determined by intrabatch SGE test was 2.247 I/mL. The mean SD and CV on each plate for the intrabatch test were 0.283 and 12.59% respectively. The overall SD and CV were 0.327 and 14.56%. For interbatch reproducibility test of SGE method, the mean MIC was 2.150 I/ML. The mean SD and CV on each plate were 0.353 and 16.41% respectively. The overall SD and CV were 0.399 and 18.56% respectively. The results of the interbatch reproducibility test of SAD method are shown in Table 17. The mean MIC was 3.33 I/ML. The overall SD and CV were 1.033 and 30.98% respectively.

The overall results of the reproducibility test on SGE method and standard reference methods are as shown in Table 18. The overall CV of the SGE test ranged from 7.17% to 18.56%, whereas the CV of the standard dilution test ranged from 30.98% to 63.8%, which is much higher than the SGE test.

Optimization of the SGE test

Agar depth (volume of agar in 15cm Petri dish).

The one-way ANOVA output is shown in Tables19 a-d. From the output, the p value for the Levine test for the homogeneity of variance for the MICs in low range plates, MICs in high range plates and average MICs is greater than 0.05 showing that there is no significant difference for variance. The p value

for the F test are 0.322, 0.09 and 0.164 for the MICs in low range plates, high range plates and average MICs respectively, which is greater than 0.05 and there was no significant difference for the MICs when the volume of agar (agar depth) varied. However, in the Duncan post hoc test, there was a tendency for MICs in high range plates and average MICs to increase when the volume of agar increased

Although there was no significant difference in MICs when the volume of agar changed, variation in MICs could occur when the agar depth varied. To avoid difference due to technical variation, the volume of agar would be arbitrary set at 50mL .

Standing time before inoculation of organism after the antibiotic deposition.

The one-way ANOVA together with the descriptive statistic and test for homogeneity of variance are shown in the Tables.20a-d. From the output, the p values for the Levine test for the homogeneity of variance were 0.062, 0.209 and 0.095 for the Low range MICs, high range MICs and average MICs respectively. They are greater than 0.05 and show that there was no significant difference for variances. The p value of F test for the low range MICs, high range MICs and average MICs are 0.068, 0.085 and 0.911 respectively which are greater than 0.05 and there was no significant difference in low range MICs, high range MICs, and average MICs when the standing time before inoculation of organisms varied from 1 to 8 hours.

Variation in MIC can occur due to change in standing time, although there was no significant difference in MICs when the standing varied between 1 to 8 hours. Moreover, variation in standing time before inoculation can lead to technical differences in laboratories, which may decrease the precision of the test. So, a standard standing time between 1 to 8 hours should be set in laboratories to perform the SGE test. One hour was chosen as the standing time before inoculation as it would be more convenient and rapid to finish the test.

Incubation time

The one-way ANOVA, together with the Levene test for homogeneity of variance and descriptive statistic are shown in the Tables 21a-d. As shown in the output, the p values for the Levene test for the homogeneity of variance are both greater than 0.05 showing that there was no significant difference for variances. The p values for the F tests are <0.01 which is less than 0.05 and there was significant difference for the MICs due to variation in incubation time. From the post hoc tests, it was shown that the MICs were significantly lower when the incubation time was 18 hours. Since the acceptable range for the control strain *H. influenzae* was 2-8 ug/mL. The MICs generated in this part of experiment were within the lower limit of the acceptable range when the MICs are rounded up to next twofold dilution (i.e. 2 ug/mL). The target MIC should be 4 ug/mL, so a higher MIC was desirable. Therefore, incubation time of at least 20 hours was required. A standard incubation time of 24 hours are chosen and used since it was necessary to avoid loss in

precision due to technical variations (20 hours vs 24 hours) and there was a tendency for the MICs to increase when incubation time were increased.

Inoculum density

The different inoculum densities were made from a dilution of a 0.5 McFarland standard suspension. The initial bacterial suspensions were made by direct suspension method (Stokes *et al*, 1993). It was verified to be at McFarland 0.5 by spectrophotometer method – measured at 625nm, 1cm curette should give 0.08 – 0.10 OD (Stokes et al, 1993). For the 10^7 CFU/mL suspension, it was made by dilution of a 10^8 CFU/mL bacterial culture. For the 10^9 CFU/mL suspension, most of the organisms will clump at this high concentration. Moreover, it was difficult to standardize. To achieve a standardized and homogenized 10^9CFU/mL suspension, 10 mL of 10^8 suspension were first made. Then they were centrifuged at 3000 rpm/min for 20 minutes and the supernant were decanted. They were then vortexed at high speed and 1 mL of saline was added. The clumping was minimized and the final suspension were homogenised and well standardized. Results of statistical analysis are shown in Table 22a-d. The p value for the Levene test of homogeneity of variance are both greater than 0.05, so there is no difference for variances. The p values for the F test are all <0.01 which are less than 0.05 and so there was a significant difference in MICs when the inoculum densities are varied. From the Post Hoc test, there are 3-4 homogenous subset. It showed that there were significant differences in MICs when the inoculum densities increased from 10^6 CFU/mL to 10^9 CFU/mL.

When the inoculum density increased, the MICs would increased. Since the resultant MICs were on the lower limit of the acceptable range when rounded up to the next two fold dilution (i.e. 2 ug/mL), a higher MICs was desirable. Therefore a inoculum density of 10^9 CFU was optimal and was be used in the study to compare the reference MICs with SGE MICs.

Deposition Stock Concentration

The descriptive statistic, Levene test for homogeneity of variance and the one-way ANOVA output are shown in Table 23 a-d. The p value for the Levene test for the homogeneity of variance was 0.109, which is greater than 0.05, showing that there is no significant difference for variances. The p value for the F test was <0.001 showing there was a significant difference for the MICs when the initial deposition concentration were different.

The Post Hoc test demonstrates that when the stock concentrations were decreased, the measured MICs of the control strain would increase.

Conclusion

SGE test showed a high intrabatch and interbatch reproducibility. Results showed that the CVs were much lower when compared with that of standard dilution method.

Varying the magnitude of the parameters of SGE test did not always lead to change in MIC values. For the optimization of agar depth and standing time, there were no significant changes in resultant MICs when the condition varied.

However, to avoid variation occur due to technical difference, the parameters were arbitrarily set.

The inoculum density seemed to be one of the most important parameters that affect the result of SGE test. The position of the edge of inhibition is determined when the critical cell mass is obtained, similar to the position of the inhibition zone of disk diffusion test (Lehman, 1985). Heavy inocula would give a shorter length of inhibition and result in a shorter RA. Less time is required to reach the critical mass when the inoculum is heavy; consequently the critical concentration of drug is heavier, resulting in a shorter length of inhibition. The optimal bacterial density was selected to be more dense (10^9 cfu/mL) since in a previous study the SGE MIC results were found to be lower than the SAD method.

The inoculum density is extremely important in disc diffusion test when testing organisms that are resistant by the production of ß-lactamase. Minor changes in inoculum density result in extreme changes in results of susceptibility test. This is especially true for ampicillin, which is well known to cause a high change in susceptibility when the innocilum density change (Amsterdam, 1996). Nevertheless, in an experiment using another antibiotic (tetracycline) to perform optimization of inoculum density, the results were the same as that of ampicillin (data not shown). The optimal bacterial density (10^9 cfu/mL) could be prepared by overnight incubation of a bacterial suspension or by direct suspension and centrifugation method. However, for M. catarrhalis, clumping problem cannot be easily resolved . Tests were still

performed and a high SD and CV (23.4%) were resulted in the reproducibility test. The direct suspension was better and gave a higher MIC results. This may be because the cells in the overnight culture might all have reached stationary phrase, and the critical mass for inhibition would decrease.

It was found that when the incubation time increased, the resultant MIC would increase. This may due to changes in the character of growth at the edge of inhibition. These changes may include: 1) the appearance of delayed growth. 2) the delayed appearance of resistant variants and 3) better visualization of partially inhibited growth. For convenience, and to avoid the change in resultant MIC, the incubation time were standardised as 24 hours.

When the stock concentration of SGE test increased, the resultant MICs would decrease. This may be due to the effect of diffusion. When the concentration of stock solution increased, the absolute concentration of drugs on the plate between adjacent deposition tracks would increase and diffusion may be faster. Whereas, for a lower stock concentration the concentration difference between adjacent deposition tracks on the plates was also lower and diffusion rate would decrease. The major effect of diffusion was a lowering of the MICs due to diffusion of antibiotics from the center part of the plate to the outer rim where there was a lower concentration and the resultant antimicrobial drug concentration at any points would increase and shift the result endpoint to the outer circumference of the plates. Thereby, the RAs were increased and leading to a decrease in the resultant MICs. Since the plates with a lower stock concentration gave higher MIC results, which were

closer to the target MIC value of the control *H. influenzae*, a lower stock concentration was used for the SGE test.

When using the SGE test for the fastidious organisms, the MICs generated from the low range plate should be recognized as the immediate results. The stock concentration of the low range plates should be carefully calculated. The range of MICs should include the sensitivity category breakpoints so as to reduce the minor errors (errors that include an intermediate categorical) and very major errors (resistant misclassified as sensitive) that may occur in the original SGE method due to the diffusion problem. When the MIC of a particular strain is out of range (i.e. RA<20mm) the results on the high range plate should be used. Since many isolates resistant to an antimicrobial agent tend to cluster around the breakpoint, increasing the sensitivity of the SGE method and decreasing the effect of diffusion by a lowering in stock concentration of the SGE test reduce the number of isolates that may be variably categorized into different categorical placement that may occur during susceptibility test by dilution method.

Optimized values of the parameters were : Agar depth: 50ml; Standing time : 1 hour; Incubation time : 20-24 hours; Inoculum density : 10^9 cfu/ml (direct suspension); Stock solution concentration: as low as possible but should include the breakpoint of susceptibility.

These parameters were used for the comparison of the SGE test and standard dilution tests for MIC determinations of *H. influenzae, S. pneumoniae, M. catarrhalis & N. gonorrhoeae* reported in the following Chapter.

The results of the validation studies were comparable to those performed by Paton *et al* (1990). However, the organism used in that study is non-fastidious organisms --- *S. aureus* and *E. coli* were used. In that study, varying the time of inoculation of organism after deposition of antibiotics mattered little after the deposition of antibiotic which has the same results as the present study. Increase the inoculum density also led to an increase in the MIC. However, a reduction in calculated MIC is noted with increasing volume of agar. This seems to be contradictory with the results of the present study. The reason for the discrepancy was that the amount of varying the agar depth was different in the two studies. In the study by Paton et al (1990), the amount of variations in the agar volume (46.5%) were much larger than the present study (25%). Moreover, since Paton *et al* (1990) used 9cm diameter plated, this contributed to much greater difference in agar depth in the studies of Paton *et al* (1990), so it is not surprising that the MIC would have significant difference in that study, Another discrepancy between the two studies were that the time of reading of the plater made little difference to the calculated MIC in the study of Paton et al (1990), whereas in the present study, the MICs were significant difference for the MIC due to variation in inoculation time. This may be due to the fact that the organisms were difference in the two studies. Longer incubation time may results in division of some cells and lead to an increase in endpoint MIC.

Table 5: Stock concentration used for low range plates and high range plates of SGE method and the corresponding MIC measured for each organisms

Organism	Plates Range	Stock Concentration	MIC Range Tested
H. influenzae	Low range	1,000 ug/mL	0.0083-6.22 µg/mL
	High range	12,000 ug/mL	0.10-74.62 µg/mL
S. pneumoniae **(penicillin)**	Low range	300 Iu/mL	0.0766-2.051 Iu/mL
	High range	10000 Iu/mL	0.255-68.37 Iu/mL
N. gonorrhoeae **(penicillin)**	Low range	200 Iu/mL	0.0017-1.24 Iu/mL
	High range	6,000 Iu/mL	0.10-74.62 Iu/mL
M. catarrhalis **(penicillin)**	Low range	1,500 Iu/mL	0.0017-1.24 Iu/mL

Table 6: Intrabatch reproducibility of SGE for susceptibility testing of *H. influenzae*

Plate No. / Number of replicate	A	B	C	D	E	F
1	1.387	1.524	1.754	1.689	1.721	1.600
2	1.595	1.557	1.686	1.628	1.813	1.487
3	1.778	1.496	1.557	1.687	1.755	1.544
4	1.651	1.605	1.598	1.773	1.714	1.389
5	1.571	1.671	1.689	1.518	1.585	1.485
6	1.540	1.755	1.651	1.755	1.585	1.517
7	1.540	1.689	1.712	1.689	1.585	1.747
8	1.518	1.628	1.651	1.715	1.651	1.574
mean	1.573	1.615	1.662	1.682	1.676	1.543
SD	0.112	0.088	0.0628	0.08	0.088	0.105
CV	7.12%	5.44%	3.78%	4.80%	5.30%	6.74%

Table 7: Interbatch reproducibility of SGE test for *H. influenzae*

Occation. No. of replicate	1	2	3	4	5	6
1	1.701	2.116	1.721	1.78	2.013	1.562
2	1.721	1.751	1.634	1.655	2.105	1.496
3	1.753	1.837	1.70	1.754	2.260	1.651
4	1.771	2.168	1.721	1.604	2.130	1.721
5	1.605	2.247	1.634	1.629	1.960	1.678
6	1.883	1.782	1.604	1.78	2.030	1.755
7	1.879	1.852	1.688	2.11	2.040	1.721
8	1.605	2.055	1.628	1.876	2.170	1.675
mean	1.740	1.986	1.666	1.774	2.09	1.66
SD	0.106	0.186	0.046	0.164	0.097	0.087
CV	6.12%	9.37%	2.80%	9.24	4.65%	5.28%

Table 8: Reproducibility of SAD test for *H. influenzae*

Occasion / No. of inoculum	1	2	3	4	5	6
1	2	2	4	4	2	8
2	2	2	4	4	2	8
3	2	2	4	4	2	8
4	2	2	4	4	2	8
5	2	2	4	4	2	8
6	2	2	4	4	2	8
mean	2	2	4	4	2	8
SD	0	0	0	0	0	0
CV	0%	0%	0%	0%	0%	0%

Table 9: Intrabatch reproducibility of SGE test for *S. pneumoniae*

Plate / No. of inoculum	A	B	C	D	E	F
1	0.790	0.678	0.790	0.790	0.732	0.581
2	0.678	0.628	0.732	0.540	0.790	0.581
3	0.628	0.732	0.790	0.581	0.678	0.581
4	0.790	0.790	0.755	0.628	0.790	0.540
5	0.678	0.678	0.732	0.790	0.732	0.790
6	0.648	0.732	0.678	0.581	0.581	0.628
7	0.790	0.628	0.732	0.678	0.678	0.732
8	0.790	0.540	0.678	0.732	0.732	0.540
mean	0.724	0.675	0.735	0.665	0.714	0.622
SD	0.072	0.078	0.043	0.098	0.068	0.092
CV	10.0%	11.4%	5.84%	14.7%	9.52%	14.7%

Table 10: Interbatch reproducibility of SGE method for susceptibility of *S. pneumoniae.*

Occa / Number of replicate	1	2	3	4	5	6
1	0.628	0.921	0.921	0.853	0.79	0.790
2	0.628	0.79	0.853	0.678	0.732	0.628
3	0.732	0.732	0.853	0.853	0.790	0.790
4	0.853	0.678	0.79	0.853	0.732	0.628
5	0.732	0.853	0.853	0.75	0.790	0.732
6	0.581	0.921	0.995	0.732	0.790	0.732
7	0.678	0.732	0.995	0.853	0.678	0.732
8	0.732	0.732	0.853	0.853	0.678	0.581
mean	0.696	0.795	0.889	0.803	0.747	0.702
SD	0.0856	0.093	0.074	0.072	0.050	0.079
CV	12.3%	11.7%	8.3%	9.0%	6.66%	11.3%

Table 11: Interbatch reproducibility test of standard broth microdilution method for *S. pneumoniae*.

Occasion No. / Number of replicate	1	2	3	4	5	6
1	4	4	4	2	2	2
2	4	4	4	2	2	2
3	4	4	4	2	2	2
4	4	4	4	2	2	2
5	4	4	4	2	2	2
6	4	4	4	2	2	2
7	4	4	4	2	2	2
8	4	4	4	2	2	2
mean	4	4	4	2	2	2
SD	0	0	0	0	0	0
CV	0%	0%	0%	0%	0%	0%

Table 12: Intrabatch reproducibility of SGE test on susceptibility testing of *N. gonorrhoeae*.

Plate No. / Number of replicate	A	B	C	D	E	F
1	0.649	0.549	0.540	0.503	0.625	0.592
2	0.573	0.517	0.548	0.562	0.539	0.595
3	0.526	0.544	0.556	0.553	0.587	0.580
4	0.526	0.508	0.576	0.631	0.508	0.613
5	0.581	0.488	0.605	0.580	0.539	0.629
6	0.571	0.511	0.564	0.531	0.582	0.646
7	0.557	0.567	0.525	0.557	0.630	0.606
8	0.562	0.584	0.531	0.571	0.646	0.590
mean	0.568	0.534	0.556	0.561	0.582	0.606
SD	0.038	0.033	0.026	0.037	0.050	0.022
CV	6.8%	6.1%	4.7%	6.06%	8.6%	3.6%

Table 13: Interbatch reproducibility test of SGE method of susceptibility testing of *N. gonorrhoeae* by SGE test.

Occ / Number of replicate	1	2	3	4	5	6
1	0.483	0.623	0.499	0.468	0.704	0.490
2	0.541	0.679	0.489	0.458	0.546	0.488
3	0.489	0.672	0.555	0.527	0.550	0.490
4	0.494	0.661	0.509	0.513	0.596	0.516
5	0.474	0.658	0.556	0.495	0.632	0.549
6	0.499	0.708	0.570	0.458	0.641	0.641
7	0.488	0.638	0.497	0.435	0.632	0.549
8	0.502	0.739	0.509	0.501	0.609	0.563
X mean	0.496	0.672	0.523	0.482	0.614	0.536
SD	0.020	0.037	0.032	0.032	0.051	0.052
CV	4.5%	5.54%	6.10%	6.60%	8.39%	9.73%

Table 14: Reproducibility of SAD of susceptibility testing of *N. gonorrhoeae.*

Occasion No. / Number of replicate	1	2	3	4	5	6
1	0.5	1	0.5	0.5	1	1
2	0.5	1	0.5	0.5	1	1
3	0.5	1	0.5	0.5	1	1
4	0.5	1	0.5	0.5	1	1
5	0.5	1	0.5	0.5	1	1
6	0.5	1	0.5	0.5	1	1
7	0.5	1	0.5	0.5	1	1
8	0.5	1	0.5	0.5	1	1
mean	0.5	1	0.5	0.5	1	1
SD	0	0	0	0	0	0
CV	0%	0%	0%	0%	0%	0%

Table 15: Intrabatch reproducibility of SGE method for the MIC measurement of *M. catarrhalis*

Plate No. / Number of replicate	A	B	C	D	E	F
1	2.025	2.106	1.847	1.847	2.684	1.850
2	2.852	2.376	2.000	2.524	2.524	2.520
3	2.237	2.376	2.376	2.524	2.106	1.709
4	2.852	2.524	2.237	2.852	2.025	2.000
5	2.680	2.025	2.376	2.376	1.847	2.106
6	2.237	2.237	1.847	2.237	2.106	2.000
7	2.852	2.237	2.000	2.376	2.684	1.847
8	2.237	2.025	1.847	2.000	2.852	1.847
X mean	2.497	2.238	2.066	2.342	2.354	1.985
SD	0.346	0.180	0.231	0.317	0.375	0.249
CV	13.85%	8.06%	11.12%	13.52%	15.92%	12.54%

.Table 16: Interbatch reproducibility of SGE method for the MIC measurement of *M. catarrhalis*.

occ / Number of replicate	1	2	3	4	5	6
1	1.850	2.52	1.709	2.000	2.106	2.000
2	2.520	2.11	1.582	2.106	2.524	1.847
3	2.110	1.709	1.464	2.524	1.709	2.106
4	1.710	2.24	2.106	2.852	2.106	2.000
5	2.520	2.11	1.355	1.847	2.000	1.847
6	2.000	2.85	2.380	2.524	1.847	2.380
7	2.680	2.85	1.464	2.106	2.000	2.524
8	2.850	2.85	2.380	2.524	1.709	2.106
mean	2.246	2.500	1.805	2.288	1.986	2.090
SD	0.417	0.430	0.422	0.343	0.265	0.241
CV	18.3%	18.1%	23.4%	15.0%	13.1%	11.5%

Table 17: Interbatch reproducibility of SAD test for the susceptibility testing of *M. catarrhalis.*

Occasion No. / Number of replicate	1	2	3	4	5	6
1	4	4	2	2	4	4
2	4	4	2	2	4	4
3	4	4	2	2	4	4
4	4	4	2	2	4	4
5	4	4	2	2	4	4
6	4	4	2	2	4	4
7	4	4	2	2	4	4
8	4	4	2	2	4	4
X mean	4	4	2	2	4	4
SD	0	0	0	0	0	0
CV	0%	0%	0%	0%	0%	0%

Table 18: Summary of Reproducibility Test Results

Organism	Test	Mean MIC	Mean SD & CV on each plates	Over all SD	Over all CV
H. influenzae	Intrabatch (SGE)	1.625 ug/ML	0.0893 (5.56%)	0.101	6.22 %
	Interbatch (SGE)	1.817 ug/ML	0.114 (6.29%)	0.201	11.06 %
	Interbatch (Standard dilution)	3.667 ug/ML	0 (0%)	2.338	63.8 %
S. pneumoniae	Intrabatch (SGE)	0.689 Iu/ML	0.0752 (10.9%)	0.083	12.1 %
	Interbatch (SGE)	0.772 Iu/ML	0.0756 (9.79%)	0.100	8.49 %
	Interbatch (Stand dilution)	3.00 Iu/ML	0 (0%)	1.095	36.5 %
N. gonorrhoeae	Intrabatch (SGE)	0.568 Iu/ML	0.034 (6.07)	0.041	7.17 %
	Interbatch (SGE)	0.554 Iu/ML	0.0.37 (6.76%)	0.078	14.02 %
	Interbatch (Stand dilution)	0.750 Iu/ML	0 (0%)	0.274	36.52 %
M. catarrhalis	Intrabatch (SGE)	2.247 Iu/ML	0.283 (12.59%)	0.327	14.56 %
	Interbatch (SGE)	2.150 Iu/ML	0.353 (16.41%)	0.399	18.56 %
	Interbatch (Standard dilution)	3.33 Iu/ML	0 (0%)	1.033	30.98 %

Table 19 Tests of significance for Optimisation of Agar Depth

a. Descriptives

			N	Minimum	Maximum	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
									Lower Bound	Upper Bound
Low range plate MIC	Agar Depth	Agar Vol. = 45 mL	32	1.622	2.400	1.88072	.14937	2.64E-02	1.82687	1.93457
		Agar Vol. = 50 mL	32	1.619	2.402	1.93881	.16079	2.84E-02	1.88084	1.99678
		Agar Vol. = 55 mL	32	1.612	2.242	1.88947	.15453	2.73E-02	1.83376	1.94518
		Agar Vol. = 60 mL	32	1.640	2.140	1.92334	.10210	1.80E-02	1.88653	1.96015
		Total	128	1.612	2.402	1.90809	.14388	1.27E-02	1.88292	1.93325
High range plate MIC	Agar Depth	Agar Vol. = 45 mL	32	1.222	1.536	1.36638	9.98E-02	1.76E-02	1.33038	1.40237
		Agar Vol. = 50 mL	32	1.223	1.610	1.36219	.10128	1.79E-02	1.32567	1.39870
		Agar Vol. = 55 mL	32	1.256	1.580	1.38928	7.78E-02	1.38E-02	1.36122	1.41734
		Agar Vol. = 60 mL	32	1.243	1.612	1.41319	7.56E-02	1.34E-02	1.38594	1.44043
		Total	128	1.222	1.612	1.38276	9.07E-02	8.02E-02	1.36689	1.39862
Average MIC	Agar Depth	Agar Vol. = 45 mL	32	1.454	1.811	1.62355	7.41E-02	1.31E-02	1.59682	1.65028
		Agar Vol. = 50 mL	32	1.421	1.861	1.65050	8.58E-02	1.52E-02	1.61957	1.68143
		Agar Vol. = 55 mL	32	1.466	1.876	1.63938	9.58E-02	1.69E-02	1.60485	1.67390
		Agar Vol. = 60 mL	32	1.544	1.809	1.66827	6.43E-02	1.14E-02	1.64508	1.69145
		Total	128	1.421	1.876	1.64542	8.16E-02	7.21E-02	1.63115	1.65969

b. Test of Homogeneity of Variance

	Levene Statistic	df1	df2	Sig.
Low range plate MIC	1.566	3	124	.201
High range plate MIC	2.343	3	124	.076
Average MIC	1.393	3	124	.248

c, ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Low range plate MIC	Between Groups	7.272E-02	3	2.424E-02	1.176	.322
	Within Groups	2.556	124	2.062E-02		
	Total	2.629	127			
High range plate MIC	Between Groups	5.312E-02	3	1.771E-02	2.214	.090
	Within Groups	.992	124	7.988E-03		
	Total	1.045	127			
Average MIC	Between Groups	3.401E-02	3	1.134E-02	1.733	.164
	Within Groups	.811	124	6.541E-03		
	Total	.845	127			

d. Post Hoc Tests

Homogeneous Subsets

Low range plate MIC

	Agar Depth	N	Subset for alpha = .05 1
Student-Newman-Keel S[a]	Agar Vol. =45 mL	32	1.88072
	Agar Vol. =50 mL	32	1.88947
	Agar Vol. =55 mL	32	1.92334
	Agar Vol. =60 mL	32	1.93881
	Sig.		.368
Duncan	Agar Vol. = 45 mL	32	1.88072
	Agar Vol. = 50 mL	32	1.88947
	Agar Vol. = 55 mL	32	1.92334
	Agar Vol. = 60 mL	32	1.93881
	Sig.		.142

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

High range plate MIC

	Agar Depth	N	Subset for alpha = .05	
			1	2
Student-Newman-Keel S[a]	Agar Vol. = 45 mL	32	1.36219	
	Agar Vol. = 50 mL	32	1.36638	
	Agar Vol. = 55 mL	32	1.38928	
	Agar Vol. = 60 mL	32	1.41319	
	Sig.		.102	
Duncan[a]	Agar Vol. = 45 mL	32	1.36219	
	Agar Vol. = 50 mL	32	1.36638	
	Agar Vol. = 55 mL	32	1.38928	1.38928
	Agar Vol. = 60 mL	32		1.41319
	Sig.		.256	.285

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

Average MIC

	Agar Depth	N	Subset for alpha = .05	
			1	2
Student-Newman-Keul S[a]	Agar Vol. = 45 mL	32	1.62355	
	Agar Vol. = 50 mL	32	1.63938	
	Agar Vol. = 55 mL	32	1.65050	
	Agar Vol. = 60 mL	32	1.66827	
	Sig.		.120	
Duncan[a]	Agar Vol. = 45 mL	32	1.62355	
	Agar Vol. = 50 mL	32	1.63938	1.63938
	Agar Vol. = 55 mL	32	1.65050	1.65050
	Agar Vol. = 60 mL	32		1.66827
	Sig.		.211	.179

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

Table 20 Tests of significance to assess the variation due to change in standing time (ST)

a. Descriptives

		N	Minimum	Maximum	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
								Lower Bound	Upper Bound
Low range plate	Standing time	Standing time = 1 hr.	32	1.661	2.020	1.80600	.10957	1.94E-02	1.76650	1.84550
		Standing time = 2 hrs.	32	1.661	2.020	1.78800	8.63E-02	1.53E-02	1.75689	1.81911
		Standing time = 4 hrs.	32	1.661	1.969	1.81694	7.17E-02	1.27E-02	1.79108	1.84280
		Standing time = 6 hrs.	32	1.557	1.969	1.78366	.10728	1.90E-02	1.74498	1.82233
		Standing time = 8 hrs.	32	1.661	2.011	1.84509	8.89E-02	1.57E-02	1.81305	1.87714
		Total	160	1.557	2.020	1.80794	9.53E-02	7.53E-02	1.79307	1.82281
High range MIC	Standing time	Standing time = 1 hr.	32	1.187	1.610	1.40047	.10265	1.81E-02	1.36346	1.43748
		Standing time = 2 hrs.	32	1.223	1.660	1.42025	.10938	1.93E-02	1.38081	1.45969
		Standing time = 4 hrs.	32	1.187	1.610	1.37050	.11638	2.06E-02	1.32854	1.41246
		Standing time = 6 hrs.	32	1.187	1.610	1.42647	9.00E-02	1.59E-02	1.39402	1.45892
		Standing time = 8 hrs.	32	1.223	1.610	1.37484	7.51E-02	1.33E-02	1.34777	1.40192
		Total	160	1.187	1.660	1.39851	.10114	8.00E-02	1.38271	1.41430

Average MIC	Standing time	Standing time = 1 hr.	32	1.524	1.701	1.60323	4.55E-02	8.05E-02	1.58682	1.61965
		Standing time = 2 hrs.	32	1.475	1.752	1.60412	6.62E-02	1.17E-02	1.58027	1.62798
		Standing time = 4 hrs.	32	1.457	1.790	1.59372	7.74E-02	1.37E-02	1.56581	1.62163
		Standing time = 6 hrs.	32	1.419	1.790	1.60506	8.33E-02	1.47E-02	1.57505	1.63508
		Standing time = 8 hrs.	32	1.522	1.731	1.60997	5.73E-02	1.01E-02	1.58932	1.63062
		Total	160	1.419	1.790	1.60322	6.67E-02	5.27E-02	1.59281	1.61363

b. Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Low range plate MIC	2.293	4	155	.062
High range plate MIC	1.485	4	155	.209
Average MIC	2.015	4	155	.095

c. ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Low range plate	Between Groups	7.848E-02	4	1.962E-02	2.229	.068
	Within Groups	1.364	155	8.800E-03		
	Total	1.443	159			
High range MIC	Between Groups	8.329E-02	4	2.082E-02	2.091	.085
	Within Groups	1.543	155	9.957E-03		
	Total	1.627	159			
Average MIC	Between Groups	4.481E-02	4	1.120E-03	.247	.911
	Within Groups	.702	155	4.531E-03		
	Total	.707	159			

d. Post Hoc Tests

Homogeneous Subsets

Low range plate

	Standing time	N	Subset for alpha = .05	
			1	2
Student-Newman-Keuls[a]	Standing time = 1 hr.	32	1.78366	
	Standing time = 2 hrs.	32	1.78800	
	Standing time = 4 hrs.	32	1.80600	
	Standing time = 6 hrs.	32	1.81694	
	Standing time = 8 hrs.	32	1.84509	
	Sig.		0.67	
Duncan[a]	Standing time = 1 hr.	32	1.78366	
	Standing time = 2 hrs.	32	1.78800	
	Standing time = 4 hrs.	32	1.80600	1.80600
	Standing time = 6 hrs.	32	1.81694	1.81694
	Standing time = 8 hrs.	32		1.84509
	Sig.		.200	.116

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

High range MIC

	Standing time	N	Subset for alpha = .05	
			1	2
Student-Newman-Keul S[a]	Standing time = 1 hr.	32	1.37050	
	Standing time = 2 hrs.	32	1.37484	
	Standing time = 4 hrs.	32	1.40047	
	Standing time = 6 hrs.	32	1.42025	
	Standing time = 8 hrs.	32	1.42647	
	Sig.		.164	
Duncan[a]	Standing time = 1 hr.	32	1.37050	
	Standing time = 2 hrs.	32	1.37484	1.37484
	Standing time = 4 hrs.	32	1.40047	1.40047
	Standing time = 6 hrs.	32	1.42025	1.42025
	Standing time = 8 hrs.	32		1.42647
	Sig.		.068	.058

Means for groups in homogeneous subsets are displayed.
b. Uses Harmonic Mean Samples Size = 32.000

Average MIC

	Standing time	N	Subset for alpha = .05
			1
Student-Newman-Keul S[a]	Standing time = 1 hr.	32	1.59372
	Standing time = 2 hrs.	32	1.60323
	Standing time = 4 hrs.	32	1.60412
	Standing time = 6 hrs.	32	1.60506
	Standing time = 8 hrs.	32	1.60997
	Sig.		.871
Duncan[a]	Standing time = 1 hr.	32	1.59372
	Standing time = 2 hrs.	32	1.60323
	Standing time = 4 hrs.	32	1.60412
	Standing time = 6 hrs.	32	1.60506
	Standing time = 8 hrs.	32	1.60997
	Sig.		.400

Means for groups in homogeneous subsets are displayed.
b. Uses Harmonic Mean Samples Size = 32.000

Table 21 a-d Statistical assessment of the variation due to change in incubation time (IT)

a. Descriptives

		N	Minimum	Maximum	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
								Lower Bound	Upper Bound
Low range MIC	Incubation time	Incubation time = 18 hrs.	32	1.423	1.773	1.54625	.10520	1.86E-02	1.50832	1.58418
		Incubation time = 20 hrs.	32	1.661	1.969	1.78431	8.36E-02	1.48E-02	1.75416	1.81446
		Incubation time = 24 hrs.	32	1.661	1.969	1.79838	8.66E-02	1.53E-02	1.76714	1.82961
		Total	96	1.423	1.969	1.70965	.14787	1.51E-02	1.67968	1.73961
High range MIC	Incubation time	Incubation time = 18 hrs.	32	1.100	1.492	1.23994	.10508	1.86E-02	1.20205	1.27782
		Incubation time = 20 hrs.	32	1.320	1.737	1.50284	8.79E-02	1.55E-02	1.47114	1.53455
		Incubation time = 24 hrs.	32	1.382	1.737	1.52816	9.00E-02	1.59E-02	1.49572	1.56059
		Total	96	1.100	1.737	1.42365	.16102	1.64E-02	1.39102	1.45627
Average MIC	Incubation time	Incubation time = 18 hrs.	32	1.279	1.632	1.39309	9.40E-02	1.66E-02	1.35920	1.42699
		Incubation time = 20 hrs.	32	1.522	1.752	1.64358	6.27E-02	1.11E-02	1.62097	1.66619
		Incubation time = 24 hrs.	32	1.555	1.815	1.66327	6.34E-02	1.12E-02	1.64042	1.68611
		Total	96	1.279	1.815	1.56665	.14409	1.47E-02	1.53745	1.59584

b. Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Low range MIC	1.641	2	93	.199
High range MIC	.465	2	93	.630
Average MIC	2.737	2	93	.070

c. ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Low range MIC	Between Groups	1.285	2	.642	75.372	.000
	Within Groups	.793	93	8.522E-03		
	Total	2.077	95			
High range MIC	Between Groups	1.630	2	.815	91.021	.000
	Within Groups	.833	93	8.955E-03		
	Total	2.463	95			
Average MIC	Between Groups	1.452	2	.726	129.743	.000
	Within Groups	.520	93	5.596E-03		
	Total	1.972	95			

d. Post Hoc Tests

Homogeneous Subsets

Low range MIC

	Incubation time	N	Subset for alpha = .05	
			1	2
Student-Newman-Keuls[a]	Incubation time = 18 hrs.	32	1.54625	
	Incubation time = 20 hrs.	32		1.78431
	Incubation time = 24 hrs.	32		1.79838
	Sig.		1.000	.544
Duncan[a]	Incubation time = 18 hrs.	32	1.54625	
	Incubation time = 20 hrs.	32		1.78431
	Incubation time = 24 hrs.	32		1.79838
	Sig.		1.000	.544

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

High range MIC

	Incubation time	N	Subset for alpha = .05	
			1	2
Student-Newman-Keul S[a]	Incubation time = 18 hrs.	32	1.23994	
	Incubation time = 20 hrs.	32		1.50284
	Incubation time = 24 hrs.	32		1.52816
	Sig.		1.000	.287
Duncan[a]	Incubation time = 18 hrs.	32	1.23994	
	Incubation time = 20 hrs.	32		1.50284
	Incubation time = 24 hrs.	32		1.52816
	Sig.		1.000	.287

Means for groups in homogeneous subsets are displayed.

c. Uses Harmonic Mean Samples Size = 32.000

Average MIC

	Incubation time	N	Subset for alpha = .05	
			1	2
Student-Newman-Keul S[a]	Incubation time = 18 hrs.	32	1.39309	
	Incubation time = 20 hrs.	32		1.64358
	Incubation time = 24 hrs.	32		1.66327
	Sig.		1.000	.295
Duncan[a]	Incubation time = 18 hrs.	32	1.39309	
	Incubation time = 20 hrs.	32		1.64358
	Incubation time = 24 hrs.	32		1.66327
	Sig.		1.000	.295

Means for groups in homogeneous subsets are displayed.

c. Uses Harmonic Mean Samples Size = 32.000

Table 22 a-d Analysis of effect of inoculum concentration

a. Descriptives

		N	Minimum	Maximum	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
								Lower Bound	Upper Bound
Low Inoc-rangeulation MIC density	Density = 10^6	32	1.423	1.727	1.58703	8.97E-02	1.59E-02	1.55468	1.61938
	Density = 10^7	32	1.517	1.969	1.63188	9.43E-02	1.67E-02	1.59789	1.66586
	Density = 10^8	32	1.619	1.969	1.78091	.11180	1.98E-02	1.74060	1.82121
	Density = 10^9 (Overnight suspension)	32	1.844	2.160	1.99291	8.07E-02	1.43E-02	1.96381	2.02200
	Density = 10^9 (Direct suspension)	32	1.969	2.248	2.05566	9.97E-02	1.76E-02	2.01971	2.09160
	Total	160	1.423	2.248	1.80968	.21068	1.67E-02	1.77678	1.84257
High Inocul rangeation MIC density	Density = 10^6	32	1.100	1.425	1.33900	7.31E-02	1.29E-02	1.31265	1.36535
	Density = 10^7	32	1.281	1.610	1.37422	7.45E-02	1.32E-02	1.34735	1.40108
	Density = 10^8	32	1.382	1.660	1.52450	8.87E-02	1.57E-02	1.49252	1.55648
	Density = 10^9 (Overnight suspension)	32	1.492	1.876	1.61131	8.24E-02	1.46E-02	1.58161	1.64102
	Density = 10^9 (Direct suspension)	32	1.610	2.026	1.88231	9.61E-02	1.70E-02	1.84768	1.91695
	Total	160	1.100	2.026	1.54627	.21227	1.68E-02	1.51313	1.57941
Aver Inocul age ation MIC density	Density = 10^6	32	1.262	1.555	1.46302	6.52E-02	1.15E-02	1.43950	1.48653
	Density = 10^7	32	1.399	1.676	1.50305	6.34E-02	1.12E-02	1.48020	1.52590

Density = 10^8	32	1.555	1.790	1.65270	6.81E-02	1.20E-02	1.62814	1.67727
Density = 10^9 (Overnight suspension)	32	1.716	1.990	1.80211	5.88E-02	1.04E-02	1.78090	1.82332
Density = 10^9 (Direct suspension)	32	1.815	2.137	1.96898	6.93E-02	1.23E-02	1.94398	1.99398
Total	160	1.262	2.137	1.67797	.19965	1.58E-02	1.64680	1.70915

b.Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Low range MIC	1.701	4	155	.153
High range MIC	1.228	4	155	.301
Average MIC	.411	4	155	.801

c.ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Low range MIC	Between Groups	5.635	4	1.409	153.505	.000
	Within Groups	1.422	155	9.177E-03		
	Total	7.057	159			
High range MIC	Between Groups	6.086	4	1.522	218.752	.000
	Within Groups	1.078	155	6.956E-03		
	Total	7.164	159			
Average MIC	Between Groups	5.681	4	1.420	335.238	.000
	Within Groups	.657	155	4.237E-03		
	Total	6.338	159			

d.Post Hoc Tests

Homogeneous Subsets

Low range MIC

	Inoculation density	N	Subset for alpha = .05			
			1	2	3	4
Student-Newman-Keul S[a]	Density = 10^6	32	1.58703			
	Density = 10^7	32	1.63188			
	Density = 10^8	32		1.7809 1		
	Density = 10^9 (Overnight suspension)	32			1.9929 1	
	Density = 10^9 (Direct suspension)	32				2.0556 6
	Sig.		.061	1.000	1.000	1.000
Duncan[a]	Density = 10^6	32	1.58703			
	Density = 10^7	32	1.63188			
	Density = 10^8	32		1.7809 1		
	Density = 10^9 (Overnight suspension)	32			1.9929 1	
	Density = 10^9 (Direct suspension)	32				2.0556 6
	Sig.		0.61	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

High range MIC

	Inoculation density	N	Subset for alpha = .05			
			1	2	3	4
Student-Newman-Keul S[a]	Density = 10^6	32	1.33900			
	Density = 10^7	32	1.37422			
	Density = 10^8	32		1.52450		
	Density = 10^9 (Overnight suspension)	32			1.61131	
	Density = 10^9 (Direct suspension)	32				1.88231
	Sig.		.091	1.000	1.000	1.000
Duncan[a]	Density = 10^6	32	1.33900			
	Density = 10^7	32	1.37422			
	Density = 10^8	32		1.52450		
	Density = 10^9 (Overnight suspension)	32			1.61131	
	Density = 10^9 (Direct suspension)	32				1.88231
	Sig.		.091	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

Average MIC

	Inoculation density	N	Subset for alpha = .05				
			1	2	3	4	5
Student-Newman-Keul S[a]	Density = 10^6	32	1.46302				
	Density = 10^7	32		1.50305			
	Density = 10^8	32			1.65270		
	Density = 10^9 (Overnight suspension)	32				1.80211	
	Density = 10^9 (Direct suspension)	32					1.96898
	Sig.		1.000	1.000	1.000	1.000	1.000
Duncan[a]	Density = 10^6	32	1.46302				
	Density = 10^7	32		1.50305			
	Density = 10^8	32			1.65270		
	Density = 10^9 (Overnight suspension)	32				1.80211	
	Density = 10^9 (Direct suspension)	32					1.96898
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

Table 23a –d Assessment of effect of to change in deposition stock concentration

a. Descriptives

		N	Minimum	Maximum	Mean	Std Deviation	Std. Error	95% Confidence Interval for Mean	
								Lower Bound	Upper Bound
Stock concentration	Stock conc. = 500 ug/mL	32	1.933	2.453	2.18384	.12249	2.17E-02	2.13968	2.22801
	Stock conc. = 1000 ug/mL	32	1.893	2.248	2.03209	8.90E-02	1.57E-02	2.00002	2.06417
	Stock conc. = 5000 ug/mL	32	1.692	2.177	1.83119	.10199	1.80E-02	1.79442	1.86796
	Stock conc. = 10000 ug/mL	32	1.493	1.879	1.65006	8.13E-02	1.44E-02	1.62074	1.67939
	Total	128	1.493	2.453	1.92430	.22535	1.99E-02	1.88488	1.96371

b. Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
MICs	2.058	3	124	.109

c.ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
MICs	Between Groups	5.211	3	1.737	173.989	.000
	Within Groups	1.238	124	9.984E-03		
	Total	6.450	127			

d. Post Hoc Tests

Homogeneous Subsets

MICs

	Stock concentration	N	Subset for alpha = .05			
			1	2	3	4
Student-Newman-Keul S[a]	Stock conc. = 500 ug/mL	32	1.6500 6			
	Stock conc. = 1000 ug/mL	32		1.8311 9		
	Stock conc. = 5000 ug/mL	32			2.0320 9	
	Stock conc. = 10000 ug/mL	32				2.1838 4
	Sig.		1.000	1.000	1.000	1.000
Duncan[a]	Stock conc. = 500 ug/mL	32	1.6500 6			
	Stock conc. = 1000 ug/mL	32		1.8311 9		
	Stock conc. = 5000 ug/mL	32			2.0320 9	
	Stock conc. = 10000 ug/mL	32				2.1838 4
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Samples Size = 32.000

Chapter 5

Evaluation of Spiral Gradient Endpoint technique for determination of MICs of fastidious organisms

Introduction

Screening tests for resistance in fastidious organisms may be performed using variations of the qualitative disk diffusion tests (NCCLS, 2000), which are simple to perform and provide results which are readily interpreted by clinicians.

However, MIC results are required when the organism is isolated from a life-threatening infection or the disc tests give unclear or unreliable results. The MIC result is also desirable when there is an unexpected treatment failure. MIC levels are used for interpretations of disc diffusion tests and selection of "cut-off" values for these tests. (Amsterdam, 1996)

Both dilution tests and the E-test have disadvantages, as have been discussed in Chapter 1. Automated systems have major advantages of good reproducibility of results and high precision compared with reference methods (Schernbra *et al*, 1993; Ferraro *et al*, 1995; Nolte *et al*, 1998). However, most of the automated systems are not suitable for susceptibility testing of fastidious organisms. In addition, the cost of running the automated machine is relatively high.

SGE is an alternative method for MIC, utilizing a spiral plater to deposit a small amount of sample agent in an Archimedes spiral on the surface of a rotating agar plate thereby affording continuous, logarithmic dilution rather than incremental dilution. Although originally used for bacterial enumeration, spiral plating can be used to distribute antimicrobial agents to achieve a continuous concentration gradient. Test strains are inoculated on the surface of the agar by streaking a radial line across the concentration gradient and after incubation, visible growth along the radial streak is observed to cease when the concentration of antibiotic reaches bactericidal / bacteriostatic concentration.

SGE test is especially suitable for the AST of fastidious organisms, as it utilizes pre-poured plates, allowing use of supplemented media, and incubation in a different atmosphere and/or temperature. While SGE has been shown to have excellent agreement with standard dilution test for AST of aerobic bacteria (Hill & Schakowsky, 1990; James, 1990; Paton *et al*, 1990; Hill & Schakowsky, 1991; James, 1991; Wexler *et al*, 1991; Wexler *et al*, 1996), few studies have investigated its use for fastidious organisms.

The results of the reproducibility tests of SGE, reported in the previous chapter, have shown that it has excellent reproducibility and a smaller CV than the standard dilution test. In this study, SGE testing was compared with the standard dilution test for AST of clinical isolates of *H. influenzae, S. pneumoniae, N. gonorrhoeae*, and *M. catarrhalis,* using the optimized parameters determined.

SGE was evaluated as a practical alternative for the determination of MICs of fastidious organisms.

Materials and Methods

A) Antimicrobial Agents

Standard antibiotic powders (Sigma Chemical Co., St. Louis, MO) were used to prepare stock antibiotic solutions for dilution test. Table 24 at the back of this chapter show the antimicrobial agents tested and concentration ranges for reference dilution test.

Fluoroquinolones antibiotics are recommended by the CLSI (CLSI, 2005b) as the first line drugs and they have a high rate of resistance for pneumococci in Hong Kong (Ho *et al*, 2004). However, they were not included in the present studies. FQ are not prescribe for children under 12 years old in Hong Kong. They were not used for pneumococci as the first line drugs locally, since it may lead to the selection of more resistant strains. In the present studies, it was only a preliminary research and I will try to involved more classes of antibiotics.

These concentration ranges were selected because they included the breakpoint concentration and the highest MICs expected for these organisms. The concentration range chosen for the SGE test was even broader for each antibiotic so that the SGE test range would include the standard dilution test range. Master

stock solutions of the drugs were prepared according to the NCCLS (2000a) recommendations. The diluent used for all drugs was distilled water.

B) Bacterial Strains

Clinical isolates of *H. influenzae, S. pneumoniae* (representing each susceptibility category for penicillin) and *M. catarrhalis* (around 50 of each), and 20 isolates of *N. gonorrhoeae* from various sites of infection were tested. There were variation in number of strains tested since many strains were lost in the Protect kit during the course of experiments. It was because the Protect kit may not tolerate several freeze and thraw procedures. The clinical isolates were obtained from Kwong Wah Hospital, Ruttonjee Hospital, Pamela Youde Nethersole Eastern Hospital and Sai Ying Pun Polyclinic. Isolates were identified as *H. influenzae* on the basis of XV factor requirement, as *S. pneumoniae* based on susceptibility to optochin and solubility in bile salt, as *M. catarrhalis* by using Tributyrin diagnostic tablet (Oxoid, UK) and oxidase reaction, and as *N. gonorrhoeae* by QuadFERM+ kit (bioMerieux Vitek, Hazelwood, MO). Isolates were stored frozen at -70° in Protect Kit (Technical Consultant Ltd., UK) and subcultured at least twice on horse blood agar or chocolate agar prior to testing. After the Protect Kit was removed from the refrigerator and brought back to room temperature, isolates were removed from the kit and streaked onto 5% horse blood agar or chocolate agar. The plates were incubated for 18 to 24 hour at 35°C in 5% CO_2. After incubation, one isolated colony was picked from the plate, streaked to a new agar plate and incubated for another 18 to 24 hours at 35°C. A suspension of growth

from the isolate was prepared in 10 mL of peptone water adjusted to equal the turbidity of 0.5 McFarland Standard and then centrifuged and made to a 10^9 CFU/mL suspension. SGE susceptibility testing was performed using this adjusted inoculum.

Quality control strains used were *S. pneumoniae* ATCC 49619, *H. influenzae* ATCC 49247, and *N. gonorrhoeae* ATCC 49226, which were included in all batches of susceptibility testing.

C) Reference MIC Determination

Conventional methods of SBD and SAD as described by the CLSI (NCCLS, 2003a, b; 2004b; CLSI, 2005b) were used as the 'gold standard' whereby the reference MICs were determined by diluting the antibacterial agents into appropriate broth or agar to give a series of doubling dilutions.

MH broth (Oxoid) supplemented with Haemophilus Test medium supplement (code SR158E; Oxoid) (HTM broth) was used for the susceptibility testing of *H. influenzae*. They were homemade by the procedures recommended by the CLSI (2005b) and the Oxiod manual. Cation–adjusted MH broth (Oxoid) supplemented with 5% lysed horse blood was used for *S. pneumoniae.* For *N. gonorrhoeae* GC agar base (Oxoid) with 1% defined growth supplement (Vitox, Oxoid) and soluble Hemoglobin powder (Oxoid) were used whereas MH agar supplemented with 5% lysed horse blood was used for susceptibility testing of *M. catarrhalis* .

Standard Agar Dilution

For SAD, the standard bacterial suspension (0.5 MacFarland Standard) was diluted 1:10 in peptone broth, and inoculated onto antimicrobial containing plates with a Steers replicator, delivering inocula of 1μL (10^4 colony-forming-unit/spot). Tests for *N. gonorrhoeae* were incubated at 35°C for 20 to 24 hours in 5% CO_2 and for *M. catarrhalis* at 35°C for 18 hours in ambient air. MICs were read by visual inspection and defined as the lowest concentration totally inhibiting growth. The procedures of the SAD were as follows:-

Preparation of stock solution using the formula:

$$\frac{1000}{P} \times V \times C = Wt$$

P	=	potency given by manufacturer
V	=	volume in mL required
C	=	final concentration (multiples of 1000)
Wt	=	weight of antibiotic in mg

GC agar base with defined growth media (Jones et al, 1989), for *N. gonorrhoeae*; and MH agar supplemented with 5% lysed horse blood for *M. catarrhalis*, were used for susceptibility testing. The pH of each batch of agar was checked by allowing a small amount of agar to solidify around the tip of a pH meter in a beaker and the pH was adjusted to between 7.2 and 7.4 if necessary. 18mL of molten agar was distributed into screw-capped universal bottles before sterilization, sterilized by autoclaving at 121°C for 15 minutes, and allowed to equilibrate to 48°C to 50°C in a preheated water bath. After the agar had equilibrated, appropriate supplements, and appropriate dilutions of antimicrobial solutions were added to molten test agars (details are provided in the Appendix I;

NCCLS, 1997). The agar and antimicrobial solution were thoroughly mixed and then poured into 90 mm diameter petri dishes on a level surface, resulting in an agar depth of 3mm to 4mm. The agar was allowed to solidify at room temperature and the surface of the plates was completely dried by placing them in the incubator for approximately 30 minutes.

Inoculum of control strains and test strains was prepared by making a direct saline suspension of isolated colonies selected from an 18 to 24 h agar plate. The density of suspension was adjusted to match the 0.5 McFarland turbidity standard and the inoculum standardized using a spectrometer. To confirm viability, a direct plate count was performed. The 0.5 McFarland suspensions were diluted 1:10 in sterile saline or broth to obtain the desired inoculum concentration of 10^7CFU/mL, and the adjusted suspension used for final inoculation within 15 min of preparation. An aliquot of each well mixed bacterial suspension was placed into the corresponding well in the replicator inoculum block. The plates with antimicrobial agents were inoculated with 1 to 2 μL of the bacterial suspension using a multi-point inoculator. The final inoculum on the plate contained approximately 10^4CFU/mL

A growth control plate with no antimicrobial agent was inoculated first and then plates containing increasing concentrations of antibiotic. Finally, a second growth control plate was inoculated to ensure there was no contamination or carry over of

antimicrobial agent. A sample of each inoculum was streaked onto an agar plate and incubated to detect contamination.

After inoculation, plates were allowed to stand at room temperature until the inoculum had been absorbed into the agar. The plates were incubated at 35°C for 16 to 20 hours. Plates for *N. gonorrhoeae* were incubated in an atmosphere of 5% CO_2. After incubation, the MICs were recorded as the lowest concentration of antimicrobial agent which completely inhibited growth. A single colony or a faint haze caused by the inoculum was disregarded. The plates were examined on a dark and non-reflecting surface.

The test was repeated if two or more colonies persist in concentrations beyond an obvious endpoint, or there is no growth at lower concentrations but growth at higher concentration.

Standard broth microdilution test

Micro-dilution trays (Falcron) were freshly prepared with antibiotics on each occasion. (For dilution scheme, please see appendix II) Each well contained 0.1mL of broth and antimicrobial agents.

The inocula were prepared by making a direct MH broth suspension of isolated colonies from 18 to 24 h non-selective agar plates. The suspension was adjusted to match the 0.5 McFarland standard. The concentrations of the inocula were

confirmed by spectrophotometer OD_{625} reading. The 0.5 McFarland suspension was diluted one in ten in sterile MH broth to obtain an inoculum concentration of 10^7 CFU/mL.

Plastic replicators were used to transfer 5μL of the inoculum from the diluted suspensions to the wells of the microbroth dilution trays. Microbroth dilution trays were inoculated within 15-20 minutes of inoculum preparation. An aliquot of the diluted bacterial suspension was subcultured to a non-selective agar plate (Chocolate agar for *H. influenzae*, blood agar for the other fastidious organisms) and incubated for 18-24 hours to check the purity of the isolates.

An uninoculated well of broth served as a negative growth control. Each inoculum was added to a well of growth medium without antimicrobial agent, which served as a growth control. After inoculation, each microbroth dilution tray was covered with a tight-fitting plastic cover. The trays were not stacked more than 4 high, and the trays were incubated at 35°C for 20 hours in an ambient air incubator.

After incubation, the growth control well was examined for viability. Acceptable growth (≥2mm button or definite turbidity) must have occurred in the positive control well for the tests to be considered valid. MICs obtained with the control strains were regularly performed to monitor accuracy. The antibiotic-containing wells were examined for growth. Endpoint MICs were the lowest concentrations

of antimicrobial agent that completely inhibited growth as detected by unaided eyes.

As endpoints may trail for bacteriostatic antimicrobial agents, the endpoint for these drugs was read as an 80% to 95% decrease in growth, compared with growth in the control well. If there was a single agent with skipped wells, the test was repeated.

D) SGE Test

For the SGE test, a WASP (Whitley Automatic Spiral Plates) (Don Whitley Co. Ltd., Shipley, UK) model spiral plates was used to dispense 50μL of a stock concentration of antibiotic in an Archimedes spiral onto a rotating plate. The deposition of antibiotic commenced at a point 13mm from the centre and continued outwards towards the edge.

The instrument utilizes a variable cam-activated syringe to create a continuous gradient of antibiotic by dispensing the antimicrobial stock solution at a slower rate (decreasing logarithmically) the further away the dispensing stylus moves from the starting point. After the antibiotic solution had been absorbed into the agar surface, the plates were left a room temperature for sometime to allow antibiotic diffusion to create the concentration gradient. The optimisation study showed inoculation could be performed from 1 hour to 8 hours after the deposition of antibiotics. In this study, after the deposition of antibiotics, the

plates were allowed to stand for 1 hour before the inoculation of organisms. The bacterial suspension (10^9 CFU/mL) was streaked radially from the periphery of each plate to a distance of 20mm from the centre, using a sterile cotton swab. A pair of streaks were made opposite to each other for each test strain. This was done to compensate for non-level plates.

Immediately after streaking the plates were incubated at 35°C in 5% CO_2. The plates were read after 20-24 hours of incubation and the distance from the commencement of the antibiotic deposition to the endpoint of growth was measured, this distance being referred to as the radial advance (RA). The larger the RA, the lower the MIC values. The volume of antibiotic solution deposited by the spiral plater at any location on the plate is referred to as the deposition factor (DF) and this together with the RA was used to calculate the endpoint MIC by the following equation:

$$\text{MIC } (\mu g/mL) = \frac{\text{DF at RA x Stock concentration of antibiotic}}{\text{Depth of agar}}$$

The detailed procedure of the SGE test, in general was based on information in the existing users' manual (Schalkowsky, 1985). 15cm diameter plates with supplemented agar media were used. Leveled agar plates with constant thickness were required. Control strains of *S. pneumoniae* ATCC 49619, *H. influenzae* ATCC 49247 and *N. gonorrhoeae* ATCC 49226 were included in each batch of SGE testing of the corresponding bacterial species.

Two plates with different concentration ranges that overlapped in the midrange were needed to flank the standard dilution test range, and cover the range of MICs expected. The MICs generated from the low range plate would be used if the level could be estimated from this plate. The stock concentration of the low range plate included the sensitivity category break points so as to reduce the likelihood of error. When the MIC of a particular strain was out of range (i.e. RA ≤20mm) the result on the high range plate was used. For a more direct comparison with the standard dilution method, endpoint MICs of the SGE test were rounded up to next higher two-fold dilution value corresponding to that of the dilution test.

On each plate, the highest concentration for reliable endpoint estimation occurred at an RA of 7 mm and the lowest agar concentration occurred at an RA of 60 mm. The DF of a WASP model spiral plater was the same as that of a model D spiral plater, so the table of DF given in the users' guide for antimicrobial susceptibility testing using SGE was employed. (Schalkowsky, 1985).

The corresponding SGE stock concentrations and the range of MICs covered are shown Table 25.

E) SGE Method (Schalkowsky, 1985)

HTM was used for susceptibility testing of *H. influenzae*; MH agar for *M. catarrhalis*; GC agar base with 1% special supplement for *N. gonorrhoeae*, and MH agar supplemented with 5% horse blood for testing of *S. pneumoniae.*

To prepare level agar plates with constant agar depth, 50mL of the appropriate melted agar media was dispensed into a glass bottle and sterilized. After the agar had cooled to about 50°C, the media was poured to a 15cm petri dish to give an agar depth of 3.247mm. The agar plates were allowed to set on a completely level bench to ensure that the plates had a smooth surface and uniform depth over the entire plate. The plates were dried in the incubator for immediate use or stored at 4°C for future use.

The WASP spiral plater was set to 15cm plate option resulting in 50μL log dispensing, fast speed, manual operation and value filling mode. The stylus was washed at least twice by pressing the "Wash" button before dispensing any antibiotic solution. The "Sample" button was pressed to position the stylus ready for the uptake of antibiotic stock solution into the syringe and stylus assembly. The corresponding antibiotic stock solution was placed in the sample position and "Fill" button depressed for approximately 5 seconds, ensuring the air slug had passed through the sight glass before completing the filling exercise. The 15cm diameter petri dish with appropriate susceptibility test medium was placed on the turntable ensuring that it was correctly centered between the jams. The "Plate" button was pressed to initiate the deposition of antimicrobial stock solution. On

completion of the plating routine, the stylus returned to the park position automatically. The plating procedure was repeated for two more plates as 3 plates with the same concentration of deposition can be produced after a single sample uptake in value mode. After deposition of the antibiotic stock solution, the stylus was washed by pressing the "Wash" button. Washing was performed 4 times between inoculating plates of different concentrations and /or different antibiotics.

The plates with antibiotic solution were left at room temperature for 1 h to allow antibiotic diffusion to create the gradient. A test culture suspension with concentration of 4 McFarland Standard (10^9 CFU/mL) was prepared by centrifugation of 10^8 CFU/mL suspension and the inoculum concentration verified by comparing with McFarland standard and plate count. The test culture was applied to agar surface, using a template to guide the applications. The edge of the petri dish was marked and the mark aligned with number 1 line when placing the dish over the template. A sterile swab was dipped in the culture, squeezed lightly against the side of the container, and streaked on the agar following the template guideline, starting the streak with the swab at the outer edge of the plate and stopping 20mm from the centre. On the opposite side of the diameter, another streak of the same test culture was deposited, using the same swab without re-dipping. Up to 8 test cultures in duplicate were placed on each plate. One control strain was included on each plate.

The plates were incubated for 20-24 h at 35 - 37°C. For tests of *H. influenzae, S. pneumoniae* & *N. gonorrhoeae,* the plates were incubated in 5% CO_2, and for *M. catarrhalis* in ambient air. Radial Advance (RA) was measured after incubation by subtracting 26mm from the distance (D) between endpoints of growth on opposite sides of the plate and the result divided by 2.

$$RA = \frac{D - 26}{2}$$

The end point growth was distinguished by viewing the plate against a dark background to highlight the presence of small colonies. Larger separate colonies were ignored, and the point marked where visible continuous growth ended.

F) Statistical Analysis

To determine the degree of agreement between standard dilution and SGE tests, the distribution of difference in the $\log_2$ dilution MIC results among the strains was examined, and the percentage of isolates which yielded identical results within the accuracy limit of the gold standard test (± 1 $\log_2$ dilution) was calculated. Using the definition of errors category discrepancies described by Thornsberry and Gavan (1980), categories of very major error, major error and minor error were used to describe errors of false susceptibility, false resistance, or response involving an intermediate result respectively. Contingency tables were constructed and the chi-square test was used to analyze if there was a statistical difference in the population proportions of the three categories when the MIC was determined by standard dilution and SGE method. The decision level was set at a

significance level of 0.05 for rejecting HA. Regression analysis was also carried out and Pearson correlation coefficient was calculated for each organism to measure the overall association between the $\log_2$ dilution MIC results of the SGE method and the standard dilution method.

Finally, to see whether there was a trend for the SGE method to produce significantly higher or lower results than the standard dilution method, Wilcoxon signed rank test was performed on the difference of $\log_2$ MIC of the SGE test results compared with standard dilution test. MIC results within ± 1 $\log_2$ dilution were assumed to be equivalent for this test.

Results

1. H. influenzae

The MIC of cefuroxime, tetracycline, ampicillin and chloramphenical were determined using SBD as recommended by the NCCLS (2000) and the SGE test (Schalkowsky, 1985). On all experimental occasions, the control strain of *H. influenzae* was well within the acceptable reference range.

Differences in the $\log_2$ dilution between the SGE MIC values and the corresponding SBD MIC values, and the percentage number of comparisons for each of the antibiotics are shown in Table 26. Notably 53.8% of the MIC values all four antibiotics were the same for both the SGE and the reference method. Overall 96.1% of the SGE MICs were within ± 1 $\log_2$ dilution of the SBD method

and thus could be considered equivalent, with 100% of the SGE MICs being within ±2 dilutions of the reference method. The lowest agreement (within ±1 $\log_2$ dilution) occurred with tetracycline (94.6%) and the highest agreement with cefuroxime (97.8%).

The dilution test sensitivity category vsSGE test sensitivity category for the antimicrobial agents are shown in Table 27.

Susceptibility category changes were observed for 13 (6.2%) tests using SGE for susceptibility testing. All of these were minor errors involving the intermediate resistance category.

1. 1. Cefuroxime

For cefuroxime, 97.8% of the MIC values determined by the SGE method agreed within ±1 $\log_2$ dilution to the MIC values determined by SBD. There was only 2.2% minor error: a susceptibility category change was observed for 1 isolate when SGE was used, the isolate which appeared sensitive by the reference test was intermediately resistant. This discrepancy represented a difference of one dilution and occurred at the breakpoint between intermediately resistant and sensitive.

1.2. Tetracycline

For tetracycline, 57.1% of the MIC values were the same for both methods. The MICs were in agreement within the range ±1 $\log_2$ dilution for 94.6% of organisms

and within 2 dilutions for 100%. Susceptibility category changes were observed for 4 (7.14%) isolates (Table 27): the SGE method interpreted 1 isolate as intermediate resistant which was found by SBD as sensitive; and there were also three discrepancies (5.4%) for isolates categorized as intermediate resistant by SBD but as highly resistant by the SGE method. These were all minor errors occurring at the breakpoint between 2μg/mL to 4μg/mL, and 4μg/mL to 8μg/mL representing a difference of one dilution.

To determine whether the SGE method could accurately categorize the isolates into different susceptibility categories, chi-square test was used to test whether the number of isolates in each susceptibility category as determined by SGE method differed from that determined by SBD ($df = 2$, $X^2 = 2.672$, $p = 0.263$), showing no significant difference between the methods.

Wilcoxon signed-rank test (Lehman, 1985) showed that the SGE method produced MIC values that were not significantly different from the MICs by SBD by more than 1 $\log_2$ MIC dilution for tetracycline ($p = 0.285$).

1.3 Ampicillin

Of the ampicillin MIC assays performed, 27 (50.9%) of the SGE method MICs were the same as the MIC obtained with SBD, and 51 (96.2%) agreed within 1 $\log_2$ dilution of the MICs by SBD. All MICs obtained by SGE method agreed within 2 $\log_2$ dilution with those obtained using SBD.

SBD classified 34 isolates as sensitive, 17 isolates as resistant, and 2 as intermediately resistant to ampicillin. Susceptibility category changes were observed for 3 (5.7%) of these isolates when the SGE method was used to determine the MICs, with one isolate (1.9%) categorized as intermediately resistant by the reference method labelled as highly resistant, and 2 isolates (3.8%) categorized as sensitive by the reference method as intermediately resistant by SGE. So there were a total of 5.7% minor errors. There may be a lot of BLNAR strains that lead to committed of errors, however by now it was difficult to recognize such strains and thus it was not elaborated. It may be a good tropic for further studies.

Chi-square test showed that the number of isolates in each susceptibility category of ampicillin determined by SGE method was not significantly different from that determined by SBD ($X^2 = 0.559$, $p = 0.756$).

Wilcoxon Signed–Rank Test (Lehman, 1985) also showed that SGE did not produce significantly lower or higher results than 1 $\log_2$ dilution than the reference method ($p = 0.180$).

1.4 Chloramphenicol

For chloramphenicol, SGE MICs were the same for SBD method for 25 strains (45.5%), within one doubling dilution for 53 strains (96.4%) and within two doubling dilutions for all strains.

The results of susceptibility testing of chloramphenicol are shown in Table 27. No very major error or major error occurred with the SGE method, however, 6 (10.9%) minor errors were observed: 5 (9.0%) of all strains intermediately resistant by SBD were interpreted as highly resistant by the SGE test. These errors were with strains exhibiting MIC values which clustered near the breakpoint between intermediately resistant and highly resistant. The SGE MICs were higher by 1 $\log_2$ dilution when compared with standard methods. Moreover, 1 resistant strain (1.8%) by SBD was interpreted as intermediately resistant by SGE. This discrepancy also occurred at the breakpoint and represented a difference of 1 $\log_2$ dilution.

Chi-square test showed no significant difference in distribution of resistance categories between the SBD and SGE ($X^2 = 0.50$, $p = 0.779$). The Wilcoxon signed-ranks test also showed that there was no significant trend to either higher or lower MIC values with SGE ($p = 0.180$)

.1.5 Overall

Regression analysis was carried out and Pearson correlation coefficient was calculated to measure the overall association between the $\log_2$ dilution MIC results of the SGE test compared with the SBD. The adjusted R^2 was found to be 0.913. Logarithm to the base 2 of the MIC measurements of standard dilution method and SGE method were plotted with the line of best fit (Fig. 5).

ANOVA analysis ($F = 2206.774$, $P<0.001$) showed that the model could significantly improve the prediction of the dependent variable. The regression coefficient for SGE MIC is 0.954 (SE = 0.020, $P<0.001$), indicating that SGE MIC is a significant predictor of the true MIC. and SGE MIC is associated with the standard dilution MIC (Table 28). The regression equation may be written as

Expected dilution MIC = 0.323 + 0.954 SGE MIC

Application of the chi-square test revealed that that there was no significant difference in numbers of isolates in the resistance categories between SBD and SGE method ($X^2 = >5.99$, $p = 0.191$). The Wilcoxon signed-rank test showed no significance trend to higher or lower MIC values if the SGE test was used ($p = 0.674$)

2. *S. pneumoniae*

All the result for MICs of the control strain *S. pneumoniae* ATCC 49619 were well within the acceptable reference ranges. The $\log_2$ dilution differences of the

SGE MIC values and the corresponding broth micro-dilution are shown in Table 29.

It was observed that 56% of the MIC values were the same for the SGE method and the reference method. Notably, 98.1% of the SGE MICs were within ±1 $\log_2$ dilution of the SBD method, and 99.5% of the SGE MICs were with ±2 $\log_2$ dilution. The highest agreement was noted with vancomycin (100%) and the lowest with erythromycin (94.7%).

The SGE test sensitivity category compared with standard dilution test sensitivity category for all the antibiotics are shown in table 30.

When SGE test was used for susceptibility testing, category changes were observed for 13 (6.3%) of the tests. No very major or major error occurred, all of the errors were minor involving the intermediate resistance category. All of the errors occurred at the breakpoint of susceptibility category and represented a decrease or increase of 1 $\log_2$ dilution compared with the standard dilution method.

A regression analysis that utilized all of the 207 direct comparisons of SGE method and standard dilution method obtained from tests of the 4 antimicrobial agents was performed which is shown in Fig 6.

Regression analysis yielded r^2 = 0.922 and the Regression Equation

Expected dilution MIC = 0.366 + 0.993 SGE MIC

ANOVA (F value = 2425.32, r = 0.960, P<0.001) showed there was a significant association between SGE and standard dilution MIC values. Chi square analysis of the numbers of strains in each of the resistance categories revealed that there was no significance difference between SBD and SGE (X^2 = 0.310, p = 0.856). Wilcoxon Signed-Ranks Test revealed that the two sided p value was 0.674, indicating no trend to higher or lower values if SGE was used to determine MICs.

2.1 Penicillin G

Penicillin MICs were the same in 49.1% of strains for both methods, and all MICs agreed within 1 $\log_2$ dilution of the MICs determined by the SBD.

When SGE was used for susceptibility testing, category changes were observed for 2 (3.5%) strains, both were only minor errors and occurred at the breakpoint between intermediate resistant and highly resistant. The minor errors represented an increase of 1 $\log_2$ dilution MIC by the SGE test compared with the SBD. These changes were not found to be significant (X^2 = 0.339, p = 0.844).

2.2. Erythromycin

For erythromycin, 47.4% of the MIC values were the same for the SGE method and SBD, 94.8% within 1 $\log_2$ dilution, 98.2%, within 2 $\log_2$ dilution, and 100% within 3 $\log_2$ dilution. Susceptibility category changes were observed for 4 (7.0%) of these isolates when SGE method was used to determined the MICs (Table 30), the SGE method interpreting 3 isolates categorized as intermediate resistant by SBD as sensitive, and 1 isolate categorized by SBD as highly resistant, as intermediate resistant. So there were a total of 4 (7%) minor errors, all of which occurred at the breakpoint between sensitive and intermediate resistant, or between intermediate resistant and highly resistant, and represented a change of only 1 $\log_2$ dilution by the SGE method compared with SBD. Statistical analysis showed no significant difference in numbers of strains in each resistance category ($X^2 = 1.129$, $p = 0.569$), nor any trend to increased or decreased values (Wilcoxon signed rank test, $p = 0.102$).

2.3. Vancomycin

Of the vancomycin MIC assays performed, 65.2% of the SGE MICs were the same as the MIC obtained with SBD. All of the MICs of the SGE method agreed within 1 $\log_2$ dilution of the MICs determined by SBD. Since there were no vancomycin-resistant strains of *S. pneumoniae* for vancomycin, chi-square test for categories was not performed. As all SGE MICs were not greater or smaller than 1 $\log_2$ dilution compared with standard dilution method, it can be concluded that

there were no significant difference between the MIC values produced by SGE and SBD for susceptibility testing of vancomycin.

2.4 Cefotaxime

Since most of the strains were not iosolated from CSF, the non-meingitis breakpoint were used to categorize the susceptibility categorises. There were 66.0% of the MIC values were the same for the SGE method and SBD, 97.9% were within the range ±1 $\log_2$ dilution, and 100% were within 2 dilutions. The SBD method classified 6 isolates as cefotaxime resistant, 12 with intermediate resistance, and 29 as susceptible. Susceptibility category changes were observed for 7 (14.9%) of these isolates when SGE method was used to determine the MICs (Table 30), all changes being minor errors. SGE interpreted 3 (6.4%) isolates categorized as being intermediate resistance to cefotaxime by SBD as sensitive. There were 3 discrepancies (6.4%) for isolates categorized as sensitive by SBD but classified as intermediate resistant by the SGE method. One (2.2%) isolate of the six classified as resistant to cefotaxime by SBD was interpreted as being intermediately resistant to cefotaxime by SGE method. The differences in resistance categories was shown not to be significantly different ($X^2 = 0.250$, $p = 0.822$) Since only one isolates (2.1%) had an MIC difference of ≥ 2 $\log_2$ dilution between SBD and SGE test, it was clear that SGE did not produce significantly lower or higher results than 1 $\log_2$ MIC result that standard dilution test., so Wilcoxon signed-ranks test was not performed.

3. *M. catarrhalis*

A control strain of *Staphylococcus aureus* ATCC 29213 was included in each batch of susceptibility testing. All the MIC values of the control strain were within the acceptable limit as described by NCCLS (1998).

The $\log_2$ dilution differences of the SGE MIC values relative to SBD and the resistance classifications of the strains tested by both methods are shown in Table 31.

It was noted that 42.3% of the MIC values were the same for the SGE and SAD. Overall 90% of the SGE MIC values were within ±1 dilution of the standard method MIC values and thus were considered equivalent; and 98.2% of the SGE MICs were within ±2 dilution of the standard dilution MICs. Erythromycin SGE MIC testing were found to have higher agreement (within 1 $\log_2$ dilution) (96.4%) than penicillin (83.6%) when compared with SAD.

Using the breakpoint of ≤0.12 μg/mL as sensitive and ≥0.25 μg/mL as resistant for penicillin, and ≤0.5 μg/mL as sensitive and ≥8 μg/mL as resistance for erythromycin, there was no significant difference in numbers of isolates in every susceptibility categories compared with reference test (X^2 test, $P>0.001$). Regression analysis was carried out and Pearson correlation coefficients were

calculated to measure the overall association between the $\log_2$ dilution MIC results of SGE test compared with standard dilution test. The r2 value was 0.930 and ANOVA analysis showed there was significant association between the values obtained by SGE and SAD ($f = 1454.12$, $p = <0.001$). The regression equation is shown below:

Dilution MIC = 0.045 + 0.856 SGE MIC

The data was plotted on a scatter plot (Fig.7).

Wilcoxon signed-ranks test (Lehman, 1985) showed that there was no trend for SGE to produce significantly lower or higher MIC results than the SAD ($p = 0.286$). Differences of ± 1 $\log_2$ dilution were neglected since the accuracy limits of the SAD are within ± 1 $\log_2$ dilution.

3.1 Penicillin

There was a high level of agreement of MIC values determined by the SGE and SAD method, within ± 1 dilutions (83.6%). Agreement within ± 2 log dilutions was 98.2%. There was no trend for higher or lower values for MIC determined by SGE (Wilcoxon signed-ranks test, $p = 0.109$).

3.2 Erythromycin

For erythromycin, 55.4% SGE MIC values were the same as the SADmethod, within ±1 log_2 dilution for 96.4%, and within ±2 log_2 dilution, for 98.2%. Once again, there was no evidence of higher or lower values of MIC using SGE (Wilcoxon signed-ranks test, p = 0.655).

4. *N. gonorrhoeae*

All MICs of the control strain of *N. gonorrhoeae* ATCC 49226, were well within the acceptable reference range suggested by the NCCLS (1998). The difference between the log_2 MICs by SGE and SAD of tested strains are shown in Table 32

Overall, 62.5% of the MIC values were the same for the SGE method and SAD method, with 97.5% of the SGE MICs being within ±1 log_2 dilution of the SAD method, and 100% of the SGE MICs within ±2 log_2 dilution.

The SGE test susceptibility categories compared with SAD test are shown in Table 33. Susceptibility category change was observed for 2 (5%) strains when SGE was used, both being minor errors and representing a difference of 1 log_2 dilution at the sensitivity category breakpoint.

A regression analysis utilizing all 40 direct comparisons of SAD and SGE MICS was performed. The r^2 value was 0.953 and ANOVA analysis showed a significant association between the values obtained by the two methods (f = 375.159, p = <0.0001) The distribution of the MICs is shown in Fig 8 .

Regression equation

Expected Dilution MIC = 0.406 + 1.073 SGE MIC

The categories of susceptibility assigned by the two methods were shown not to be significantly different ($X^2 = 0.129$, $P = 0.937$). This result was also observed for individual antibiotics (penicillin $X^2 = 0.393$, $P = 0.822$; tetracycline $X^2 = 0.343$, $P = 0.842$).

Wilcoxon signed-ranks test was not performed since ± 1 $\log_2$ MIC values were assumed to be the same, and there were only a very low number of MIC differing by >1 $\log_2$ dilution.

Discussion

With increasing resistance of fastidious organisms to antimicrobial agents, accurate and rapid assessment of the susceptibility of fastidious organisms has become a major concern. Problems of interpretation with the Kirby-Bauer disk diffusion test make an MIC determination desirable for patients with life threatening infections, or when there is unexpected failure of treatment or an unusual resistance pattern observed.

For *S. pneumoniae*, the disc diffusion test cannot precisely detect the degree of penicillin resistance (Brown, 1994) and isolates with an oxacillin inhibition zone of ≤19mm require testing with a MIC method to determine the degree of resistance (Jorgenson *et al*, 1994). Non-meningeal foci of infection may be adequately treated with high dose penicillin in infections caused by relatively resistant pneumococci (Friedland & McCraken, 1994), whereas high dose penicillin therapy may lead to treatment failure in meningitis caused by relatively resistant strains (Friedland & McCraken, 1994). The disc diffusion method has not been standardized for testing of broad spectrum cephalosporins such as cefotaxine or ceftriaxone (Jorgenson *et al*, 1993).

The SBD is labour intensive, cumbersome, requires skilled technique, and the inherent errors of the test may result in a number of ambiguous results. The SBD techniques are difficult to perform and commercial tests are not commercially available for most fastidious organisms. Vitek 2 provides a commercial automated test for AST of S. pneumoniae, but the initial investment for the machine is very high. Both E test and SGE test seem to offer good alternatives to the conventional MIC method. However, although the methodology of E test is very simple, there are few parameters that can be standardized or controlled by the user, preventing thorough evaluation. So, the performance of the E test cannot be monitored and depends entirely on the quality of the E test strips, which may be subject to batch variation. Moreover, the retail price of E test is relatively high, especially in developing countries meaning the E test is not an economic

method for testing a number of drugs on each isolate. There is also a storage problem associated with the E-test strips, as the strips have to be kept at -20°C but extreme care must be taken to avoid any moisture (AB Biodisk 1994). There may be condensation of water when the strips are taken out from the refrigerator, and some drugs may be subject to deterioration in a short time, making it difficult to ensure an accurate result. On the other hand,, SGE must be carefully evaluated before the system is adopted for routine use.

In the present study, the results have shown the SGE test is a precise and accurate method for MIC measurement of fastidious organisms. It is easy to perform in a routine laboratory and the equipment required is minimal. Moreover, the price per 100 tests was only a few hundred Hong Kong dollars and was the cheapest method amongst the others (SAD, E test, Vitek 2 and FCM-AST) (see Appendix ix). The only requirement for SGE was antibiotic stock solution and the test could be performed at any time at short notice. Comparison of the results of SGE tests with the 'gold standard' in common laboratory practice of standard doubling dilution tests, showed that associations between the two methods were excellent. The Pearson correlation coefficient between the standard dilution method and the SGE test on the four fastidious organisms was between 0.956 to 0.965 and there were significant associations between the two methods for all organisms tested ($P<0.001$).

In an earlier study by Paton et al (1990) on non-fastidious organisms, in which 81 Gram negative and Gram positive aerobic bacteria were tested by SGE method and standard SADdilution method using8 different antibiotics, the Pearson correlation coefficient was found to range from 0.86 to 0.96. The present study's results show higher correlation. Paton *et al* (1990) showed that the percentage agreement between the SGE test and standard dilution test range was 46% to 88% within a twofold dilution, and 88% to 100% agreement within two twofold. The present study showed agreement between the 2 methods ranging from 83.7% to 100% agreement within 1 $\log_2$ dilution of the reference method, and 96.4% to 100% within two twofold dilutions.

In an earlier study on *S. pneumoniae* (Pong *et al*, 1998), without optimization and using the suggested test protocol (Schalkowsky, 1985), the percentage of susceptibility category agreement with penicillin, cefotaxime and cefoperazone for the SGE test ranged from 80% to 87.5% when compared with the standard dilution test. There was no significant differences in the number of isolates in each susceptibility category when susceptibility tests were performed by standard dilution test and SGE test (X^2 test, $P>0.05$).

In the present study, following optimization, the SGE MIC values were somewhat higher, resulting in much better agreement of MIC values, and the percentage of susceptibility category agreement of SGE testing of *S. pneumoniae* with penicillin, erythromycin, and cefotaxime ranged from 85.1% to 96.5% compared with the

standard dilution test. There was also no significant difference in numbers of isolates in each susceptibility category (X^2 test, $P>0.05$). All the interpretive errors in the present study were minor. For comparison, for cefotaxime there were 12.5% to 15% minor errors and 5% very major errors in the previous study (Pong *et al*, 1998), but all errors in the present study represented a difference of only 1 $\log_2$ dilution in the MICs of the isolates at breakpoint between susceptibility categories. In this study, the percentage agreement between the two methods ranged from 96.5% to 100% agreement within 1 $\log_2$ dilution,. and 100% within two twofold dilutions compared with 87.5% to 95% agreement within 1 $\log_2$ dilution of the reference method and 95% to 100% within two twofold dilutions in the previous non-optimized study (Pong et al, 1998). Category discrepancies in the previous study were largely due to the tendency of the MICs to be slightly lower using SGE test and the MICs for penicillin, cefotaxime, and cefoperazone were significantly lower than those achieved by the reference standard dilution method (Wilcoxon signed-ranks test, $P<0.05$). This was attributed to the diffusion of drugs in the plates. At the correct endpoint location on the plate, the antimicrobial concentration had increased due to diffusion from the higher concentration region, so the radial advance (RA) became larger with a lower deposition factor (DF) and the endpoint MICs were lowered. This diffusion problem was counteracted in the present study, where SGE test parameters were adjusted higher up the SGE MIC, and the SGE MIC of penicillin, erythromycin, cefotaxime, and vancomycin were shown not to be significantly higher or lower

than 1 $\log_2$ dilution of the MIC of standard dilution test (Wilcoxon signed-ranks test, P>0.05).

For other fastidious organisms, the SGE test was also found to have excellent correlation with standard dilution tests. For *H. influenzae,* the percentage of susceptibility category agreement with all the drugs tested ranged from 93.8% to 97.8% compared with SBD and there was no significant difference in the number of isolates in each susceptibility category between tests (X^2 test, P>0.05). All the interpretive errors were minor. The percentage of MICs in agreement with the reference method ranged from 94.6% for tetracycline to 97.8% for cefuroxime within one twofold dilution, and 100% within two dilutions.

These results are comparable to those of Jorgensen *et al* (1991), who evaluated the E-test for antimicrobial susceptibility testing of *H. influenzae* using both HTM and PDM ASM II chocolate agar. E-test MICs for a total of 10 antibiotics were compared with SBD MICs. The overall agreement between E-test MICs measured on HTM agar and SBD MICs ranged from 76.5% for ampicillin to 99% for erythromycin. Giger at al (1996) also evaluated E-test for susceptibility testing of *H. influenzae*, using eight antimicrobial agents. Although overall agreement was good, E-test on HTM failed to detect one of the nine BLNAR strains and failed to categorize this strain as ampicillin- resistant. In the present study, all BLNAR strains were categorized correctly by the SGE test as ampicillin-resistant. The

overall performance of the SGE is therefore at least as good as E test for susceptibility testing of *H. influenzae*.

Monitoring of the antimicrobial susceptibility of *N. gonorrhoeae* is essential in an environment of rapidly changing resistance patterns. There are currently no commercial methods to determine the MICs of isolated strains of *N. gonorrhoeae* other than the E-test. However, E-test MICs have been reported to be substantially lower than SAD determinations for gentamicin, penicillin, tetracycline, ciprofloxacin, and chloramphenicol (Sanchez *et al*, 1992; Yeung *et al*, 1993; Van Dyck *et al*, 1994; Daly *et al*, 1997). In contrast, 70% of erythromycin MICs were twofold higher using E-test (Baker *et al*, 1991; Sanchez *et al*, 1992). There was also a trend for cefuroxime and cefotaxime E test MICs to be 1 to 2 $\log_2$ dilutions higher than the reference SAD MIC. The quantitative accuracy of E-test was reduced by 55% to 80% (Schchez *et al*, 1992). The total quantitative accuracy (± 1 $\log_2$ dilution step) obtained between the E test and agar dilution results ranged from 77.4% to 97.5% (Baker *et al*, 1991; Sanchez *et al*, 1992; Yeung *et al*, 1993; Van Dyck *et al*, 1994; Daly, 1997). A significant relationship ($P<0.001$) between E-test and SAD was observed, with correlation coefficients of 0.76, 0.87, 0.88, 0.94 and 0.99 for ciprofloxacin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, and penicillin respectively (Van Dyck *et al*, 1994). The low correlation coefficient of ciprofloxacin (0.76) is of concern due to a high resistance rate of ciprofloxacin in Asia. This low correlation of coefficent may be due to the comparison of a continuous scale (Etest) with a discrete scale (SAD).

Although ciprofloxacin was not included in the present study, it was expected to be an accurate test as extrapolated from the performance of the SGE test on other drugs In the present study, the Pearson correlation for SGE test and SAD for penicillin and tetracycline were 0.953, which are comparable to the coefficients for penicillin and tetracycline in the E-test evaluation. Moreover, SGE test did not produce significantly lower or higher than 1 $\log_2$ dilution MIC result than SAD.

One study evaluating the use of E-test for *N. gonorrhoeae*, reported 9 strains categorized as resistant by SAD to be wrongly classified as susceptible, which constituted 1.4% very major errors (Van Dyck *et al*, 1994). In another study (Sanchez *et al*, 1992), the minor error rates for penicillin and tetracycline were 25% and 20% respectively. Use of SGE produced only minor errors and these represented only 5% of all strains tested. So, overall SGE test seems to produce more accurate results.

The accuracy and reproducibility of SGE have shown this method to offer an acceptable alternative to dilution methods or E-test for determination of MICs of fastidious organisms. The major disadvantage is the initial cost of the spiral plater and the initial correlation of results with the standard method. However, the cost of a spiral plater is not too high a price to be purchase. Once this has been completed the method offers the advantage of extreme flexibility of antimicrobial agents to be tested, allowing rarely used drugs to be tested. This can be cost saving as E-test requires the minimum purchase of 100 strips with limited shelf

life after opening. Plates can be set up rapidly and results achieved within the same or shorter time scales than standard methods or E-test. The reduced cost allows SGE to be used for all strains of pneumococci thus reducing the time for recognition of PRSP to one day as the intermediate step of oxacillin testing may be omitted.

Table 24. Antimicrobial agents tested and Concentration ranges for reference dilution test.

Organism	Standard M	D	Range
S. pneumoniae	Broth Micro-dilution (SBD)	Penicillin G	0.03 – 16 IU/mL
		Cefotaxime	0.075 – 16 µg/mL
		Erythromycin	0.015 – 32 µg/mL
		Vancomycin	0.015 – 16 µg/mL
H. influenzae	Broth Micro-dilution (SBD)	Ampicillin	0.03 – 64 µg/mL
		Tetracycline	0.03 – 128 µg/mL
		Chloramphenicol	0.03 – 16 µg/mL
		Cefuroxime	0.03 – 16 µg/mL
N. gonorrhoeae	Agar dilution (SAD)	Penicillin G	0.065 – 32 Iu/mL
		Tetracycline	0.06 – 16 µg/mL
M. catarrhalis	Agar dilution	Penicillin G	0.15 – 16 Iu/mL
		Erythromycin	0.03 – 16 µg/mL

Table 25. SGE stock concentrations and the ranges of MIC covered

Organism	Antimicrobial	Low stock (μg/mL)	High range st (μg/mL)	Assay range (μg/mL)
H. influenzae	Ampicillin	700	10,000	0.0179-68.37
	Cefuroxime	2,400	8,000	0.061-54.20
	Tetracycline	1,300	14,000	0.033-95.72
	Chloramphenicol	1,300	14,000	0.033-95.72
S. pneumoniae	Penicillin G	400	10,000	0.01-68.37
	Erythromycin	200	5,000	0.005-34.19
	Vancomycin	1,200	--	0.03-8.130
	Cefotaxime	400	10,000	0.01-68.37
M. catarrhalis	Penicillin G	200	8,000	0.005-54.20
	Erythromycin	600	5,000	0.015-34.19
N. gonorrhoeae	Penicillin G	600	5,000	0.015-34.19
	Tetracycline	600	5,000	0.015-34.19

Table 26: Differences in log_2 MIC between SGE and SBD for susceptibility testing of *H. influenzae*. Antimicrobial agent *Difference in log_2 MIC Cross-tabulation

Antimicrobial A		Difference in log_2 MIC (log_2 SGE - log_2 dilution M)					Total
		-2.00	-1.00	.00	1.	2.00	
Cefuroxime	Count % within	0 .0%	4 8.7%	29 63.0%	12 26.1%	1 2.2%	46 100.0%
Tetracycline	Count % within	2 3.6%	6 10.7%	32 57.1%	15 26.8%	1 1.8%	56 100.0%
Ampicillin	Count % within	0 .0%	14 26.4%	27 50.9%	10 18.9%	2 3.8%	53 100.0%
Chloramphenicol	Count % within	0 .0%	5 9.1%	25 45.5%	23 41.8%	2 3.6%	55 100.0%
Total	Count % within	2 1.0%	29 13.8%	113 53.8%	60 28.6%	6 2.9%	210 100.0%

Table 27: Dilution test sensitivity category vs SGE test sensitivity category *Antimicrobial agent Cross tabulation for *H. influenzae*

Antimicrobial agent			SGE test sensitivity category			Total
			Sensitive	Resistant	In ediate	
Cefuroxime	Dilution test Sensitivity category	Sensitive	45	0	1	46
	Total		45		1	46
Tetracycline	Dilution test Sensitivity category	Sensitive	34	0	0	34
		Resistant	0	14	0	14
		Intermediate	1	3	4	8
	Total		35	17	4	56
Ampicillin	Dilution test Sensitivity category	Sensitive	34	0	0	34
		Resistant	0	15	2	17
		Intermediate	0	1	1	2
	Total		34	16	3	53
Chloramphenicol	Dilution test Sensitivity category	Sensitive	46	0	0	46
		Resistant	0	2	1	3
		Intermediate	0	5	1	6
	Total		46	7	2	55

Table 28: **Regression analysis to measure the overall association between the MIC result of SGE test compared with dilution test for *H. influenzae***

i) **ANOVA[b]**

Model		Sum of Squares	df	Me	F	Sig.
1	Regression	1041.461	1	1041.461	2206.774	.000[a]
	Residual	98.163	208	.472		
	Total	1139.624	209			

b Predictors: (Constant), SGE MIC (log_2)

a. Dependent Variable: Dilution MIC (log_2)

ii) **Coefficients[a]**

Model		Unstandardized Coefficients		Standardized Co ts	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.323	.049		6.546	.000
	SGE MIC (log_2)	.954	.020	.956	46.976	.000

a. Dependent Variable: Dilution MIC (log_2)

Table 29: **The log_2 dilution MIC difference of SGE test and broth micro-dilution test for susceptibility test of *S. pneumoniae:* Antimicrobial agent* Difference in log_2 MIC Cross tabulation**

Antimicrobial a		Difference in log_2 MIC					Total
			.00	1.00	2	3.00	
Cefuroxime	Count % within	8 17.0%	31 66.0%	7 14.9%	1 2.1%	0 .0%	47 100.0%
Vancomycin	Count % within	1 2.2%	30 65.2%	15 32.6%	0 .0%	0 .0%	46 100.0%
Erythromycin	Count % within	7 12.3%	27 47.4%	20 35.1%	2 3.5%	1 1.8%	57 100.0%
Penicillin G	Count % within	16 28.1%	28 49.1%	13 22.8%	0 .0%	0 .0%	57 100.0%
Total	Count % within	32 15.5%	116 56.0%	55 26.6%	3 1.4%	1 .5%	207 100.0%

Table 30: Dilution test sensitivity category* SGE sensitivity cat (number)* Antimicrobial agent Cross tabulation for *S. pneumoniae*

Antimicrobial agent			SGE test sensitivity cat (number)			Total
			Sensitive	Resistant	Intermediate	
Penicillin G	Dilution test Sensitivity category	Sensitive	9	0	0	9
		Resistant	0	27	0	27
		Intermediate	0	2	19	21
	Total		9	29	19	57
Erythromycin	Dilution test Sensitivity category	Sensitive	15	0	0	15
		Resistant	0	33	1	34
		Intermediate	3	0	5	8
	Total		18	33	6	57
Vancomycin	Dilution test Sensitivity category	Sensitive	46			46
	Total		46			46
Cefotaxime	Dilution test Sensitivity category	Sensitive	26	0	3	29
		Resistant	0	5	1	6
		Intermediate	3	0	9	12
	Total		29	5	13	47

Table 31 : Comparison by log_2 dilution of MICs obtained by SGE method versus the dilution method for the clinical isolates of *M. catarrhalis*

Antimicrobial agent		Difference in Log2 MIC					Total
		-1	-2	0	1	2	
Penicillin G	Count % within	1 1.8%	1 1.8%	16 29.1%	26 47.2%	1 1.8%	55 100.0%
Erythromycin	Count % within	1 1.8%	15 26.8%	31 55.4%	8 14.3%	1 1.8%	56 100.0%
Total	Count % within	2 1.8%	16 14.4%	47 42.3%	34 30.6%	2 1.8%	111 100.0%

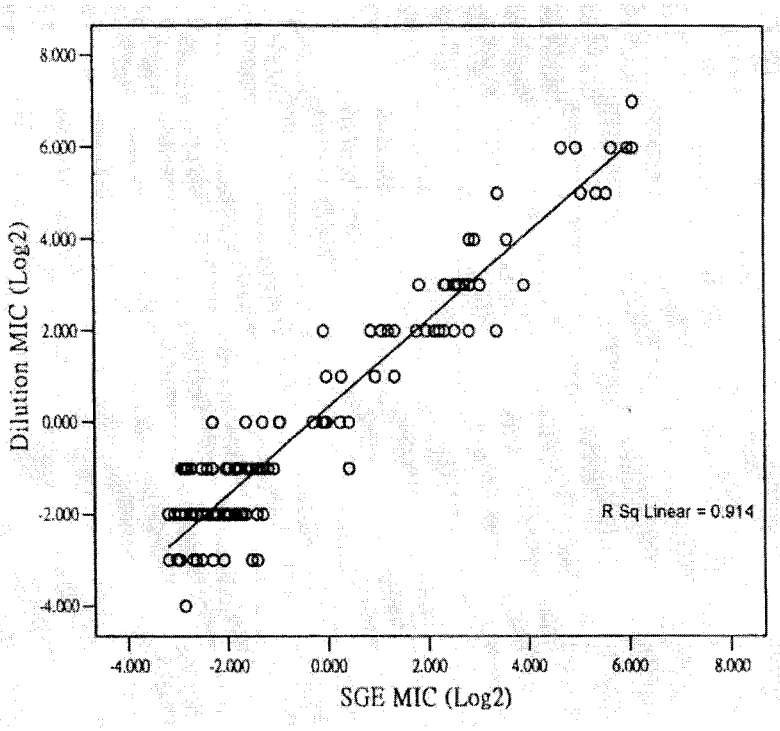
Table 32: **The log_2 MIC difference of SGE Test and SAD Test for susceptibility testing of *N. gonorrhoeae***

Antimicrobial Agent		Difference in log_2 MIC				Total
		-1.00	.00	1.00	2.00	
Penicillin G	Count %	40 10.0%	11 27.5%	4 10.0%	1 2.5%	20 50.0%
Tetracycline	Count %	3 7.5%	14 35.0%	3 7.5%	0 .0%	20 50.0%
Total	Count %	7 17.5%	25 62.5%	7 17.5%	1 2.5%	40 100.0%

Table33 Dilution test sensitivity category vs. SGE test sensitivity category for *N. gonorrhoeae*

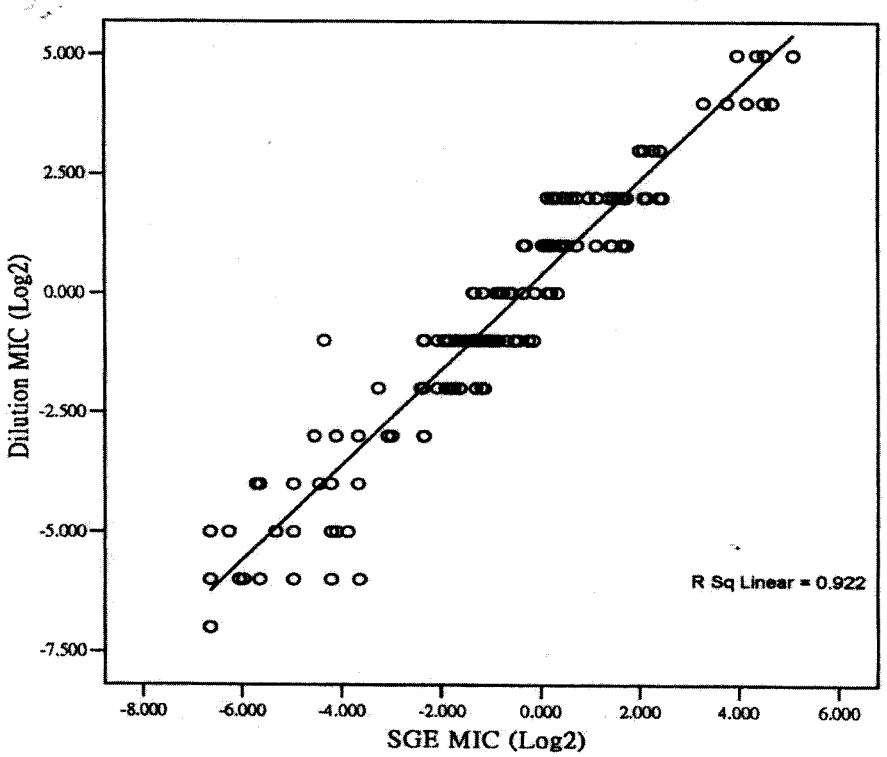
Antimicrobial agent			SGE test sensitivity category			Total
			Sensitive	Resistant	Intermediate	
Penicillin G	Dilution test Sensitivity category	Sensitive	6	0	1	7
		Resistant	0	9	0	9
		Intermediate	0	0	4	4
	Total		6	9	5	20
Tetracycline	Dilution test Sensitivity category	Sensitive	8	0	0	8
		Resistant	0	7	0	7
		Intermediate	0	1	4	5
	Total		8	8	4	20

Figure 5: Log_2 dilution MIC vs log_2 SGE MIC for *H. influenzae*



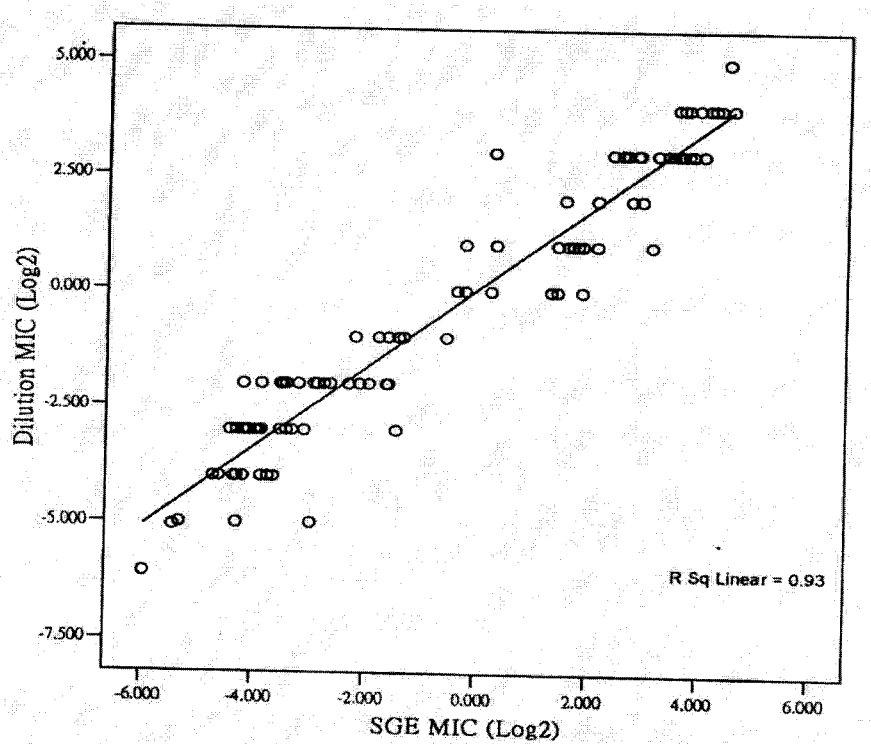
From the regression analysis, $r^2 = 0.914$.

Figure 6: Scatter Plot of log_2 dilution MIC vs log_2 SGE MIC for *S, pneumoniae*



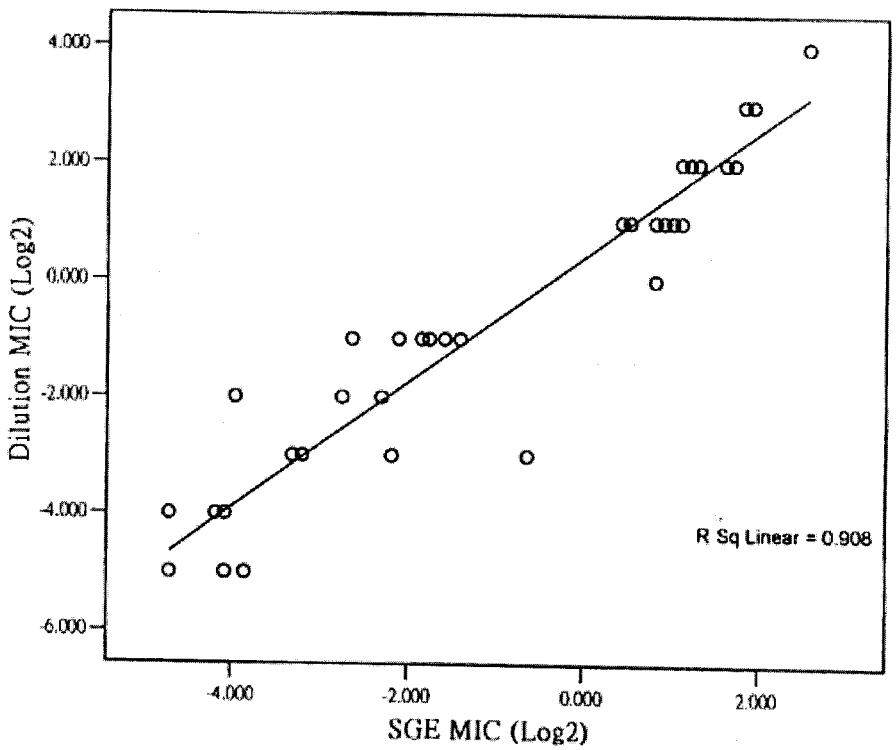
From the regression analysis, $r^2 = 0.922$.

Figure 7: Scatter plot of log_2 dilution MIC vs log_2 SGE MIC for *M. catarrhalis*



From the regression analysis, $r^2 = 0.93$

Figure 8:Scatter plot of log_2 dilution MIC vs log_2 SGE MIC for *N. gonorrhoeae*



From the regression analysis, $r^2 = 0.908$.

Chapter 6

Determination of Optimal Conditions for Flow Cytometric Studies on *Haemophilus influenzae*

Introduction

Haemophilus influenzae continues to cause considerable levels of morbidity and mortality and is responsible for several serious infections, including pneumonia and meningitis (Peltola, 2000). In the past, this organism did not require susceptibility testing since it was known to be consistently susceptible to certain antimicrobial agents such as ampicillin and chloramphenicol. However, more recently, resistant strains have emerged (Campos *et al*, 2004; Ho *et al*, 2004; Beekmann *et al*, 2005). These produce challenges for the clinician because previously employed empirical therapy for these organisms is now inadequate. Laboratory susceptibility testing is now required to ensure successful therapy.

Fastidious bacteria sometimes grow too slowly, or require supplemental nutrients or modified incubation conditions, and, therefore, cannot be tested by standard methods without modifications. Nevertheless, dilution MIC tests and disk diffusion tests have been standardized by the CLSI for these organisms (NCCLS

2003a, b; CLSI, 2005b). MIC methods such as the E test strip (PDM Epsilometer; AB Biodisk, Solna, Sweden) (Jorgenson *et al* 1994(a); Sanchez *et al* 1992; Hughes *et al* 1993; Macias *et al* 1994; Jorgenson *et al* 1994(b); Skulnick 1995) and the SGE method described in the previous chapters were found to yield highly reproducible and accurate results when compared with reference methods for susceptibility testing of fastidious organisms. However, in the routine laboratory in Hong Kong and elsewhere, only results of qualitative susceptibility testing are required. So, disk diffusion tests are used. Furthermore, all the above mentioned antimicrobial susceptibility tests require considerable time for results to be available, since for a sufficient number of cell divisions to occur to allow visible colonies or turbidity to develop, overnight incubation is required. Consequently, empirical antimicrobial therapy is usually initiated before the arrival of laboratory susceptibility reports. Such therapy may not optimal for the infected patients.

Rapid methods for AST may have a considerable clinical impact (Bauer, 1966; Gleason *et al*, 1999; Klugman *et al*, 2004). The reduction of time for issuing of AST results for at least 10 hours would enable physicians to choose more

appropriate, narrower spectrum and less costly anti-microbial therapy at the initiation of therapy.

The use of the FCM for determination of AST would allow rapid detection of antimicrobial effects on individual organisms as measured by fluorescent probes and does not rely on growth inhibition. Furthermore, thousands of cells could be analysed in a short time providing statistically reliable results which would be available after a few hours.

Although limited work on development of susceptibility testing using FCM has been reported (Steen *et al*, 1982; Martinez *et al*, 1982; Gant *et al*, 1993, Durrdie *et al*, 1995; Pore, 1994; Walberg *et al*, 1997), to date there have been no published studies on the ability of the FCM to detect fastidious organisms such as *H. influenzae* or to determine antimicrobial susceptibility results for these organisms. However, if shown to be capable, it may provide AST results more rapidly than current standard methods.

In this study, the ability of the FCM to detect *H. influenzae* cells was examined and the conditions necessary for the optimal discrimination of viable and non

viable cells were determined. These included optimization of the concentration of Propidium iodide (PI) and $DiBAC_4(3)$ and of the incubation time for maximum signal from the FCM. Several media, Haemophilus Test Medium (HTM) media, Tris-HCL (5mM pH7.5), Phosphate Buffer Saline (pH7.4) and distilled water were also investigated to determine the optimal medium for staining. The results obtained were used in Chapters 7 and 8 to determine ASTs of *H. influenzae* and *S. pneumoniae* by FCM.

Materials and Methods

A. Bacterial Strains and Growth Condition

A *H. influenzae* control strain (ATCC 49247) was used for this optimization study. It was a BLNAR strain. Prior to each experiment, three small colonies from an overnight culture on chocolate agar were inoculated in to 20ml pre-warmed cation adjusted Mueller Hinton broth supplemented with 15mg/L NAD and 15mg/L Haematin. The culture was then incubated at 35°C on an orbital shaker at 250 r.p.m. for 3 hours to reach exponential growth phase, indicated by an optical density (OD600nm) of 0.04. All liquid culture media was filtered (pore size 0.22 μm, Millipore, Molsheim) at least three times before use.

B. Flow Cytometry

FCM was performed using a Coulter EPICS Elite ESP flow cytometer (Coulter Corporation, Miami, FL, USA). The light source of the instrument is an air-cooled argon laser lamp operated at 488nm with an intensity of 15mw. The system is run under standard conditions with a sheath pressure of 82.7 Kpa (12psi). The instrument had disposable self-bleeding in-line sheath filter cartridges (Millipore, 0.2 μm pore size, Millipore, MA., U.S.A.) installed in parallel close to the flow cell to minimize particle background noise. Fluorescence signals (green) from $DiBAC_4(3)$ were collected through a 525-nm band pass filter, whereas red fluorescence from PI was collected through a 610-nm band pass filter. All detector amplifiers were set on logarithmic gain mode. Minimum gate discriminators on side scatter (SS) criteria were set on logarithmic gain mode, and to exclude sub-cellular debris and optical 'noise'. To monitor the cell flow, a dot plot of log forward scatter (FS) against log SS was set with an analysis gated around the bacterial population. Log fluorescence histograms were simultaneously generated from the gated data by the Coulter Elite Software Version 4.5 Revision B. Sample flow rate was adjusted to give a data rate of less than 500 cells/s. All samples were allowed to run for 1 min before data acquisition. Data from approximately 10,000 cells was accumulated for each run. Median channel fluorescence was the parameter used for analysis.

C. Calibration of the Machine

The optical and fluidics systems were aligned to maximize the detection of fluorescence and scatter signals before daily operation. Uniform fluorospheres of 10 μm (Flow-Check Fluorospheres; Beckman Coulter Ltd., USA) were used to verify optical alignment. The Half-Peak Coefficient of Variance (HPCV) and peak position for FS, SS, and each of the appropriate fluorescence parameters were set to <2%.

All detectors were used with logarithmic amplification. The maximum emission wavelengths of PI and $DiBAC_4(3)$ are 617 and 516 nm respectively. Fluorescence detection was gated by light scatter parameters and was carried out with a filter block with the following characteristic excitation: 470-490 nm; band stop 510, and emission >520 nm. At all times, the flow rate was limited to 200-500 events/sec.

A flow check YG size range calibration kit (green emission) (Polyscience Inc. Warrington, PA, USA) containing beads with diameters of 0.5, 1.0 and 2.0 μm was used daily to calibrate FS, SS, and green fluorescence parameters, and to

ensure the position of organisms to be analysed. Alcohol-fixed organisms were added, stained with PI, and gated to fluorescence photomultipliers to calibrate the red fluorescence detector (617 nm).

To discriminate the bacterium from background noise, fluorescent beads or PI stained, alcohol-fixed organisms were added. The instrument was then triggered for fluorescence and the voltage adjusted so that the beads (or PI stained alcohol fixed cells) were displayed in the top right quadrant of a SS versus fluorescence graph. The threshold for detection was then set.

For each run, an equal volume (500µl) of Flow-Count Fluorospheres (Coulter Co. Miami USA) was added to the test sample to determine the absolute count of viable bacteria.

D. Preparation of Positive Control (Alcohol-Fixed) Cells

1. A broth culture of *H influenzae* control strain ATCC 49247 was grown to mid log phase (OD_{600} of 0.2) (approx 3 h).

2. The culture was centrifuged at 14000 x g at room temperature for 1 minute. The supernatant was discarded and the pellet re-suspended in sterile filtered distilled water.

3. Ice-cold 80% ethanol was added to give a final concentration of 70% and the mixture was held for a minimum of 60 minutes at 4°C. Final cell concentration was OD_{600nm} 0.04.

   Fixed samples could be stored in 70% ethanol at -20°C for several months without noticeable deterioration. Fixed samples were analysed within 2 h of removal of fixative.

For each FCM cytometric run, the fixed cells were centrifuged, the supernatant discarded, and the pellet re-suspended in filtered broth. The sample was washed twice with broth and re-suspended in filtered staining media. Dye solution at the appropriate concentration as described later, was added. The mixture was incubated and then examined by FCM.

E. Chemicals, Buffers and Fluorescent Probes Used

A stock solution (1 mg/ml) of PI (Sigma Co. St. Louis MO, USA) was prepared using Milli-Q water. This could be stored for up to 3 months at -20°C, if protected from light. Before use, the stock solution was further diluted with Milli-Q water to obtain a working solution of 100 μg/ml. .

$DiBAC_4(3)$ (Molecular Probes, Eugene, OR, USA) was dissolved in absolute ethanol to give a stock solution of 1 mg/ml. This was stored in aliquots of 1 ml

at -20°C for up to 12 months protected from light. Before use, the stock solution was diluted using 70% (V/V) ethanol to obtain a working solution of 100 μg/ml. Valinomycin (Sigma Co. St Louis MO. USA) was re-constituted using Milli-Q water to a 25 μg/ml final concentration and stored at -20C. Milli-Q water was obtained from a Millipore ultra pure water system (Millipore Corp., USA). The sheath fluid Isoton II and the enzymatic cleaner Coulter Clenz required for running the FCM were purchased from the manufacturer (Coulter Cooperation, Miami, FL., USA).

F. Optimization Studies

Determination of Optimal Cell Concentration

A range of concentrations of alcohol-fixed *H. influenzae* control cells (10^5CFU/ml to 10^9CFU/ml) were stained using PI and $DiBAC_4(3)$, and fluorescence signals were measured by the FCM to determine the optimal bacterial concentration. An exponential phase culture was diluted to the appropriate concentration using filtered, pre-warmed culture broth. The culture concentration was confirmed by measuring the turbidity using a spectrophotometer.

Determination of Optimal Dye Concentration and Length of Incubation Time of Dye with Fixed Cells

Various concentrations of PI and $DiBAC_4(3)$ over a range of 0.1 to 50 μg/ml were used to stain alcohol-fixed organisms using optimal cell concentrations determined above. Fluorescent signals were measured at five-minute intervals, from 5 minutes to 30 minutes, to determine the optimal incubation time. In addition, equivalent concentrations of both dyes were added to live cells, so as to ensure negligible staining occurred and that they can be used as a negative control (see below). The concentrations of PI and $DiBAC_4(3)$ determined were used for subsequent experiments.

Staining media

The optimal media for staining was determined by comparison of the cell staining intensities of alcohol-fixed *H. influenzae.* Using the optimal conditions derived from 1 and 2 above, FCM tests were performed on fixed cells using (a) Haemophilus test medium (HTM), (b), Tris-HCL buffer (5 mmol/L, pH 7.5), (c) phosphate buffered saline (PBS, pH 7.4), and (d) distilled water. All the media are homemade from powder form and sterilized before used. They were filtered three times to minimize background signals. Each condition was repeated five times.

G. Controls used in this Study

Several controls were included in these experiments:

(a) Viable cells from a culture grown to early exponential phase and stained with the relevant dye were used as negative controls.

(b) To differentiate depolarized cells from normal cells, isolates were treated with the ionophore, valinomycin (25 μg/ml). This should result in the depolarization of the bacterial membrane and allow the entry of $DiBAC_4(3)$ into the cell resulting in intense intracellular fluorescence.

H. Toxicity test

In order to determine whether or not the dyes have harmful effect on the control cells, toxicity tests were performed. Suspension of live control cells were treated with the determined optimal concentration of dyes for the determined optimal tme. The control cells were then washed three time and resuspensed in normal saline. 100uL were then added to 10 mL of saline, well mixed and then 10 uL were plated out on chocolate agar. The plates were then incubated for 24 hours in CO_2 incubator at 35°C and counted. (Figure 10a)

Results

A. Cell Concentration

The results of dot plots indicated that concentrations of 10^6 or 10^7 CFU/ml of cells gave the most clear cut scatter signal. Typical results for 10^7 CFU/ml are shown in Figure 9. In the case of cell concentrations of 10^5CFU/mL, two peaks were observed on histograms. The first peak seemed to be background noise while the second peak was fluorescence from the cells. Although a faster rate of analysis would seem to be expected at higher cell concentrations such as 10^8CFU/mL or greater, the results in this study suggest that the cells are too closely packed as they flow through the laser beam resulting in a false high signal .

A cell concentration of 10^7CFU/mL was chosen for the remaining optimization studies because it would require no further dilution of cells when initial bacterial concentrations were determined by OD measurement. Signal-to-noise ratio was also better for a higher concentration of cells.

B. Dye Concentration and Staining Media

Propidium Iodide (PI)

The uptake of PI by 10^7CFU/mL fixed cells reached saturation at a concentration of ≥ 5 μg/mL, and when the dye concentration was increased beyond 5 μg/mL, the time taken to reach equilibrium or plateau of fluorescence decreased (Fig. 9b). The maximum signals were almost the same for dye concentrations 5 and 10 μg/mL. However, with dye concentrations of 1 and 5 μg/mL, it took a longer time to reach steady signal compared with a dye concentration of 10 μg/mL, which took approx 15 minutes to reach steady state. With PI concentrations of 50 μg/mL quenching occurred due to the high concentration and the fluorescent intensity decreased. Furthermore, dye concentrations ≥20 μg/mL caused staining of the tubing of the cytometer making it difficult to wash. This led to an unfavorable background signal. Using concentrations of 0.1 and 0.5 μg/mL, resulted in weak fluorescence signals which took a long time to reach equilibrium. This suggested that a dye concentration of 10 μg/mL and incubation time of 15 minutes was optimal. For consistency, these times and concentrations were used for all subsequent experiments. In addition, a toxicity test was performed by the addition of 10 μg/mL PI to viable organisms and incubating for 15 minutes prior to plating them out. PI was found to have no effect on viability (Fig. 10a), so appeared to have no unwanted effects on the organisms at the dye concentration used.

Staining media to be used with PI

Results of the comparison of cell staining intensities for alcohol-fixed *H. influenzae* cells suspended in Tris-HCL (5mM pH 7.5), original medium, PBS (pH7.4) or distilled water, and stained with PI at a concentration of 10 μg/mL for 15 minutes are shown in Table 34. Results were analysed using one way ANOVA was performed. From the output, the Levene test ($p = 0.973$) showed there was no significant difference for variances. There was no significant differences in fluorescent intensity between the media (F test $p = 0.194$).

$DiBAC_4(3)$

The results for testing $DiBAC_4(3)$ fluorescence at different concentrations with measurements taken at five minute intervals are shown in Fig 10. As with PI, when steady fluorescent intensity had been reached, it was found that the fluorescent intensity of the control strain was low at dye concentration of 0.5 and 1 μg/mL. The maximum fluorescent intensity occurred at dye concentrations of 5 μg/mL and 10 μg/mL, both of which reached similar steady state fluorescent intensities. Concentrations of 20 μg/mL and 50 μg/mL resulted in the fluorescence intensities lower than those of 5 μg/mL and 10 μg/mL. At low concentrations of $DiBAC_4(3)$ (0.5 μg/mL and 1 μg/mL), the cell population took a

long time to reach fluorescence equilibrium, whereas at dye concentrations ≥5 μg/mL, only 5 minutes was required to reach steady intensity.

Thus, for $DiBAC_4(3)$, a concentration of 5 μg/mL, and a incubation time of 5 minutes were determined to be the optimal conditions to be used for subsequent experiments. It is important for the fluorescence intensity to reach steady state quickly if reproducible results are required. Use of 10 μg/mL was not chosen, since it would required more dye, and back ground signal was high which would lead to an unfavorable high noise to signal ratio.

As with PI, a toxicity test was performed by incubating *H influenzae* control cells with 5μg/ml $DiBAC_4(3)$ for 5 minutes. Viability was determined by plating on chocolate agar. There was no loss of viability observed for the organism Results are shown in figure 10a.

Staining media to be used with $DiBAC_4(3)$

Use of different suspension media for $DiBAC_4(3)$ staining resulted in differences in intensity of fluorescence. (Table 35). Oneway ANOVA was performed and the results are shown in Table 36. The Levene test for the homogeneity of variance showed no significant difference ($p = 0.088$) There was a significant difference for the fluorescent intensity when $DiBAC_4(3)$ staining was performed in different

media (F test p = <0.0001). As Tris-HCL, 5mM, pH 7.5 gave the most intense fluorescence, this medium was used as the staining medium for subsequent experiments involving both $DiBAC_4(3)$ and PI.

Discussion

The volume of a bacterial cell is three orders of magnitude smaller than that of a typical mammalian cell (Shapiro, 2003d). It is not surprising therefore, that the development of FCM capable of providing useful information on bacteria is a comparatively recent event. Despite the advent of FCMs theoretically sensitive enough to detect bacteria, few studies on this subject have been published. This is surprising since FCM has the potential to yield valuable information on the characteristics of both naturally occurring and pathogenic bacteria. Of even greater relevance is the ability of the FCM to determine effects of antimicrobial agents on bacterial cells.

One reason for inadequate use of FCM for bacteria is that most FCMs are at the margin of sensitivity for detection of bacteria. Sensitivity can be defined either from the fluorescence threshold or from its resolution (Shapiro, 2003c). The fluorescence threshold is the lowest signal from a fluorescent particle that can be distinguished from background noise; the resolution is the degree to which the

instrument can distinguish non-fluorescent and fluorescent populations in a mixture. A number of factors including noise, exposure time, excitation light flux, bleaching, light collection efficiency, optical filtration and fluorochrome amplification contribute to the sensitivity of a FCM. The sensitivity of a FCM may be defined in several ways: a widely used technique for determination of FCM sensitivity expresses detection limits in terms of Molecules of Equivalent Soluble Flurochrome, or MESF Units (Schwartz and Fernandez-Repoller, 1993).

A second reason why researchers were slow to use the FCM for bacterial cell studies is that the mode of action of the fluorescent dyes used in FCM studies were better understood for mammalian systems in comparison to their action in microbial systems, as there was a great deal of research performed for the development and optimisation of fluorescent probes appropriate for mammalian cells. It appears there were few links between microbiologists and those interested in fluorochome measurements.

A third factor was the cost of instrumentation, which includes the high price of the machine, purchase of fluorescence beads, and the need for a skillful operator.

In this study, it was demonstrated that the Coulter EPICS ELITE FCM can detect alcohol-fixed stained and unstained *H. influenzae* cells and differentiate them from optical and electronic "noise". The sensitivity of the machine was able to cover the size of *H. influenzae* cells, which is > 0.5 μm. The instrument settings were similar to those used for the analysis of the smallest mammalian cells, except that logarithmic amplification of photomultiplier output was used, and the flow rate of the samples was much reduced (<500/S as opposed to 1000/S for mammalian cells) (Nebe-Von Caron, 1994). Additionally, for the present study, two disposable self-bleeding in-line sheath filter cartridges were installed in parallel, close to the flow cell to minimize background noise. In fact, the sensitivity of the FCM used here could be further enhanced by the use of a photomultiplier for FS instead of a diode, however this was not done due to cost.

Stains most commonly used for assessment of viability of bacteria included fluorescein diacetate (FDA), SYTO –13, SYTO – 17, membrane potential sensitive dyes such as $DiOC_5$, rhodamine 123 (Rh 123), Bis (1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$), and the membrane exclusion dyes, propidium iodide (PI) and ethidium bromide.

$DiBAC_4(3)$ is a lipophilic anionic oxonol dye which has a low binding affinity for intact polarized membranes. However, when membranes become depolarized by chemical or physical means the oxonal enters the cell, binding to lipid-rich intracellular components (Waggoner, 1976), causing the cells to become increasingly fluorescent. $DiBAC_4(3)$ has the advantage of only entering depolarized cells, and thus being non-toxic to cells, whereas cationic dyes such as Rh123 has been found to be highly toxic to bacteria (Diaper et al, 1992). The use of membrane potential probes has the advantages of high sensitivity and of showing a rapid response to small changes in membrane potential. However, the user is also faced with the disadvantage that the mechanism by which probes respond to membrane potential is largely unknown, and factors other than membrane potential also govern the response of these probes. These include effects of the staining medium used, pH quenchers, and self quenching (Shapiro, 2003a).

Ethidium Bromide (EB) and PI form complexes with double-stranded DNA and RNA by intercalating between base pairs (Le Pecq, 1966). They are excluded from cells with intact cytoplasmic membranes. Only when membrane integrity is compromised will the dyes penetrate cells and bind tightly to nucleic acid.

However, for ethidium bromide, efficient efflux pumps capable of removing the dye from *Escherichia coli* have been demonstrated (Jernaes and Steen, 1994). Another limitation of ethidium bromide is that low levels of staining have been reported in some types of viable cells (Glazer et al, 1990). Thus, the ability of live cells to exclude ethidium bromide should be verified for the cell type of interest when adapting this method.

In this study, $DiBAC_4(3)$ and PI were chosen for staining of *H. influenzae* cells because both of them are excluded from viable bacterial cells. This minimizes the risk of the dyes themselves causing harm to the organism. The dyes were not included in the growth medium, but were added to the cultures prior to analysis to avoid dye-induced effects on bacterial cells or on the antibiotic-cell mechanism. This further reduced the chances of dye perturbance to the cells. Toxicity tests performed for both dyes indicated that no inhibition of live cells occurred following incubation in the presence of these dyes.

Accumulation of fluorescent dyes by cells is dependent on their concentration. Best results are obtained by adjusting the dye concentrations to achieve maximum distinct labelling of positive cells. In general, it is best to use the lowest dye

concentration giving the maximum signal. It is important to determine the optimal concentration of dyes and the optimal length of time between addition of stain and subsequent FCM analysis in order to achieve sensitive and reproducible results.

In this study, it was found that uptake of both dyes used reached equilibrium faster when high dye concentrations were used. Once the dyes enter the cells, they bind to their target site and slowly establish an equilibrium state with the free dye molecules in solution. As the concentration of dye increases, the rate of reaction increases and time to reach equilibrium is decreased.

In the case of both dyes used in this study, the florescent intensity increased when the dye concentration was increased up to a certain concentration, when increasing the dye concentration further no longer resulted in an increase in fluorescent intensity. This may be due to self quenching by the dye.

Quenching of fluorescence may occur when an excited molecule returns to ground state via any non-radioactive pathway which provides alternatives to florescence.

Loss of energy by vibration and collision, by energy transfers, and by intersystem crossing may all account for quenching (Shapiro, 2003c). Bleaching is another mechanism whereby there is sufficient chemical modification of the fluorochrome structure to destroy its fluorescent properties rendering it non -fluorescent. Quenching may occur when the fluorescent dye molecules are close enough to quench one another by energy transfer and can be caused by a high dye concentration as in this study. This is a form of self quenching is known as concentration quenching (Shapiro, 2003c).

In this study, $DiBAC_4(3)$ gave different fluorescent intensity signals in different media, being highest in Tris-HCL pH7.5 buffer of the media tested. This finding was consistent with the results of Deere and coworkers (1995), who optimized the staining media for FCM by comparison of cell staining intensities for heat-killed cultures of *E. coli* and *S, aureus* in PBS, distilled water, Tris-HCL (5mM) pH7.5 or Tris-EDTA (5 in mM, 1mM EDTA) pH7.5 at different incubation times and temperatures.

Buffer systems used for staining have a profound influence on the membrane potential itself and on the dye distribution obtained (Nebe Von-Caron and Badley,

1996). Under normal circumstances, the intracellular concentration of K^+ $[K^+]_i$ is considerably higher than the extracellular $[K^+]o$, while the intracellular concentration Na^+, $[Na^+]i$, is considerably lower than the extracellular concentration $[Na^+]o$. In isotonic NaCl, the cell maintains a membrane potential $\Delta\psi$ of –100 –200 mV. These potential differences are due in part to existence of concentration gradients of Na^+, $K^+$and Cl^- zones across the cell membrane, and in part to the operation of various electrogenic pumps (Mason *et al*, 1993).

However, in the present study, the bacterial cells used were alcohol-fixed making them freely permeable to various ions. The membrane potential $\Delta\psi$ would be zero and the dye concentration inside and outside the cell should be equal. Higher $DiBAC_4(3)$ fluorescence intensity can be obtained in such cells because:

1) the fluorescence of oxonol dyes is enhanced when they are in a hydrophobic or non-polar environment (Mason and Gant, 1995) with the result that any dye molecule bound to membranes or lipids becomes more fluorescent than it would be in aqueous solution;

2) the lipophilic character of the dye causes it to be concentrated in cells in the absence of a potential gradient because it binds to intracellular lipids and the membrane structure.

A possible explanation for Tris-HCL pH7.5 giving more intense fluorescence for alcohol fixed *H. influenzae* cells used in this study may be that this buffer may enhance the hydrophobic character of the binding site for $DiBAC_4(3)$, or it may increase the binding sites of the oxonol dye by modification of some intracellular structure.

Alcohol-fixed cells were chosen in this study to compare fluorescence intensities rather than heat-fixed cells, as there was no increase in fluorescence intensity in heat-fixed cells when compared with control cells. It is possible that the heat fixation process may destroy the binding site of the oxonol dye.

For good performance it is essential that the FCM must be kept clean, as a contaminated instrument will produce a considerable quantity of noise, which is unfavorable for any scattering measurement. One source of noise is cells left over from a previous run, and this is particularly likely to occur if there is a partial

blockage in the feed tube or any of its connections. Some fluorochromes may become adsorbed onto plastic or silicone rubber feed lines. FDA, Hoechst 33342 and acridine orange are known to be particularly problematic in this respect. Apart from 'back flushing' with the sheath fluid, washing with copious distilled water may remove some of the dye and cells from previous samples. Flushing with Coulter Clenz (Beckman Coulter Co. USA) can dissociate proteins, which are the major problem and can usually keep the tubing clean. Occasional flushing through of a dilute hypochlorite solution is very effective at removing fluorochromes adsorbed to the sample feed-tube. If these measures are ineffective, it is necessary to replace the sample feed tube.

The daily preparation of the FCM for use is time consuming, as this includes an alignment check, washing, preparation of stained sample and running the control. However the machine only requires alignment once daily and samples can then be run throughout the session. In comparison with other methods, FCM-AST still has advantages since the running time of each sample is less than 5 minutes. Although the running of samples cannot be batchwise and required to be submitted one by one. The time required for several antibiotics to be tested were still short. The preparation of the samples, which was most time consuming can be batchwise and

reduced a lot of time. It is hoped that further advances in the development of this method would lead to a specifically-designed version of the FCM exclusively for AST, similar to the Cell-Dyne haematology analyzer, requiring less preparation.

This study has shown that samples for analysis can be grown, incubated with antibiotics, and then stained, taking a total of 6h. This would allow AST to be completed within one day. The abilities of the dyes used to successfully distinguish between living and dead cells, indicated that the method had potential for development into a tool for AST of fastidious organisms.

Table 34: Median fluorescence intensity of alcohol-fixed *H. influenzae* cells stained with PI at different staining media

Medium / Experiment number	Tris-HCL (pH 7.5)	PBS	Water	HTM Broth
1	7.0	7.5	6.8	7.0
2	6.9	7.3	6.5	6.4
3	7.3	7.3	6.6	6.8
4	7.3	6.8	7.0	6.9
5	6.5	7.0	7.2	7.2

Table 35: Median fluorescence intensity of alcohol fixed *H. influenzae* cells stained with $DiBAC_4(3)$ using different staining media

Medium / Experiment number	Tris-HCL (pH 7.5)	PBS	Water	HTM Broth
1	32.4	11.7	31.2	13.9
2	33.0	11.5	28.3	12.6
3	31.8	11.8	29.1	12.1
4	34.7	12.1	28.3	13.0
5	32.3	12.5	31.2	11.9

Table 36: Oneway Anova to compare median fluorescence intensity of $DiBAC_4(3)$ in different media

i) ANOVA - Fluorescence intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1752.204	3	584.068	341.211	.000
Within Groups	27.388	16	1.712		
Total	1779.592	19			

ii) Homogeneous Subsets - Fluorescence intensity – Tukey B[a]

Media	N	Subset for alpha = .05		
		1	2	3
PBS	5	11.9200		
HTM broth	5	12.7000		
Water	5		29.6200	
Tris-HCl	5			32.2400

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 5.000.

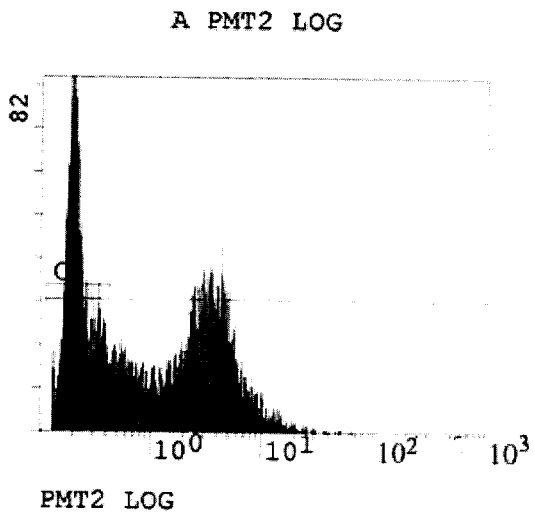


Figure 9a. When 10^5 CFU/mL of cells were used for FCM studies a second peak with very low fluorescence intensity ($DiBAC_4(3)$) was observed in single parameter histograms indicating high signal to noise ratio.

Figure 9b (i)

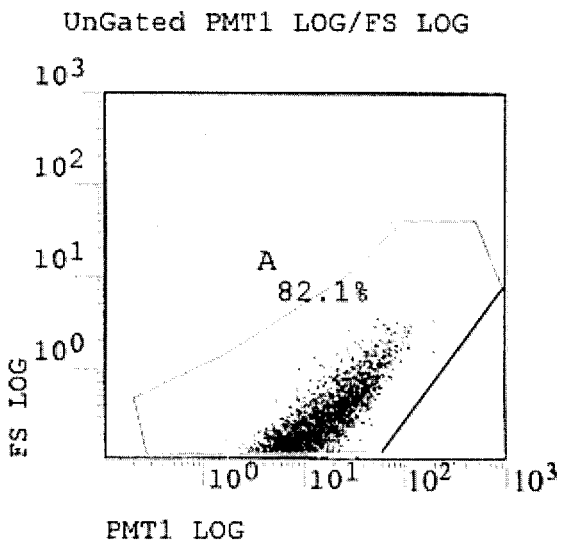


Figure 9b (ii)

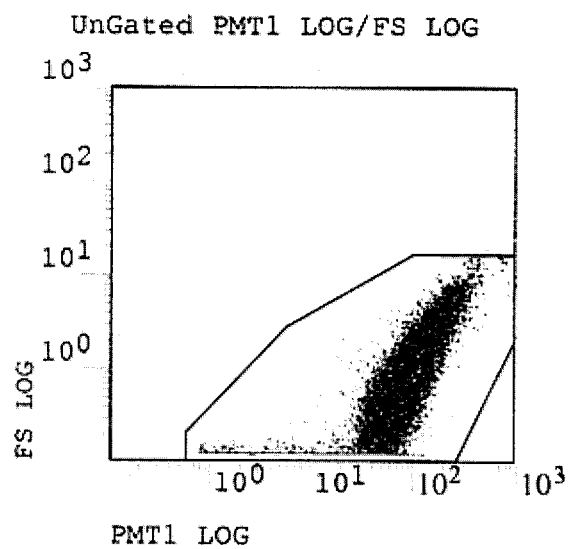


Figure 9b. Differences in forward scatter against side scatter dot plot when a concentration of 10^7 (i) 10^8 (ii) CFU/ml were used for FCM studies.

Figure 9c: Graph of fluorescence intensity against time for a range of concentrations of PI at a bacterial density of 10^7 CFU/mL.

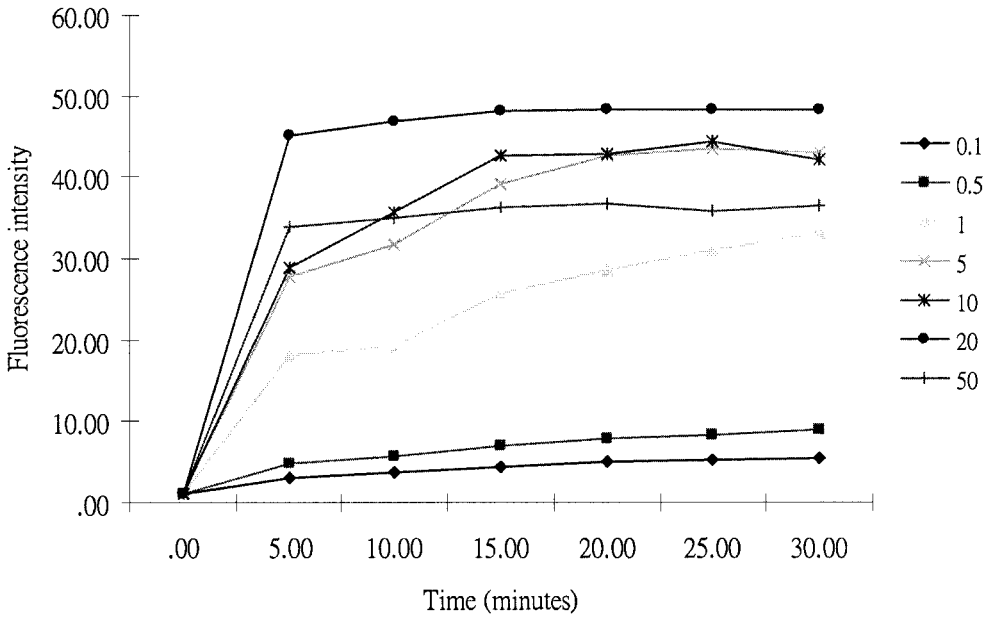


Figure 10. Graph of fluorescence intensity against time for a range of concentrations of $DiBAC_4(3)$ at a bacterial density of 10^7 CFU/mL.

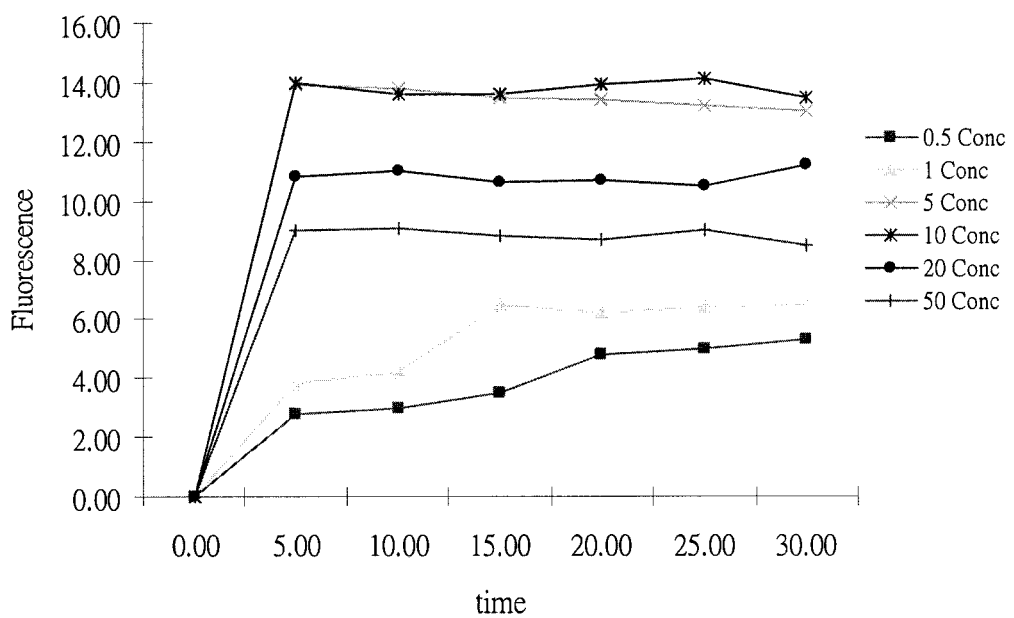
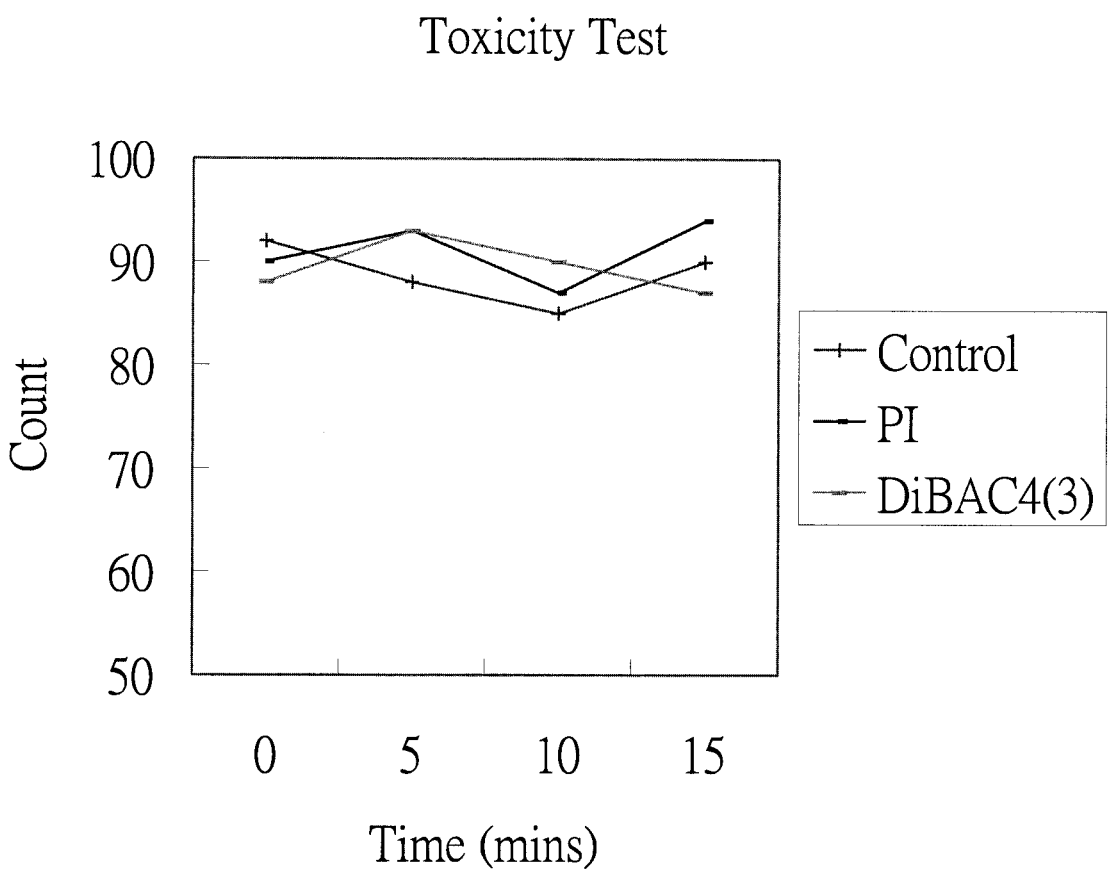


Figure 10a Cell count after treatment with dye at its optimal concentration and time



Chapter 7

Evaluation of the Effects of Antimicrobial Agents on *H. influenzae* using Flow Cytometry

Introduction

Assessment of bacterial viability or response to antimicrobial agents by classical methods has always had the major drawback of requiring many cycles of cell division to produce a result. AST of isolates requires at least 24 h before reporting, possibly leading to a corresponding delay in appropriate treatment for patients. Conventional ASTs also require skilled personnel, are laborious, tedious, and, thus, expensive. They are difficult to automate.

FCM, which has proved useful in other aspects of biomedical science (Boye *et al*, 1983; Allman *et al*, 1992) is an option that needs further assessment, as it permits rapid analysis of a given microbial population in non-synchronous cultures with excellent precision and accuracy, and can provide information relating to the heterogeneity of individual bacterial responses to antibiotics. Using FCM, AST results can be available after several hours. FCM ASTs have employed membrane potential-sensitive fluorescent probes to assess membrane damage and depolarization (Mason *et al*, 1984; Ordonez and Wehman, 1993; Pore, 1994;

Jepras *et al*, 1995; Mason *et al*, 1995), probes which detect cytoplasmic membrane integrity, and DNA content to detect the physiological characteristics of bacterial cells' responses to antibiotics.

Bis (1,3 – dibutylbarbituric acid) trimethine oxonal, or $DiBAC_4(3)$ is widely used, as it has been shown to be a robust membrane potential sensitive dye. $DiBAC_4(3)$ is an anionic lipophilic ion that undergoes membrane potential-dependent distribution between the cytoplasm and external medium, and has an increased binding affinity for depolarized membranes (Epps *et al*, 1994). Propidium iodide (PI), also a commonly used dye for membrane integrity, is a small cationic molecule that is excluded from cells having intact cytoplasmic membranes. If membrane integrity is compromised, then PI will enter the cells and bind tightly to nucleic acids. Both probes have the advantage that they bind to physiologically 'dead' cells, and thus prevent the problems of toxicity and efflux by the cells. Use of both probes for AST may provide increased sensitivity (Gauthier *et al*, 2002) and these probes have been proven to be robust and successful for AST of non-fastidious organisms. However, there have been no previous studies of AST of fastidious organisms by FCM.

The aim of this study is to develop a technique for the rapid AST of fastidious bacteria by FCM. The effects of different concentrations of ampicillin and tetracycline on a *H. influenzae* control strain using $DiBAC_4(3)$ and PI and incorporating the optimized conditions described in the previous chapter were investigated by determining the number of positively stained cells, and increases in fluorescence following antibiotic exposure. Thirty clinical isolates of *H. influenzae* were then tested by the FCM method to determine their susceptibility to ampicillin and tetracycline and the results were compared to the reference SBD.

Materials and Methods

A. Bacterial Strains and Growth Conditions

Control strain *H. influenzae* ATCC 49247 was used to determine the response of this organism to a range of antibiotic concentrations for two antibiotics and varying exposure times. Following this, 30 clinical isolates of *H. influenzae* recovered from various body sites were obtained from several district hospitals in Hong Kong, and tested for susceptibility to ampicillin and tetracycline. The results were compared to standard methods. All strains were confirmed as *H. influenzae* by Gram stain, and X and V factor requirements. Strains were stored frozen at

-70°C in Protect kits (Technical Consultant Ltd. UK) and subcultured at least twice on chocolate agar prior to testing.

B. Drugs and Fluorescent Probes Used

Ampicillin and tetracycline were obtained in powder form from Sigma Co. (St. Louis MO, USA) and antibiotic stock solutions were prepared using appropriate diluents, as described by NCCLS (2004b).

Fluorescent probes and ionophores used were as described in Chapter 6.

The buffer used as staining medium was Tris-HCL (5mM) pH7.5.

C. Reference Susceptibility Tests

Standard broth dilution susceptibility testing for fastidious organisms was performed in accordance with CLSI guidelines (CLSI, 2005b).

D. Flow Cytometry

The instrument and calibration methods used were as described in Chapter 6. Calibration beads (Flow-Check High Intensity Alignment Grade particles) (Polyscience Inc., Warrington PA, USA) were tested before the commencement of experiments, and the mean fluorescence channel did not vary by more than five channels. Fluorescence compensation for PMT2 and PMT4 was set at < 10%.

E. Flow Cytometry Test Protocol

a) To determine the response of H. influenzae ATCC 49247 to different concentrations of antibiotics.

Two to three colonies of the control strain of *H. influenzae* were transferred to pre-warmed HTM broth supplemented with 15 μg/mL of Haematin plus NAD (HTM supplement, Oxoid, UK) in an aerated bottle and grown at 35°C on an orbital shaker set to 250 r.p.m. for 3 h to reach exponential growth. The culture was then diluted with pre-warmed broth to 10^7 CFU/mL, indicated by an OD_{600} nm of 0.04. The culture was divided into five equal volumes of 25 mL. Antibiotic (ampicillin or tetracycline) was added to four of the cultures at 0.1, 1, 10, 100 times the MIC, the fifth culture serving as a control. All cultures were incubated for 3h. A 1 mL sample was removed from the control culture prior to incubation, and further 1 mL volumes of control culture and tested culture were removed after 30, 60, 120, and 180 minutes. The control cultures served as a baseline of fluorescence intensity when there was no antibiotic treatment. Each sample was centrifuged for 1 minute at 13,000 r.p.m., and the pellet was washed in buffer three times and re-suspended in fresh buffer. An aliquot (0.5 mL each) was stained with $DiBAC_4(3)$ or PI for FCM analysis. The remainder of the washed

sample was used for plate count determination of viability of the organism at different concentration and time of antibiotic exposure.

b) Determination of antimicrobial susceptibility of H. influenzae by flow cytometry

Clinical isolates of *H. influenzae* were grown as described in (a) and adjusted to an OD_{600} of 0.04 (10^7 CFU/mL). Ten μL of antibiotic (1 μg/mL ampicillin or 2 μg/mL tetracycline) was added to a 10 mL volume of culture achieving a final antimicrobial agent concentration equivalent to the CLSI MIC breakpoints for this organism (CLSI, 2005b). The cultures were incubated for 3 h at 35°C in an orbital shaker set at 250 r.p.m., centrifuged and re-suspended in 1 mL of Tris-HCL pH7.5 buffer as described in (a) above. The cultures were then washed twice and re-suspended in fresh Tris-HCL buffer. To 425 μL of the cultures, 50 μL PI (100 μg/mL) and 25 μL $DiBAC_4(3)$ (100 μg/mL) were added to make a final concentration of 10 μg/mL PI and 5 μg/mL $DiBAC_4(3)$. After incubation for 15 minutes in the dark at room temperature, the cultures were tested by FCM.

F. Separation of cells for FCM analysis

To ensure there was no cell clumping three vortexing steps (duration 1 minute each) were included in these studies.

- After inoculation of HTM medium with 2-3 colonies of organism
- After 3 hours incubation and before addition of antibiotic
- After incubation with antibiotic for 3 h and before testing by FCM

G. Interpretation Criteria for FCM-AST

Two acquisition regions were used; the first to exclude cell debris and background noise was applied to the light scattering profile (Figure 11). Data were analyzed by Coulter Elite software version 4.5 revision B. The fluorescent intensity of PI and $DiBAC_4(3)$ were only measured on the events gated within this region.

The second acquisition region was applied to fluorescent signals from PI and $DiBAC_4(3)$ (Figure 12), and this dot plot was used to exclude events that had no fluorescent signal (live cells).

A factor for comparison (F) was defined as:-

$$\mathbf{F} = \frac{\mathbf{X_T}}{\mathbf{X_C}} \qquad \text{Equation 1}$$

Where $X_T = F_T \times MFI_{DiBAC4(3)} \times MFI_{PI}$ Equation 2

F_T = % events in f1 + % events in f2 + % events in f3

MFI_{PI} = Median fluorescent channel of PI

$MFI_{DiBAC4(3)}$ = Median fluorescent channel of $DiBAC_4(3)$

X_C was the value determined in equation 2 above for untreated live control cells.

H. Control

Several controls were used in these experiments.

a) Viable cells from a culture grown to OD_{600} of 0.04 not treated with antibiotics and non-viable cells from an alcohol-fixed culture were stained with both dyes and used as negative and positive controls respectively.

b) Untreated and unstained cells were used to determine auto-fluorescence.

c) Valinomycin treated *H influenzae* control strain to test the ability of the cells to take up $DiBAC_4(3)$ after membrane depolarisation.

I. Determination of Plate Count

In all experiments performed with antibiotics, serial dilutions of liquid cultures were performed using PBS (pH 7.4) and were plated in duplicate on chocolate agar plates. The cultures were first washed once with PBS (pH7.4) and then resuspended in fresh PBS. The number of CFUs was counted after incubation for 24 h at 35°C in 5% CO_2.

J. Cell Counting by FCM

Cell counting was performed using the ratiometric method. Equal volumes of bacterial suspension and Flow-Count Fluorospheres (Coulter Co. Miami, USA) were mixed, and the ratio of cells in the specimen to fluorospheres was established.

The bacterial cells and the fluorospheres in the sample were then counted by the FCM. The absolute count of cells was determined using the following formula:

$$\text{Absolute Count} = \frac{\text{Total no. of cells counted}}{\text{Fluorospheres counted}} \times \text{Flow Count Fluorosphere Assay concentrations}$$

K. Statistical Analysis

The coefficients of variation (CV) for the fluorescent histograms were calculated using the software provided by Coulter Co. (EXPO^{TM} cytometer software). Mann Whitney U test was used to determine statistically significant differences in parameters between treated and untreated cells. Values obtained were tested by Kruskal-Wallis ANOVA test, with the level of significance set at 0.05.

Results

The ability of the FCM to identify and distinguish between viable and non-viable bacteria is illustrated in Figure 13. After staining with either PI or $DiBAC_4(3)$, non-viable fixed *H. influenzae* control strain cells were detected by FCM with high log median fluorescent channels. In contrast, live bacteria had low median channel numbers (Figure 13 d and f). Live gating was performed on *H. influenzae* cells during data acquisition to exclude background noise. Auto-fluorescence of

H. influenzae cells was found to be minimal (Figure 13 a & b) with similar results on repeated experiments.

A significant increase in $DiBAC_4(3)$-conferred cellular fluorescence was observed for the control strain following treatment with the ionophore valinomycin (Figure 14). This indicated complete membrane depolarization, allowing entry of the oxonol and confirmed that $DiBAC_4(3)$ responds to changes in membrane potential.

Comparison of the results in Figure 14 (a) and (b) showed a large increase in $DiBAC_4(3)$ fluorescence after 3 minutes of treatment with valinomycin indicating that very few live cells were present following the treatment. The cells were intact since there was little effect on the light scattering. This result was confirmed by standard plate count (data not shown).

The ability of the FCM to count individual viable cells was determined and compared to standard plate count results. The natural tendency of cells to clump might significantly affect the FCM count. Figure 15a and b shows a comparison of viable counts determined by plate count and FCM.

The plate counts and FCM showed good agreement for the first few readings, but thereafter the FCM count was slightly lower than the plate count. There was no increase in clumping with time since the light scattering intensity seemed to slightly decrease with growth.

A. Response to Ampicillin

Beta-lactam antibiotics cause changes in cell morphology of bacteria including *H. influenzae,* which is reflected as change in FS and SS in dot plot graphs. In this study ampicillin-susceptible strains showed such changes within 3 h when exposed to ampicillin at its MIC (Figure 16a and b), but ampicillin-resistant strains showed no change in light scatter profiles (Figure 16c and d). Sensitive cultures were equally shifted to give increases in both FS and SS, with few cells remaining in the part of the plot occupied by the control population.

At the same time, a tail of events with predominantly shifted SS characteristics appeared. There was a second smaller population corresponding to sub-cellular particles. These phenomena reflect the mode of action of ampicillin, which is to inhibit cell wall synthesis and cause filamentation, or enlargement of the bacteria. When the time of incubation increased, the bacteria began to lyse and a substantial amount of sub-cellular debris was seen below and to the left of the intact bacteria

(Figure 16b). The effects of ampicillin on a control strain of *H. influenzae* at 0.1, 1, 10 and 100 times of its MIC were determined and detected by $DiBAC_4(3)$ staining (Figure 17).

After incubation with antibiotic, sub-populations taking up $DiBAC_4(3)$ and PI were present. The presence of sub-populations was excluded to avoid presentation of excessive data, the simplified information providing an "average value" for the whole population.

There was only slight increase in fluorescence at 0.1 x MIC after 180 minutes incubation indicating minimal loss of viability at this concentration of ampicillin. At 1 x MIC there was an increase in median fluorescence after 60 minutes incubation. The fluorescence continued to increase until the end of the incubation period (180 minutes), when it reached the highest fluorescence intensity. At 10 x MIC, the log fluorescence median intensity increased rapidly during the first 60 minutes and then the fluorescence intensity shifted downwards. The same trend was observed for the fluorescence intensity curve at 100 x MIC antibiotic concentration. At high ampicillin concentrations, the membrane potential of the cells dropped rapidly. However, when the time of exposure increased, some of

the depolarized cells decomposed and became debris thus no longer contributing to the average fluorescence intensity. The decrease in fluorescence was more rapid when the concentration of ampicillin increased. To determine the percentage of positively stained cells after antibiotic exposure, a dot plot of SS against fluorescence intensity was divided into 4 quadrants (Figure 18). Before the commencement of test samples, a negative control was run, and the margins of the 4 quadrants were adjusted so that the number of events in F2 and F3 were fewer than 5%.

For 0.1 x MIC, the effect was evident after 180 minutes, 7.4% of cells were stained with $DiBAC_4(3)$ compared with 5% in the negative control ($P<0.05$, Mann Whitney U Test)(data not shown) For 1 x MIC 12.5% cells stained positive after 60 minutes, and 26.6% after 3 h. This was statistically significant when compared with the negative control (3%) ($P<0.005$, Mann Whitney U Test). Cell debris was also increased from 8% to about 12%. Positive stained cells in the 10 x MIC treatment increased from 14.75% after 1 h to 26.52% after 3 h, this increase being significant ($P<0.05$, Kruskal-Wallis ANOVA Test). At the same time, cell debris increased from about 10% to about 22%. 100 x MIC treatment resulted in a large increase of cell debris, from around 20% at 30 minutes to 50% at 180

minutes. At the same time, the positively stained cell percentage increased from 3.21% to 13.67% compared with 2.28% to 2.30% of positive cells in the negative control, ($P<0.05$, Mann Whitney U Test).

Fig 19 shows the change of PI fluorescence intensity of *H. influenzae* control cells over time in the present of different concentrations of ampicillin. For 0.1 x MIC, there was almost no influx of PI to the cells, and the fluorescent intensity was almost constant. The cells remained intact at this concentration of ampicillin. At 1 x MIC, the PI fluorescent intensity increased smoothly after 30 minutes and continued to increase up to 180 minutes. At 10 x MIC, the fluorescent intensity increased rapidly after 30 minutes reaching a maximum at 120 minutes, when it began to plateau. The change in fluorescent intensity at 100 x MIC followed the same trend as that in 10 x MIC, except the influx of PI to the cells was greater by 30 minutes.

At 0.1 x MIC the proportion of cells exhibiting PI – associated fluorescence was not statistically significant when compared with negative control cells (Mann Whitney U Test, $X = 0.05$). In contrast, exposure to 1 x MIC for 60 minutes resulted in 3.3% of the population exhibiting PI-associated fluorescence. The

proportion of organisms stained with PI increased to 6.06% and 6.26% after 120 minutes and 180 minutes respectively. The proportion of organisms from the control cultures rendered fluorescent by PI was approximately 1.6% and 1.5% respectively ($P<0.05$, Mann Whitney U Test). The cell debris signals ranged from approximately 17% to 22% when the incubation times were 120 minutes and 180 minutes respectively. With 10 x MIC, the percentage of stained organisms was between 3.20% to 3.95% by 60 minutes, at 120 minutes, however, 5.52% of the cells were fluorescent with PI, and by 180 minutes, the percentage of cells fluorescing was 14.9%. These increases over the control value were consistent and reached statistical significance ($P<0.05$, Mann Whitney U Test). At the same time, the cell debris signals were approximately 6%, 20% and 25% for incubation times 60, 120 and 180 minutes respectively. PI stained between 5.56% to 8.94% of the cell population exposed to 100 x MIC in the first 60 minutes, values significantly larger than those for cells from the control culture (1.58%) ($P<0.05$). By 120 and 180 minutes of incubation time with ampicillin, the percentages of stained organisms were 15.74% and 14.58% respectively, both significantly higher than control cells ($P<0.05$). At the same time, the cell debris signals increased rapidly from approximately 40% at 60 minutes, to 45% at 120 minutes, and 55% at 180 minutes.

The result of the kinetic study are shown in appendix III and IV

B. Response to Tetracycline

Tetracycline acts against susceptible micro-organisms by inhibiting protein synthesis. It is a slow bacteriocidal (bacteriostatic) drug. After entering the cell, it binds reversibly to the 30s ribosome, preventing bacterial polypeptide synthesis (Stratton, 1996). Thus there would be no growth of the bacteria after the addition of the antibiotic to the culture and the cells would later undergo autolysis.

In the present study, the FS and SS profiles did not change after addition of antibiotic during the course of the experiment. Figs 20 and 21 show the change of fluorescence intensity with time for *H. influenzae* with different concentrations of tetracycline for $DiBAC_4(3)$ and PI respectively.

The graphs of uptake of $DiBAC_4(3)$ and PI in different concentrations of tetracycline followed the same pattern. At 0.1 x MIC, there was no increase in fluorescent intensity throughout the course of the experiment. Exposure to 1 x MIC resulted in increase in fluorescent intensity starting after 60 minutes of incubation. The fluorescent intensity of both $DiBAC_4(3)$ and PI continued to increase smoothly until 180 minutes. For 10 x MIC and 100 x MIC, the

fluorescent intensity increased rapidly at 30 minutes of incubation, indicating rapid loss of viability of cells at these high concentration of drugs. After 120 minutes of incubation, the fluorescent intensity of both dyes started to slightly decrease or remain constant until 180 minutes.

Tetracycline had little effect on the florescence profiles for PI and $DiBAC_4(3)$ – stained cells exposed to 0.1 x MIC, the proportion of bacteria fluorescent being similar to those obtained with the control culture ($P>0.05$). In contrast, exposure to 1 x MIC rendered more than 4.93% of the bacteria stainable by $DiBAC_4(3)$ after 60 minutes of incubation. This increase over the control value was consistent and reached statistical significance (Mann-Whitney U Test). Stainable cells increased to 21.23% when the incubation time reached 180 minutes. PI stained between 4.03% to 5.46% of the cell population exposed to 1 x the MIC in the first 60 minutes, and 6.19% and 7.27% were stained at 120 and 180 minutes of incubation respectively, which was statistically significant ($P<0.05$) compared with negative control cells. Exposure to 10 x MIC rendered >8.0% of bacteria stainable by $DiBAC_4(3)$ by 30 minutes., and the proportion of $DiBAC_4(3)$-stainable cells continued to increase to 17.8%, 20.7% and 26.7% for incubation times 60, 120 and 180 minutes respectively. Over the same period

the cell debris signal percentage was moderately increased from 4.12% to 18.7%. For 100 x MIC, the proportion of bacteria exhibiting $DiBAC_4(3)$-associated fluorescence ranged from 10.2% to 9.84% in the first 30 and 60 minutes. This increased to 18.47% and 18.15% after exposure of the cells to tetracycline for 120 and 180 minutes respectively. The cell debris signal increased from 4.75% to 38.1% during the period of incubation.

Unlike the control culture, which took up very little PI dye, the presence of tetracycline resulted in significant numbers of events that exhibited red fluorescence. For exposure to 10 x MIC, PI positive cells ranged from 4.96% to 12.14% as the times of incubation increased from 30 to 180 minutes. At the same time, cell debris signals increased from 0.56% to 24.8%. At 100 x MIC of drug, the PI associated fluorescence ranged from 7.81% to 12.4% over the incubation period from 30 to 180 minutes. The cell debris signals increased rapidly from 1.92% after 30 minutes to 31.2% after 180 minutes of exposure. The results of the effect of tetracycline on H. influenzae cells are shown in appendix V and VI.

C. FCM - AST

After 1-3 h exposure of *H. influenzae* to ampicillin or tetracycline at its MIC, use of either dye (PI or $DiBAC_4(3)$) identified the emergence of fluorescent

sub-populations (Figure 22). The $DiBAC_4(3)$ or PI fluorescence intensity distribution after 3 h exposure suggested that a proportion of cells had received antibiotic-induced damage (Figure 22a, 22b), resulting in depolarization and increased permeability of the membrane, and subsequent entry of dye into the organism. Another sub-population with low fluorescence was also evident; these cells were unaffected bacteria with intact membrane.

After 1 to 2 h, the sub-population with increased fluorescence was fairly small, this increased considerably when incubation was continued for a further hour, so 3h exposure to antibiotics was used to determine the susceptibility of clinical isolates. After 3 h exposure it was found that the antibiotic–damaged proportion of cells indicated by PI uptake (increased permeability) was lower than that identified by $DiBAC_4(3)$ (depolarization of membrane).

Fluorescence is measured on logarithmic scale of 1024 of which corresponds to a channel number, thus the median fluorescent intensity gives an indication of the sensitivity of that population to a particular antibiotic. Ampicillin and tetracycline caused increased fluorescence in a susceptible control *H. influenzae* strain at

breakpoint MICs, but there was no response for a resistant control strain with an MIC greater than the breakpoint MIC.

However, prolonged incubation with the antibiotics or incubation with high concentrations of drugs, resulted in a tailing of the region of single organism into a region of the plot in which a peak of non-fluorescent debris was seen, corresponding to sub-cellular particles. This cell debris could take up the dyes, so had a very low fluorescence intensity. This affected the median fluorescent channel number and contributed to a lowering of fluorescence intensity when cell debris signals were high. Figs 23 and 24 show the proportion of *H. influenzae* cells fluorescing with PI and $DiBAC_4(3)$ following incubation with ampicillin and tetracycline respectively at its MIC. Corresponding CFUs of *H. influenzae* by plate count are also shown. It can be seen that a reduction in the viable counts was paralleled by an increase in $DiBAC_4(3)$ and PI fluorescent cell proportions, both dyes being able to identify growing population of damaged organisms.

Thirty clinical isolates of *H. influenzae* were tested by the FCS-AST using the optional conditions determined in previous chapter, and antibiotic concentrations of ampicillin2 μg/mL and tetracycline = 4 μg/mL. $DiBAC_4(3)$– and PI–associated

fluorescence was expected to increase following treatment with valinomycin in the presence of KCl and alcohol-fixed cells respectively. The equation described in the section of materials and methods was used to determine the susceptibility categories of the isolates.

Before running a particular antibiotic-treated strain, a negative control of the strain was tested. The number of events in F_1, F_2, F_3 and F_4 were adjusted by moving the dimension of the dual parameter dot plot of $DiBAC_4(3)$ against PI fluorescence intensity, so that <5% of cells would be for F_1, F_2 and F_4 (refer Figure 2).

H. influenzae cells resistant to ampicillin and tetracycline gave F values ranging from 0.54 to 2.29, whereas susceptible strains gave higher F values of 5.35 to 25.2. The range of F values associated with resistance and susceptibility to ampicillin and tetracycline were similar. Tables 37 and 38 show the results of FC-AST for ampicillin and tetracycline on *H. influenzae* isolates respectively. They were the typical results of 3 experiments. There were 7 ampicillin resistant strains; amongst it, 6 were shown to be β-lactamse positive, the other one was suspected to be a BLNAR strains. All of them were within range of F value of resistance category, and there were no error occurred.

Using one susceptible and one resistant control strain, FC-AST was performed after treating strains with ampicillin and tetracycline for 3 h. Significant numbers of fluorescent cells were detected on F1, F2 and F3, and the fluorescent intensity increased for both dyes for the sensitive control strain, giving an F value of ≥ 5.1 for both antibiotics. For the resistant strain, most cells remained unstained, with no increase in fluorescent intensity after 3 h. The F values were found to be ≤ 2.4.

Using the F values derived to determine the susceptibility category, only one minor discrepancy was observed compared with the conventional assays. This minor error occurred with ampicillin testing of an isolates with an MIC value of 0.125 (sensitive)was determined to have a F value of 3.6 (intermediate resistant).

Discussion

FCM is a technique based upon multi-parameter measurements of individual cells. These parameters can be FS or SS, which reflect cell size and structure, and fluorescence, which may reflect content of specific protein, DNA or membrane potential, depending on the fluorescent probe being used. In this study, the

membrane potential dye, $DiBAC_4(3)$ and a DNA probe, PI were used,. The ability of the FCM to detect effects of antibiotics as functions of drug concentration over a concentration range (0.1 to 100 x MIC) and as functions of duration of exposure to drugs (30 minutes to 180 minutes) was determined. The results showed that the independent parameters measured DNA – associated fluorescence, membrane potential associated fluorescence, and FS and SS provides statistically significant detection of effect of ampicillin and tetracycline at the range of MIC values tested over the time of incubation and confirms that this technique has a potential for routine testing of susceptibility test of *H. influenzae*.

The antibiotics used (ampicillin & tetracycline) were chosen to reflect different mechanisms of action: ampicillin is a β-lactam which acts on the cell wall and is bactericidal, tetracycline is a bacteriostatic agent which acts by inhibiting protein synthesis. The use of $DiBAC_4(3)$ in combination with cells treated with a bacteriostatic antibiotic would not be expected to show any induced fluorescence in the time course of experiments. However, in this study, the bacteriostatic agent (tetracycline) still caused changes in membrane potential and could be detected by using $DiBAC_4(3)$. This is because no matter what the mechanism of action of an

antibiotic, it will disrupt the metabolic activity of sensitive bacterial cells and result in a disruption of membrane potential.

It should be recognized that the control strain (ATCC 49247) used in these studies is a BLNAR strains. The other wild strains of H. influenzae were expected to behave the same since the size and structure was the same and there were not much susceptibility errors occurred.

In the present study, cultures exposed to ampicillin and tetracycline at 1 x MIC exhibited no change in membrane potential over the first 30 minutes, as determined by the persistently low uptake of the membrane potential probe of $DiBAC_4(3)$. After 30 minutes, the uptake of $DiBAC_4(3)$ began to increase and the fluorescent intensity continued to increase up to 180 minutes. Further time of incubation with the antibiotics for the experiments were not performed since if the time of incubation increased to 4 or 5 h, the procedures would not be considered as a rapid method. Moreover, a time span of 6 h (3 h for growth to exponential phase + 3 h of incubation with antibiotics) for a rapid test would be more suitable for routine laboratory test work flow since it is less than 8 h. The membrane

potential was extensively perturbed subsequent to exposure to 10 times and 100 times MIC of ampicillin and tetracycline. There was only a small proportion of cells in the bacterial culture showed PI – associated fluorescence at 1 time the MIC of both drugs over the 180 minutes. The reason of this is that there are 4 physiological states which can be distinguished for bacterial cells: reproductive viable, metabolic active, intact and permeabilized, it is not enough to look only at the loss of membrane potential as cell death because depolarization can be reversible (Nebe-Von Caron and Badley, 1994; Nebe-Von Caron *et al*, 1998). They can still show reproductive viability after loss of membrane potential. PI can detect membrane integrity and should be accepted as an indicator for cell death in bacteria (Nebe-Von Caron and Badley, 1994; Nebe-Von Caron *et al*, 1998). So, it is not astonishing that $DiBAC_4(3)$ – associated fluorescent cells may be different from PI – associated fluorescent cells. A depolarized cell must be further damaged or further treated with antibiotic before it can take up PI. In the culture with 10 x or 100 x the MIC, the cells were damaged further and the PI fluorescence intensity increased. This probably reflects the mechanism of action of the antibiotics, which causes loss of membrane potential and then affects membrane integrity.

It was found that for the tetracycline treated cells, the cell debris signals were low for all concentrations tested compared to those of ampicillin. This was because tetracycline is only bacteriostatic, Cells were not killed immediately, and bacterial cells would not rapidly disintegrate.

For ampicillin treated cells, the cells were rapidly killed. When the concentration of ampicillin was high (10X and 100X MIC), a lot of cell debris signals appeared resulting in a low fluorescent intensity. So the fluorescent intensity of $DiBAC_4(3)$ and PI decrease at high ampicillin concentrations.

In all experiments, only a proportion of cells in the bacterial culture showed PI or $DiBAC_4(3)$ – associated fluorescence at any given time following exposure to antibiotic. This demonstrated the dynamic and heterogeneous nature of bacterial cell populations; and has been previously described (Lloyd, 1993),

The interpretation criteria for susceptibility and resistance for the FC-AST had to be established. A measurement was sought to give a more objective determination of susceptibility. In a previous study by Gauthier *et al* (2002) a multiparametric FCM-AST was developed on clinical urine isolates and samples. PI and $DiBAC_4(3)$ were used to monitor cytoplasmic membrane integrity and

measure changes in membrane potential respectively. The measure was based on the number of cells fluorescent for both dyes and the mean fluorescent intensity of events measured in the gated region of a FS vs SS dot plot, and was compared between antibiotic treated and non-treated samples giving a ratio termed C_1. The C_1 values were used to define sensitive, resistant and intermediate resistant susceptibility categories; and were established by comparison with conventional standard tests. In the present study, *H. influenzae* cells were tested instead of urine isolates. A modified version of C_1 (F) was used to determine the susceptibility categories of *H. influenzae* isolates. In addition to the mean fluorescent intensity of PI and $DiBAC_4(3)$, the acquisition region of f_1, f_2 and f_4 which were defined on the biparametric florescent histogram (Figure 12) were also taken into account. Events recorded in these regions were fluorescent with either one of the dyes (PI or $DiBAC_4(3)$) or both dyes and allowed the exclusion of non-fluorescent events. The events on region f_1, f_2 and f_4 and the median fluorescence of the dyes were added together, and this value was compared between the live controls and the antibiotic – treated samples. The difference of the F value of the present study and C_1 value of the study of Gauthier *et al* (2002) is that events regions that were labeled with only one dye was also take into account for the calculation of F, whereas for the study of Gauthier *et al* (2002),

only events labeled with both dyes were used for the product C1. This is because I found some of the susceptible stains were only fluorescent for $DiBAC_4(3)$ but not PI. This was demonstrated by the results of the dose dependent antibiotic response (kinetic) studies in which the control cells only showed a small amount of increase of fluorescence with PI when the antibiotic concentration was 1 x time MIC and can probably be explained by the fastidious nature and thus slow growth of the organism.

The use of two mortality dyes for the calculation of F values in the present study increased the sensitivity of the FCM for AST of *H. influenzae*. Since the antibiotic effect on the cells sometimes resulted in fluorescence from only one dye (most probably $DiBAC_4(3)$), it was decided that limiting the results to inclusion of only cells that took in both dyes as for Gauthier's study (2002) would not yield best results. Other workers have used only one dye for FC-AST (Suller *et al*, 1987; Gant *et al*, 1993; Ordonez and Wehman, 1993).

In the study by Gauthier *et al* (2002), using quantified C_1 values to determine susceptibility, 328 strains were tested. Seven minor errors, 8 major errors and 5 very major errors were observed; there was 93.99% agreement with standard AST methods. In the present study, using 30 strains of *H. influenzae* and two antibiotics,

there was only one susceptibility discrepancy which was a minor one. The conventional dilution method has an allowable error of 1 $\log_2$ dilution. An isolate can easily shift from one category to another if the isolate has an MIC close to breakpoint. Thus, the AST measurement by FCM may easily be determined as an error, even if the FCM-AST was the more accurate test.

It has been shown that some bacterial cells stained only by $DiBAC_4(3)$ may have lost membrane potential but not membrane integrity as the cells could not take in non-permeable dye such as PI (Nebe-Von Caron *et al*, 1998); and that a significant proportion of these could be recovered on solid media. This means that a sub-inhibitory value of antibiotics could be detected by using a membrane potential dye such as $DiBAC_4(3)$. Antibiotics at sub-inhibitory concentration may adversely affect microorganisms in many different ways, and these effects may be clinically relevant (Shapiro, 2003). Moreover, bactericidal or bacteriostatic antibiotic, irrespective of the cell targets, would eventually cause a change in membrane potential as shown in the present study. So $DiBAC_4(3)$ is an excellent dye to detect the effect of antibiotics. However, with respect to the impermeable dyes such as PI, exclusion of the fluorescent probe by a intact cytoplasmic membrane equates with the absence of an antibiotic effect, whilst

uptake of the fluorescent probe via the antibiotic – damaged membrane is consistent with antibiotic susceptibility (Pore, 1994). Therefore use of both dyes (PI and $DiBAC_4(3)$) would be the best combination to shown the effect of antibiotics on bacterial cells.

The choice of fluorescent probes for AST by the FCM that would have the flexibility to be used by a range of organisms and antibiotics is important as it would enable standardization of the test method. So versatility is an essential property. $DiBAC_4(3)$ has been used for AST in both gram-positive and gram-negative organisms, and with different classes of antibiotics. Mason *et al* (1984) used this dye to analyze membrane damage caused by gentamicin and ciprofloxacin. Suller *et al* (1997) used it to study the effect of penicillin, methicillin and vancomycin. Jepras *et al* (1995) used $DiBAC_4(3)$ to study the effect of azithromycin, cefuroxime, and ciprofloxacin on *E. coli.* All of the results correlated well with conventional interpretative categories for AST. However, evaluation of the FC-AST for fastidious organism has never been performed.

Ordonez & Wehmen (1993) used a commercial FCM, FCScan, to develop a FCM-AST for *Staphylococcus aureus* for penicillin. A cationic membrane potential sensitive probe $DiOC_5$ (3.3' – dipentyloxacarbocyanine was used. Thus, fluorescent intensity occurred in intact cells but fluorescent intensity was lost when bacterial viability was compromised. The investigators attempted to correlate the FC-AST data with those from disc diffusion tests, MIC determination and viable count. They tried to determination the quantitative aspect of FCM-AST for the susceptibility to penicillin using a formula called the sensitivity index (SI). However, they were unable to derive MICs from the results. They were able to reliably assess susceptibility categories of *S. aureus* to penicillin G and oxacillin, 90 minutes after addition of the antibiotic. The reasons for difficulty in quantifying the effects of antibiotics by FCM-AST may be due to the inconsistency of fluorescence ratio between strains of the same species or different species as was observed in this study. The ratio of mean fluorescence between the test and control strains were subject to variation even if the MIC of the strains were the same. The reasons may be : i) the baseline fluorescence is different between different strains; ii) shifting of fluorescent intensity is also different for strains of same or different species of bacteria and iii) shifting is different for different dye concentrations as reported by Mason and Gant (1995).

For these reasons, FCM-AST will only provide a qualitative susceptibility result due the variation of responses between strains of the same species of *H. influenzae.*

Realizing the potential utility of FC-AST, several companies have developed new products to rapidly perform AST in the clinical laboratory. One of these products was the Bac-Light viability kit (Molecular Probes Inc.), which enables distinction of live and dead cells by the use of two fluorochromes. Bacteria with intact membranes allow only one dye to enter, whereas those with damaged membranes allow both dyes to enter. The kit was evaluated (Alvarez *et al*, 2000) with testing on enterococci species and *E. coli.* Antibiotics effects were rapidly detected by measuring the variations in fluorescence following 3 h of incubation with the antibiotics. However, the interpretation of heterogeneous staining patterns produced by the Bac-Light kit has not been fully defined as to which populations represent viable and non-viable cells. It is further complicated when testing antibiotics which can be bacteriostatic or bactericidal. A limitation of the kit is that it failed to identify any heterogeneity and thus can only be used as a general indicator of the adverse effect of an antimicrobial agent.

Bio-Rad Laboratories also developed a FCM-AST (FAST^{TM} – 2 kit) that relies on detection of antimicrobial effect on bacteria measured by a pair of fluorescent probes. Evaluations were performed on two models for antibiotic activity: *E. coli* and ampicillin, and *Enterococcus faecuim* and vancomycin were studied as a function of dosage and exposure time with antibiotic at various drug concentrations (Chiu *et al* ; 1997). These experiments defined the effectiveness of ampicillin against *E. coli* as a function of its concentration and *E. faecium* and vancomycin as a function of exposure time. The results have illustrated the effects of antibiotic action on bacterial cells and indicate that the FAST-2 system is a rapid, reproducible and sensitive technique to study the mechanism of antibiotic action in non-fastidious bacteria.

In the present study, the protocols were especially optimized for *H. influenzae*. The stains were ones that have been extensively studied and the susceptible and resistant sub-populations in a culture could be clearly defined. By now, we still do not know exactly what the FCM FS and SS diode or photomultiplier tube events represent. It can be assumed that FS is simply proportional to size, which should be true for bacterial cocci. There is an approximately linear relationship between light scatter properties and total protein content of individual bacteria (Tormquist

et al, 1991). For SS amplitude, it may bear a relationship to the complexity of the eukaryotic cells, in which well-defined subcellular organelles are seen through a lipid bilayer of different composition to that of bacteria. However, a bacterial cell wall may be opaque to light and SS may reflect the optical density of the cell wall. Nevertheless, filamentous cells that occurred after antibiotic treatment scattered more light in the forward direction than non-filamentous cells. Past research (Lorian and Amoral, 1990; Gant *et al*, 1993; Walberg *et al*, 1996) has confirmed that under the same experimental condition, drug response can be detected by light scatter alone. This implied that FCM-AST may be carried out without staining of the cells. In this study, I also found that there was filamentation of *H. influenzae* cells with an increase in FS intensity, especially with the β-lactam antibiotic – ampicillin. However, in some culture samples, it may be difficult to distinguish the light scatter of bacteria from that of the large amount of cell debris present. Therefore measurement of membrane potential sensitive fluorescence and nucleic acid specific fluorescence to discriminate between bacterial cells and others particulate material would be preferable.

Statistics are very much in favor of FCM counting. Counting more than 5000 cells reduced the counting error to below 1% (Shapiro, 2003b). Accurate

counting of bacteria is very important especially when physiological measurements of bacteria, including antibiotic effects, are investigated. The method used in EPICS Elite FCM is ratiometric counting. Reference beads of known concentration are mixed with an equal volume of culture sample and are discriminated in a region on the basis of size and fluorescence. The ratio of detected beads to detected bacteria is then used to calculate the absolute numbers of bacteria. This method of counting corrects for system dead time and the variations in flow rates that can occur in instruments that deliver their samples by differential pressure (Nebe-VON Caron and Badly, 1995). The tight cluster of beads also serves as an on-line alignment control.

As shown in Figure 15 a and b, the trend in CFU counts compared well with the flow viable count that was stained with $DiBAC_4(3)$. But, there was a small discrepancy between the drop in viability determined from colony counts and the proportion of cells staining with $DiBAC_4(3)$ at an intensity suggesting loss of membrane potential. Similar counts were obtained during the first few minutes, but thereafter, the FCM count remained lower than the result by the viable plate count assay. The plate counts may be higher since the samples had undergone many dilution steps before being plated onto chocolate agar. This facilitates the

breaking up of any cell clumps and may lead to higher numbers of CFU. Moreover, some of the cells may be recovered and grown into a CFU even if they had lost some of the membrane potential and taken in the $DiBAC_4(3)$ dye. However, the discrepancies were believed to be insignificant and has not effect on the FCM-AST results. Analysis of the same strain by FCM with PI and by standard plate count followed the same pattern. However, unlike the $DiBAC_4(3)$ stained cells, those that take in PI have lost membrane integrity, cannot recover and would not grow on an agar plate. This finding is consistent with the studies of Nebe-Von Caron *et al* (1998) and Kaprelyants and Kell (1992), who showed that depolarized but intact cells, i.e. cells that exclude PI but take up $DiBAC_4(3)$, can still show reproductive viability. They also showed that it is possible to distinguish depolarized and permeabilized cells, and to demonstrate substantial recovery of the depolarized but intact cells, even without use of 'recovery agents".

With DiBAC4(3), a greater fluorescence response was obtained from valinomycin-treated *H. influenzae* cells compared to heat-treated bacteria. Moreover, the fluorescence from PI and $DiBAC_4(3)$ were greater in alcohol-fixed cells than in heat-treated cells. Possibly the heat treatment destroyed some of the

PI and the $DiBAC_4(3)$ binding sites, or the heat-treated cells were viable but non-culturable.

Valinomycin has been added to the live cells to prove that the $DiBAC_4(3)$ responded to changes in membrane potential. Valinomycin is an ionophore, which is lipophilic and forms a complex with potassium ions. Potassium ions can thus be readily transported across the cell membrane. So addition of valinomycin increases the cells' potassium permeability, so that the membrane potential is determined entirely by the transmembrane potassium gradient. If extracellular K^+ concentration is low, valinomycin hyperpolarizes cells. If extracellular K^+ is high valinomycin depolarizes cells. In this study, 200mM of KCl was added to the live cells together with valinomycin, so that cells were depolarized and $DiBAC_4(3)$ could be proved to have entered the depolarized cells.

During analysis, it is important that the profiles are as sharp as possible and that only single cells or nuclei are measured. The quality of a histogram is measured by its coefficient of variation (CV). CV is defined as the standard deviation of a series of values divided by the means of those values. It can be used to assess the variation in particle characteristics within a population. It is frequently used to

assess the alignment of a FCM and the skill of the operator. Small CVs give a better resolution of small difference. The quality of a histogram is affected by i) instrument alignment; ii) sample preparation; and iii) staining procedures (Shapiro, 2003c). In this study, the most common CV values were between 10% to 30%. The instrument alignment was checked daily using fluorescent beads (Polyscience Inc. U.S.A.). The half peak CV of 2% or better was obtained for fluorescence parameters being used for analysis. Sample preparations were checked microscopically. All the FCM-ASTs were performed at least three times and the median value was used for analysis.

The limiting factors in using FCM for AST are the cost of instrumentation, the perceived complexity of analysis, safety, and skills of operators. However, the reagents used in this experiment were relatively inexpensive if a purpose built FCM for AST was available. The time required of the technician for performing the test when compared with conventional method is almost the same but the results are available more rapidly. Nevertheless, a FCM plus accessories remains expensive. However a rapid report of sensitivity test may lead to improved patient care with appropriate and cheaper antibiotics and shorter hospital stay. A one day reduction in hospital stay for one patient may save more than HK $10,000

Thus the money saved by the hospital would pay for more than 100 tests (see Appendix ix).

In these studies, time-dependent and dose-dependent effect of ampicillin and tetracycline to *H. influenzae* cells were performed. An FCM-AST was developed for *H. influenzae*, which has not previously been evaluated for FCM-AST. The studies were not an exhaustive statistical comparison with standard AST method, but were intended to evaluate the feasibility of a FCM-AST for *H. influenzae* strains. The results showed that the FCM-AST developed was reliable for susceptibility testing of *H. influenzae,* with only 1 minor error occurring in 60 tests. Further work is necessary to confirm the use of $DiBAC_4(3)$ and PI as dyes for FCM-AST and determine whether it gives useful information with another fastidious organism such as *S. pneumoniae*. It should be noted that responses may vary between bacterial species and even between strains of a single species. The fluorescence response can also depend on the dye concentration and method of cell perturbation. So FCM-AST may have be a potential candidate to replace the disk diffusion test but not the MIC tests for *H. influenzae*.. More extensive testing will be necessary to assess the value of the FC-AST for routine susceptibility testing to be used in clinical laboratory.

Table 37: Results of FC-AST for susceptibility testing of ampicillin on *H. influenzae* isolates

Strain ID	Treated Strain $F_1+F_2+F_4$	Treated Strain MFI DiBAC4(3)	Treat Strain MFI_{PI}	X_T	Control $F_1+F_2+F_4$	Control $MFI_{DiBAC4(3)}$	Control MFI_{PI}	X_C	F	MIC	S. Cat
32201	15.99	1.8	0.7	20.1	8.61	1.9	1.6	2617	0.76	128	R
44333	12.0	6.4	5.2	399.36	6.68	2.0	3.8	50.76	7.9	0.5	S
23313	22..3	4.9	2.0	2 1 8 . 5	11.06	1.6	1.0	17.69.	12.3	0.5	S
12213	29.01	4.3	2.1	262.0	11.00	1.3	1.0	14.3	18.3	0.25	S
31930	12.06	6.8	2.0	164.06	7.45	1.2	1.2	10.72.	15.3	0.125	S
32207	8.9	6.5	1.9	111.435	5.88	2.4	2.4	11.29.0	9.9	0.125	S
17022	8.2	6.1	8.0	400.16	4.71	4.7	4.7	104.0	3.8	2	I
49247	6.45	3.0	2.9	56.1	3.23	2.1	2.1	14.2	3.9	2	I
22121	7.90	3.2	1.6	40.4	3.00	1.0	0.8	2.40	16.8	0.5	S
42121	8.73	5.1	4.6	204.8	3.16	1.7	4.3	23.100	8.9	2	I
31905	31.79	5.5	3.1	$5.42x10^4$	6.02	2.3	2.1	2907.7	18.6	1	S
31933	8.05	1.2	1..9	18.4	3.90	0.8	1.4	4.36	4.2	2	I
22106	48.39	4.9	1.3	310	12.41	1.3	0.8	12.9	24.0	0.5	S
10006	13.77	3.8	1.6	83.3	8.72	1.2	0.8	8.37	10.0	1	S
32210	20.8	18.0	3.6	1347.8	5.86	2.1	2.5	30.7	43.9	2	I
10010	**8.9**	**2.4**	**4.2**	**89.7**	**5.37**	**1.7**	**2.7**	**24.64.**	**3.64**	**0.125**	**S**
29247	8.55	4.2	4.0	143..7	6.96	2.0	3.2	44.54	3.2	2	I
31920	6.75	2.9	1.4	27.4	6.44	2.1	1.2	16.22	1.69	4	R
41920	10.96	2.4	1.4	36.8	6.51	1.9	1.3	16.07	2.29	4	R

Strain ID	Treated Strain $F_1+F_2+F_4$	Treated Strain MFI DiBAC4(3)	Treat Strain MFI_{PI}	X_T	Control $F_1+F_2+F_4$	Control $MFI_{DiBAC4(3)}$	Control MFI_{PI}	X_C	F	MIC	S. Cat
32207	20.96	6.1	1.2	153.5	7.08	1.9	0.8	11.9	12.9	0.5	S
32211	14.46	2.9	2.2	92.3	7.47	2.5	1.2	22.41	4.11	2	I
32206	22.37	7.8	2.2	390	7.25	1.5	1.4	15.22	25.2	1	S
46022	9.57	2.6	1.5	37.3	4.94	1.0	1.0	4.94	7.5	0.5	S
22131	8.79	2.7	1.9	45.1	6.24	1.3	0.8	6.48	7.0	0.5	S
22108	725	1.9	1.7	2341.8	461	2.6	1.8	2157.5	1.08	128	R
22107	11.6	3.2	1.2	44.5	4.08	1.4	1.0	5.7	7.8	0.03	S
31910	16.6	1.9	2.3	72.5	15..22	2.0	1.6	48.7	1.5	4	R
31916	15.94	3.1	1.2	59.3	5.46	1.3	1.1	5.4	7.8	0.25	S
42211	4.38	3.6	1.0	15.76	5.49	3.5	1.1	21.13	0.75	128	R
31905	7.41	2.5	2.5	46.34	8.29.0	2.3	2.5	47.66	0.97	64	R

* Susceptibility category

Where $XT = F_T \times MFI_{Dibac4(3)} \times MPI_{PI}$

$XC = F_C \times MFI_{Dibac4(3)} \times MFI_{PI}$

F_T was all the number of events in F_1, F_2 and F_4 quadrants as shown in Figure 9 of chapter 6 for the tested culture.

Fc was equal to the percentage of events in the F_1, F_2 and F_4 quadrants times MFI_{PI} and $MFI_{Dibac4(3)}$ for the untreated control cells.

$MFI_{Dibac4(3)}$ and MFI_{PI} were the median fluorescence value for $DiBAC_4(3)$ and PI respectively.

Table 38: Results of FC-AST for susceptibility testing of tetracycline on *H. influenzae* isolates

Strain ID	Treated Strain $F_1+F_2+F_4$	Treated Strain $MFI_{DiBAC4(3)}$	Treat Strain MFI_{PI}	X_T	Control $F_1+F_2+F_4$	Control $MFI_{DiBAC4(3)}$	Control MFI_{PI}	X_C	F	MIC	S. Cat*
42208	12.83	4.5	2.2	127.0	7.54	4.6	2.2	76.3	1.66	8	R
42212	9.7	3.3	2.0	64.02	4.22	1.0	1.0	4.22	15.22	0.5	S
31930	3.17	2.9	2.2	20.23	3.92	2.9	2.2	25.0	0.81	128	R
32207	8.99	7.8	1.1	77.13	2.73	3.7	0.7	7.07	10.9	0.125	S
12207	7.88	4.8	1.6	60.5	3.12	2.2	1.0	6.86	8.8	0.5	S
31905	4.88	2.2	5.6	60.12	2.37	1.3	2.5	7.70	7.81	1.0	S
32213	5.36	1.6	1.0	8.58	8.02	1.3	1.0	10.42	0.82	32	R
32211	15.6	2.6	1.4	56.8	7.99	2.5	1.1	22.0	2.58	4	I
14294	11.61	5.6	3.4	220.9	5.96	2.0	2.5	29.8	7.41	2	S
32210	4.23	1.8	1.3	9.9	4.8	1.9	1.3	11.86	0.83	8	R
31933	4.49	1.8	1.0	8.08	3.86	1.9	0.8	5.87	1.37	16	R
41933	4.98	1.6	0.9	7.17	5.02	1.7	0.8	6.83	1.05	8	R
31916	16.0	1.7	1.6	43.52	5.46	1.2	1.2	7.86	5.54	1	S
42211	25.7	2.7	1.2	83.2	10.7	1.0	1.1	11.8	7.05	0.5	S
22108	7.5	0.3	1.8	4.05	7.13	0.3	1.9	4.06	1.0	8	R
22121	5.4	1.9	2.9	29.75	4.73	2.1	2.8	27.8	1.07	8	R
32212	13.35	3.2	2.2	84.0	8.35	2.5	1.1	22.96	4.09	4	I
40021	19.63	4.4	3.7	320.0	10.33	1.5	1.0	15.5	20.6	0.5	S
22107	6.22	0.5	.7	2.18	5.36	0.4	0.7	1.5	1.45	32	R

Strain ID	Treated Strain $F_1+F_2+F_4$	Treated Strain $MFI_{DiBAC4(3)}$	Treat Strain MFI_{PI}	X_T	Control $F_1+F_2+F_4$	Control $MFI_{DiBAC4(3)}$	Control MFI_{PI}	X_C	F	MIC	S. Cat*
42107	3.29	0.4	0.7	0.92	5.65	0.5	0.4	1.69	0.54	64	R
31910	5.84	2.4	0.9	12.61	5.64	2.0	1.4	15.8	0.8	16	R
31916	12.28	1.0	1.3	15.96	9.96	0.8	1.2	9.56	1.7	64	R
22131	4.65	1.3	0.8	4.84	2.52	1.2	0.8	2.42	2.0	8	R
42121	6.01	1.8	2.3	24.9	5.71	1.0	2.4	13.7	1.82	16	R
12206	26.09	3.4	1.8	160.0	14.94	2.0	1.0	29.9	5.35	0.25	S
22206	10.96.	3.0	2.1	69.1	5.58	1.1	0.9	5.52	12.5	0.5	S
22106	7.56	2.5	0.6	11.34	3.98	0.5	0.3	0.597	19.0	0.25	S
31920	7.39	5.5	1.1	44.7	4.48	2.8	1.0	12.5	3.576	4	I
32201	10.57	1.2	0.8	10.15	7.48	1.0	0.8	5.98	1.7	16	R
31916	11.95	4.5	2.5	134.4	5.24	2.9	0.9	13.67	9.83	0.125	S

* Susceptibility category

Where $XT = F_T \times MFI_{Dibac4(3)} \times MPI_{PI}$

$XC = F_C \times MFI_{Dibac4(3)} \times MFI_{PI}$

F_T was all the number of events in F_1, F_2 and F_4 quadrants as shown in Figure 9 of chapter 6 for the tested culture.

Fc was equal to the percentage of events in the F_1, F_2 and F_4 quadrants times MFI_{PI} and $MFI_{Dibac4(3)}$ for the untreated control cells.

$MFI_{Dibac4(3)}$ and MFI_{PI} were the median fluorescence value for $DiBAC_4(3)$ and PI respectively.

Figure 11: (a) Forward scatter vs side scatter

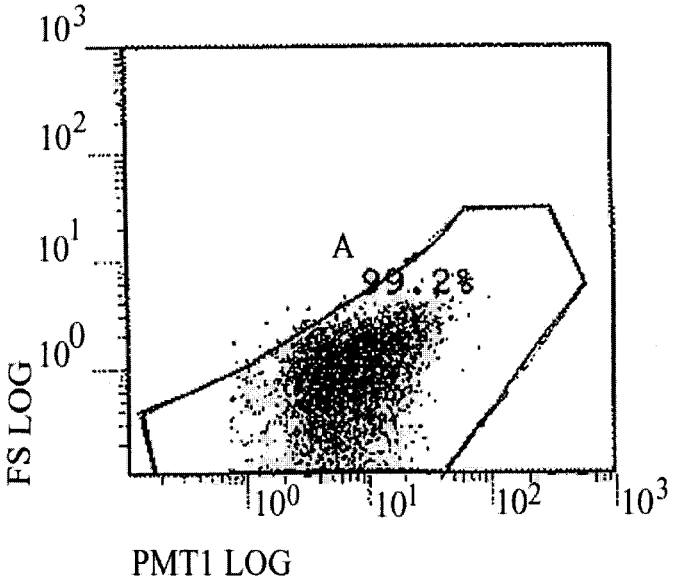


Figure 11: (b) DiBAC4(3) (PMT2)

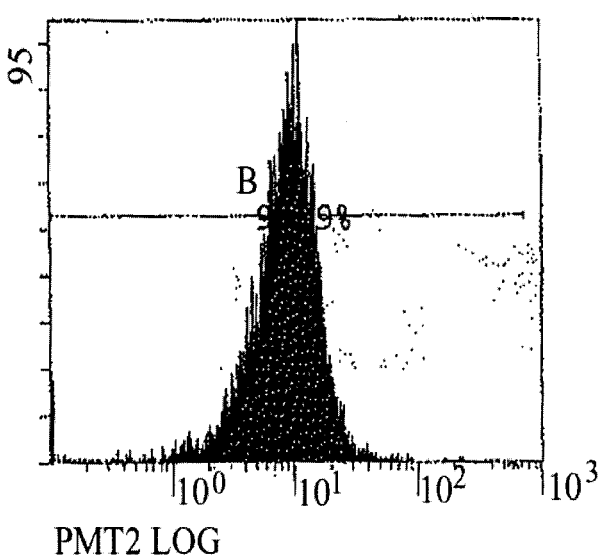


Figure 11: (c) PI (PMT4)

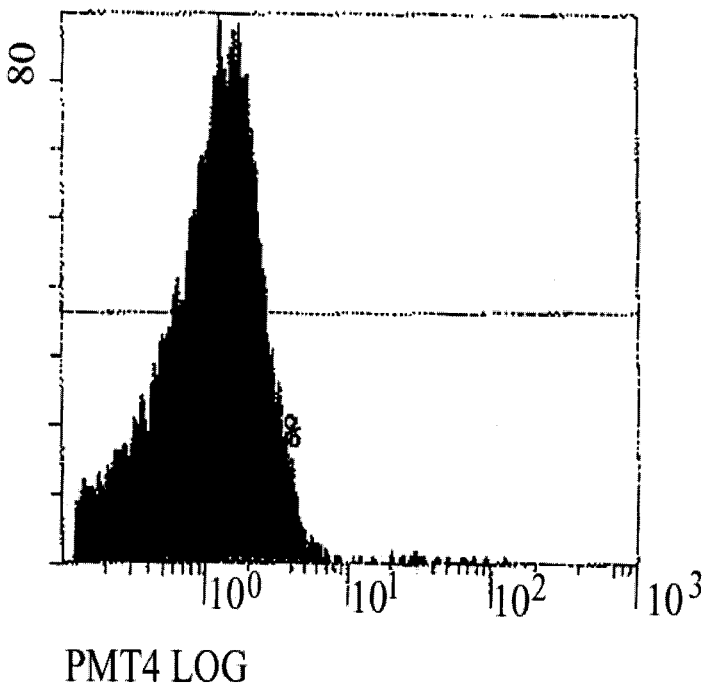


Figure 11: Untreated *H. influenzae* acquisition gating (a) light scatter profile – forward scatter (FS) versus side scatter (PMT1) with gating A. (b) Fluorescent histogram of DiBAC4(3) (PMT2) and (c) PI (PMT4) acquired on events of A.

Figure 12: (a) Untreated *H. influenzae* cells

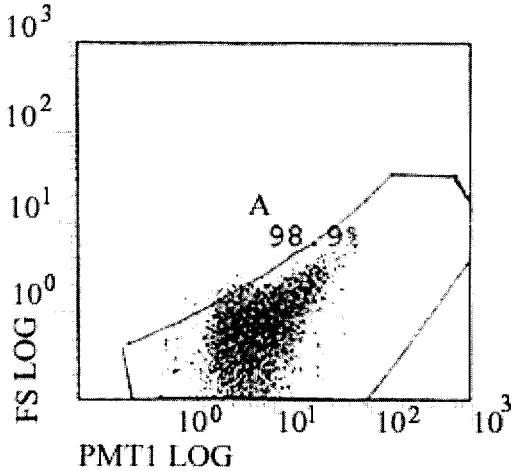


Figure 12: (b) Untreated

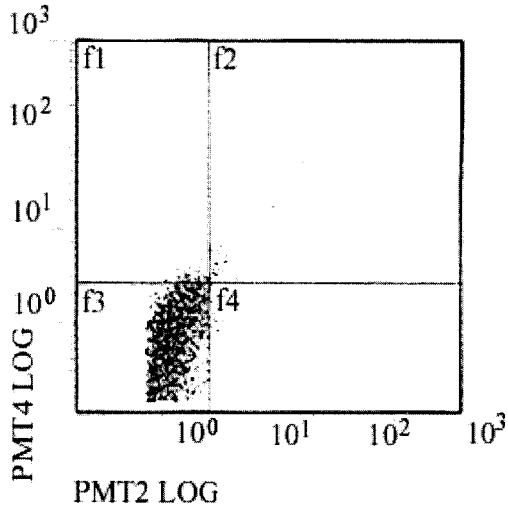


Figure 12: (c) Incubation with Amp for 3 hrs

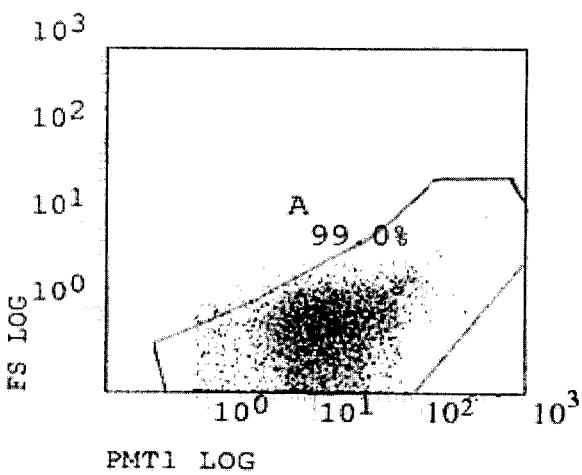


Figure 12: (d) Amp 3 hr

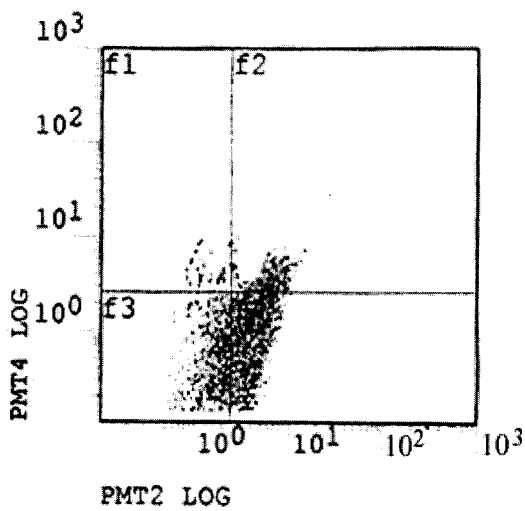


Figure 12: (e) Incubation with Tet for 3 hs

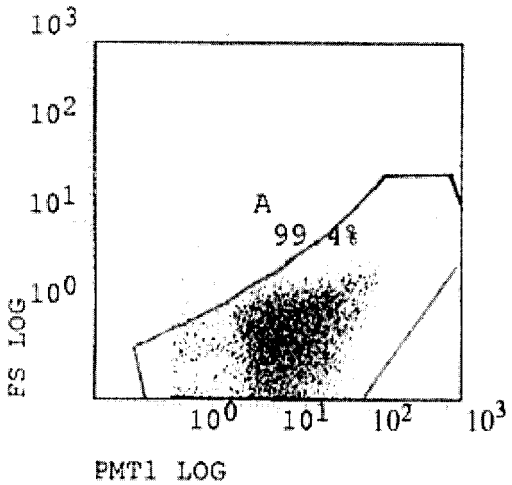


Figure 12: (f) Tet 3 hrs

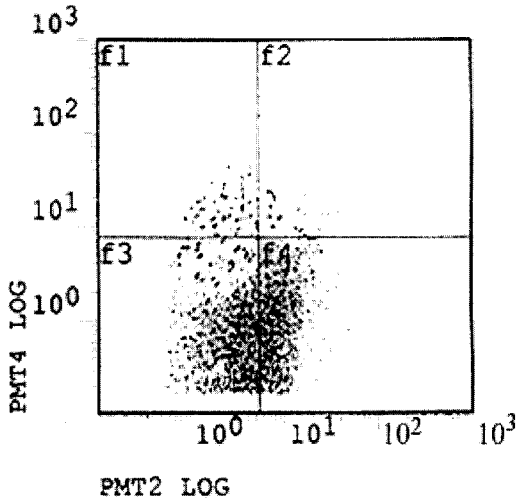


Figure 12. Light scatter profile of FS against SS (a, c, e) and biparametric dot plot of PI (PMT4) vs $DiBAC_4(3)$ (PMT2) (b, d, f) for a control strain of *H. influenzae* sensitive to ampicillin (amp) and tetracycline (tet). Untreated cells (a, b), cells treated with ampicillin for 3 hours (c, d), treated with tetracycline for 3 hours (e, f). The percentage of cells positive for fluorescence b, d, f was 31%, 68.3% and 45.4% respectively. Events on region f_1 represent cells that took up PI, on f_4 represent cells took up $DiBAC_4(3)$. F_2 contain cells that took up both dyes. Events on f_3 were cells that remained unstained.

Figure 13: (a) Unstained viable *H. influenzae* control strain plotted against fluorescence intensity of PI (PMT4).

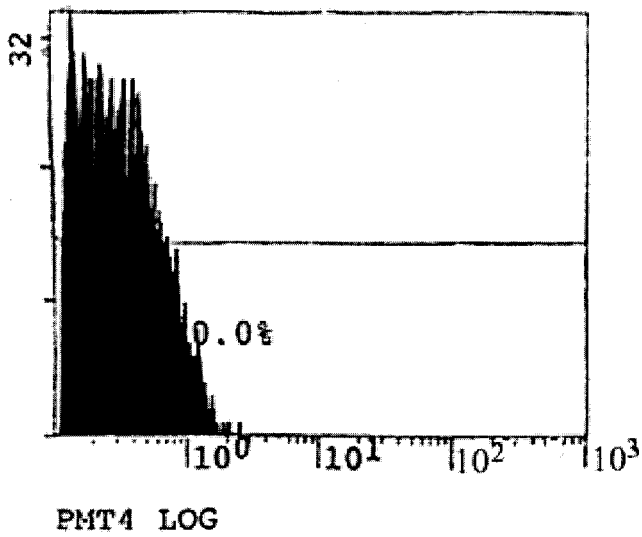


Figure 13: (b) Unstained viable *H. influenzae* control strain plotted against fluorescence intensity of $DiBAC_4(3)$ (PMT2)

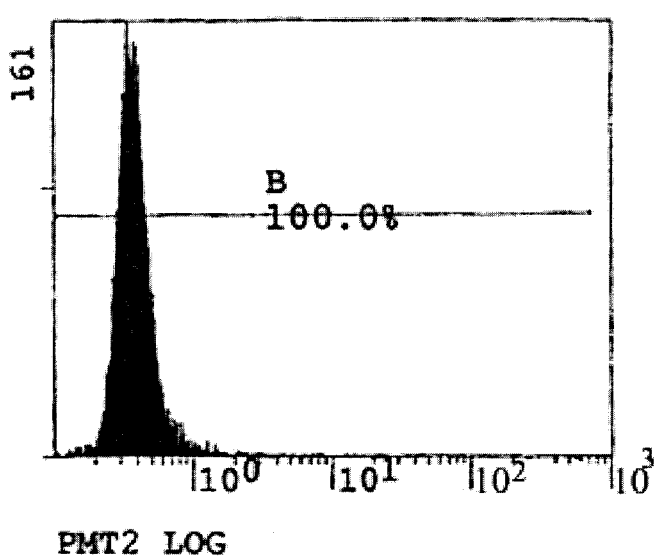


Figure 13: (c) Viable *H. influenzae* control strain cells stained with PI

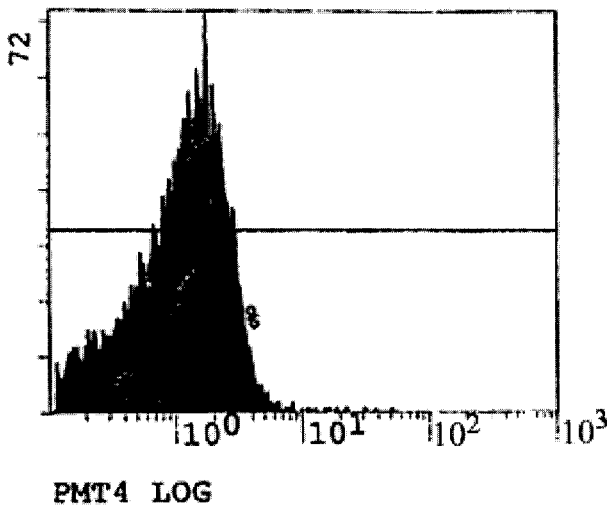


Figure 13: (d) Non viable *H. influenzae* control strain cells stained with PI

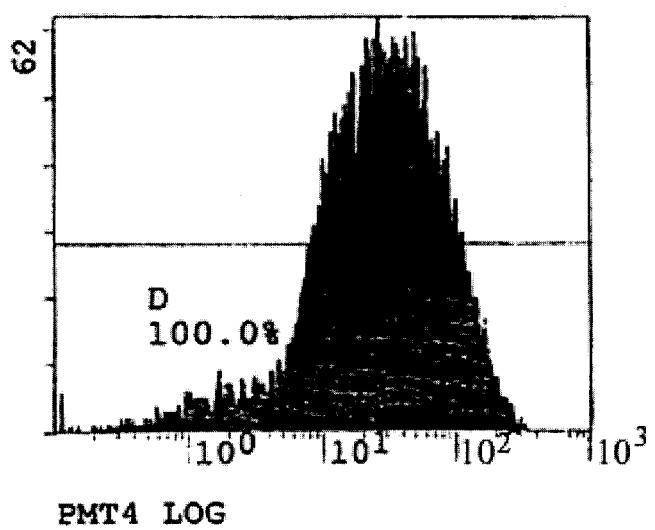


Figure 13: (e) Viable *H. influenzae* control strain cells stained with $DiBAC_4(3)$

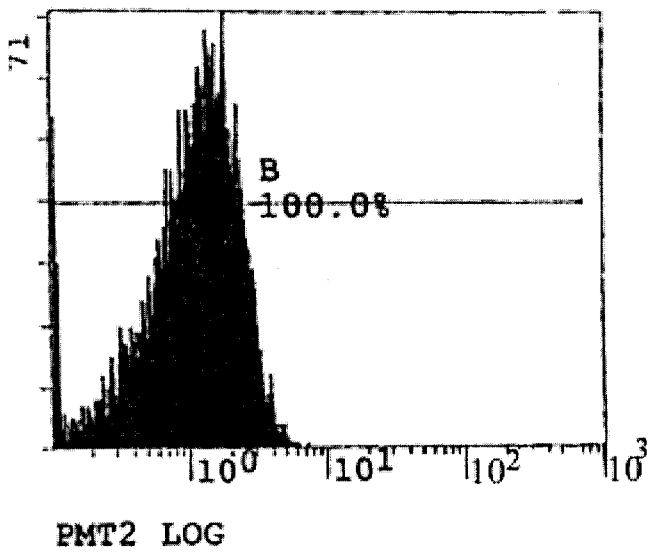


Figure 13: (f) Non viable *H. influenzae* control strain cells stained with $DiBAC_4(3)$

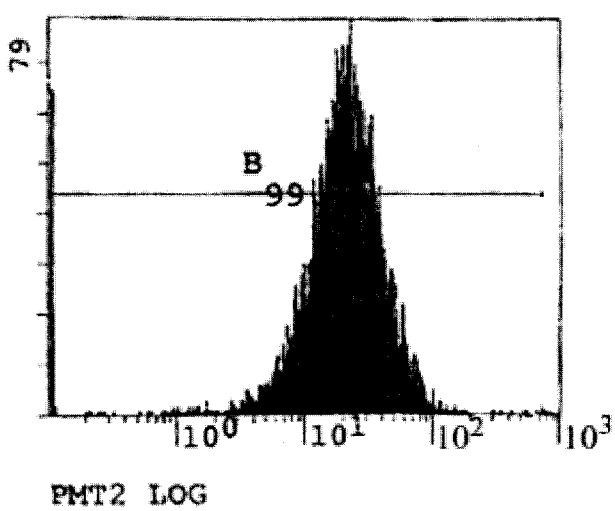
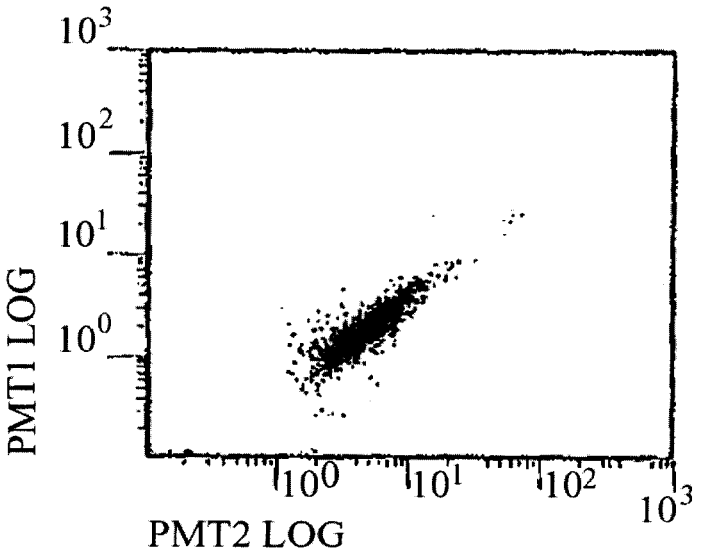


Figure 13: Histogram profiles of *H. influenzae* control strain cells in Tris-HCL pH7.5 medium with no dye (a and b); with viable cells and non-viable cells stained with PI (c and d) and with $DiBAC_4(3)$ (e and f). Unstained cells showed very low fluorescence intensity.

Figure 14 (a) UnGated PMT2 LOG/PMT1 LOG- viable cells



Fiure 14 (b) UnGated PMT2 LOG/PMT1 LOG- valinomycin -treated

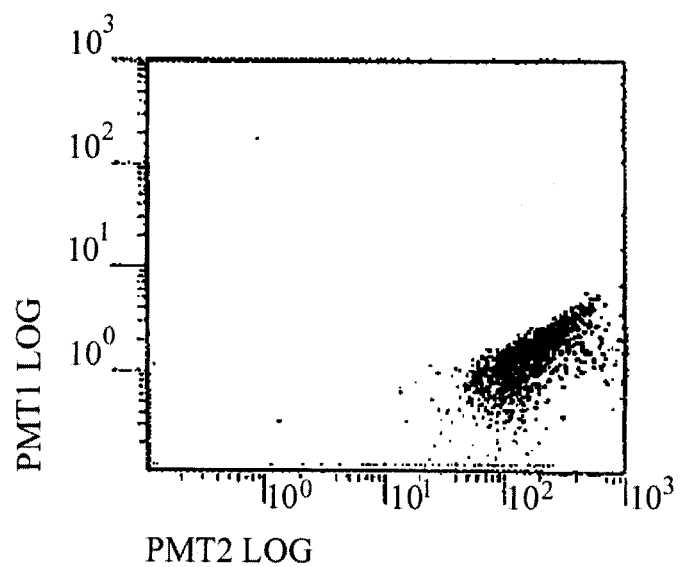


Figure 14: Dot plot figure of SS against $DiBAC_4(3)$ fluorescence for (a) viable (b) valinomycin -treated cells of *H. influenzae*.

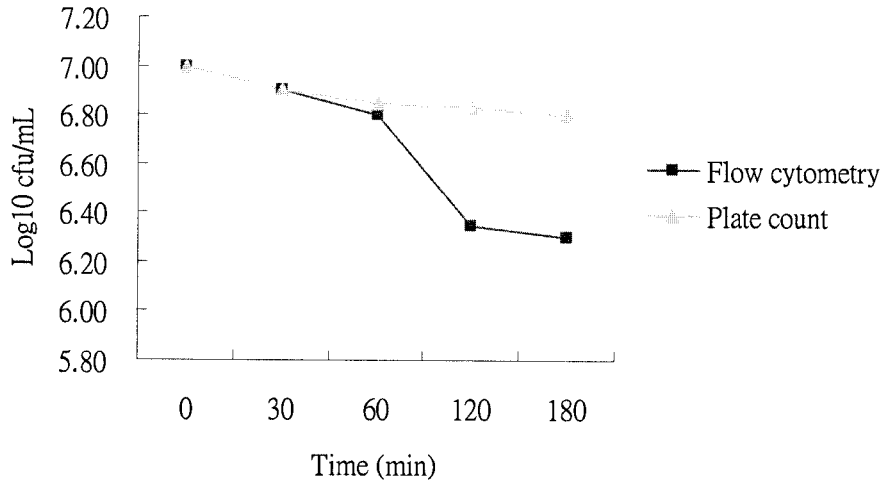


Figure 15a: Comparison of viable count (CFU/mL) of *H. influenzae* treated with 0.1x MIC determined by plate count and FCM count.

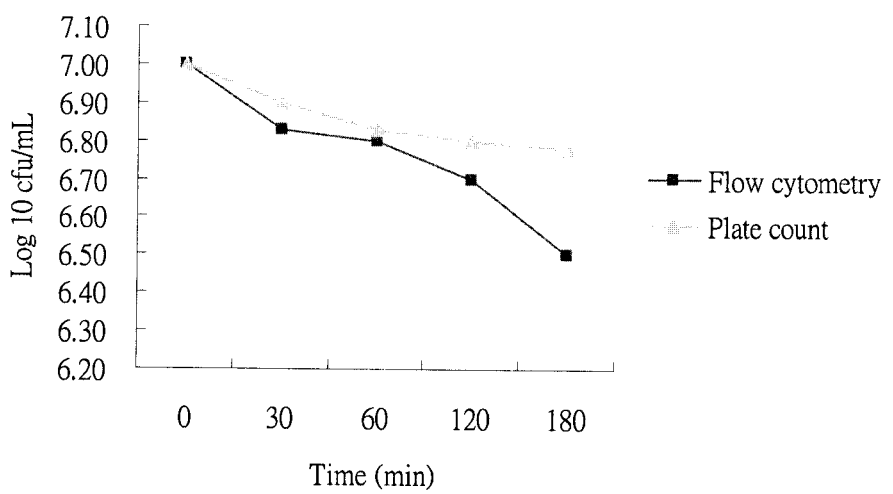


Figure 15b: Comparison of viable count (CFU/mL) of *H. influenzae* treated with 1 x MIC determined by plate count and FCM count.

Figure 16: **(a)** Untreated *H influenzae* control strain

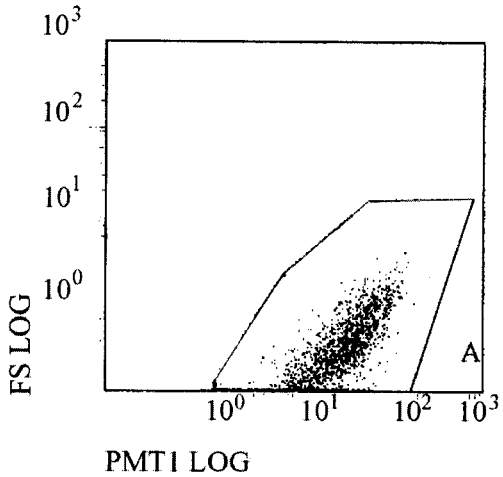


Figure 16: **(b)** Ampicillin treated *H influenzae* control strain

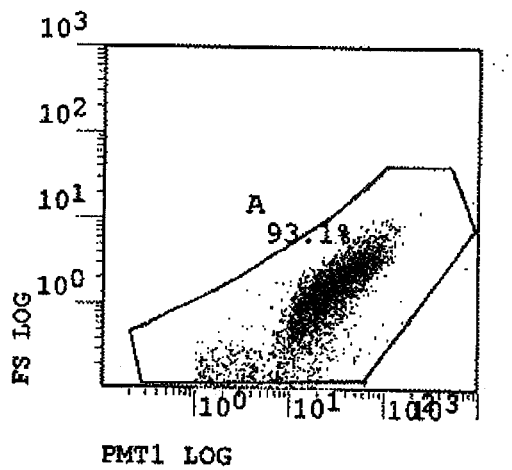


Figure 16: Two dimensional dot plot of *H. influenzae* control strain after incubation with ampicillin at its MIC for (a) 0 hour (b) 3 hours. In (b) there is a major population shift of both forward (FS LOG) and side scatter (PMT1 LOG) indicating filamentation of cells following ampicillin treatment. A small number of events were recorded close to the axis of origin in (b), which may represent sub-cellular particles.

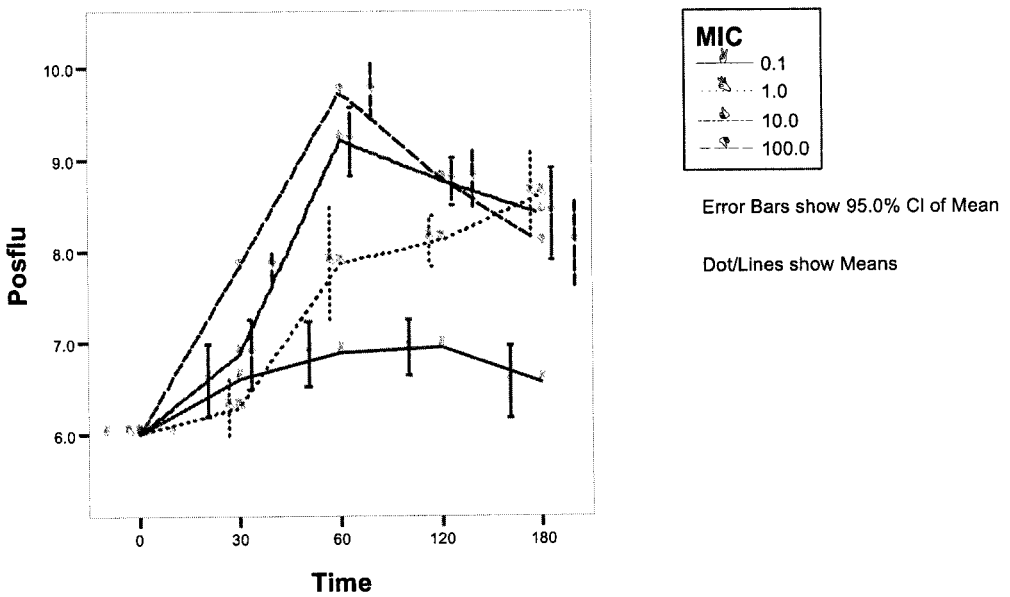


Figure 17: Uptake of $DiBAC_4(3)$ by ampicillin-treated *H. influenzae* cells at different concentrations of ampicillin and times.

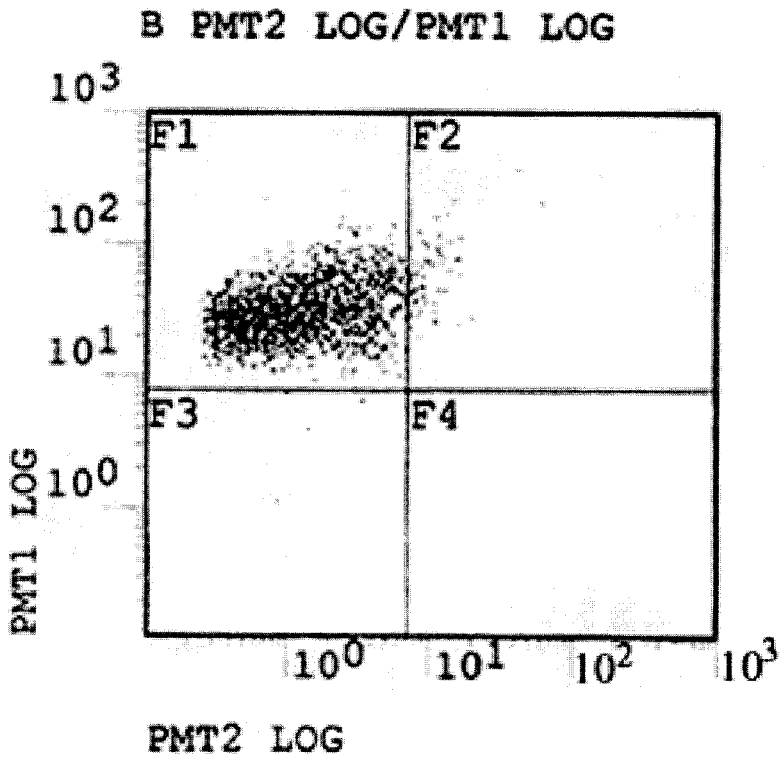


Figure 18: Dot plot diagram of side scatter (PMT1 LOG) against fluorescence intensity (PMT2 LOG; $DiBAC_4(3)$) exhibited by four sub populations of cells of *H. influenzae* ATCC 49247 . *Quadrant F1 population*: high PMT1 LOG and low PMT2 LOG were assumed to be live cells with polarized plasma membrane. *Quadrant F2 population:* positive for both PMT1 LOG and PMT2 LOG, assumed to be intact cells with depolarized membrane. *Quadrant F3 population*: negative for both PMT1 LOG and PMT2 LOG, assumed to be cell debris. *Quadrant F4 population*, positive for PMT2 LOG but low for PMT1 LOG, assumed to be entangled cell debris.

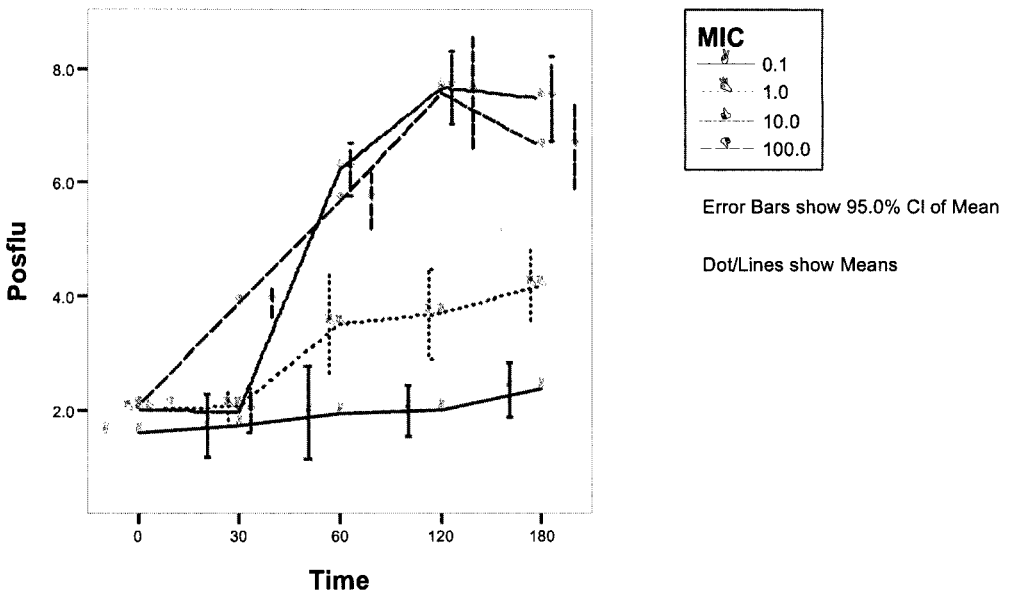


Figure 19: Graph of PI fluorescence intensity of *H. influenzae* cells at different concentrations of ampicillin and time of incubation.

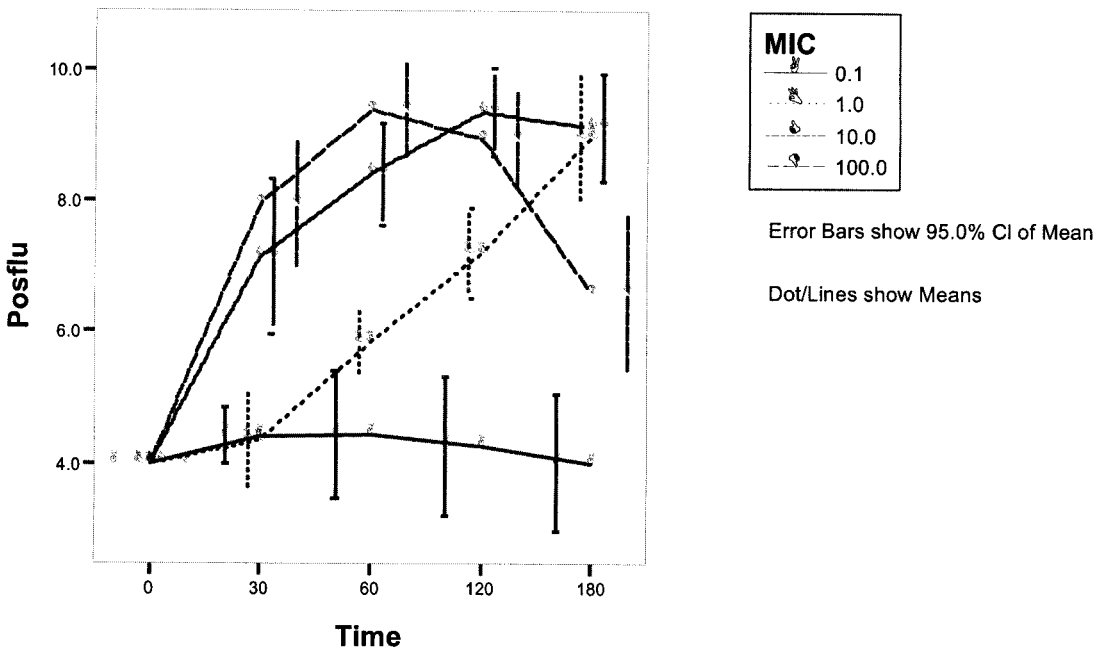


Figure 20: Effect of tetracycline on uptake of $DiBAC_4(3)$ by a *H. influenzae* control strain at different concentrations of tetracycline and incubation times.

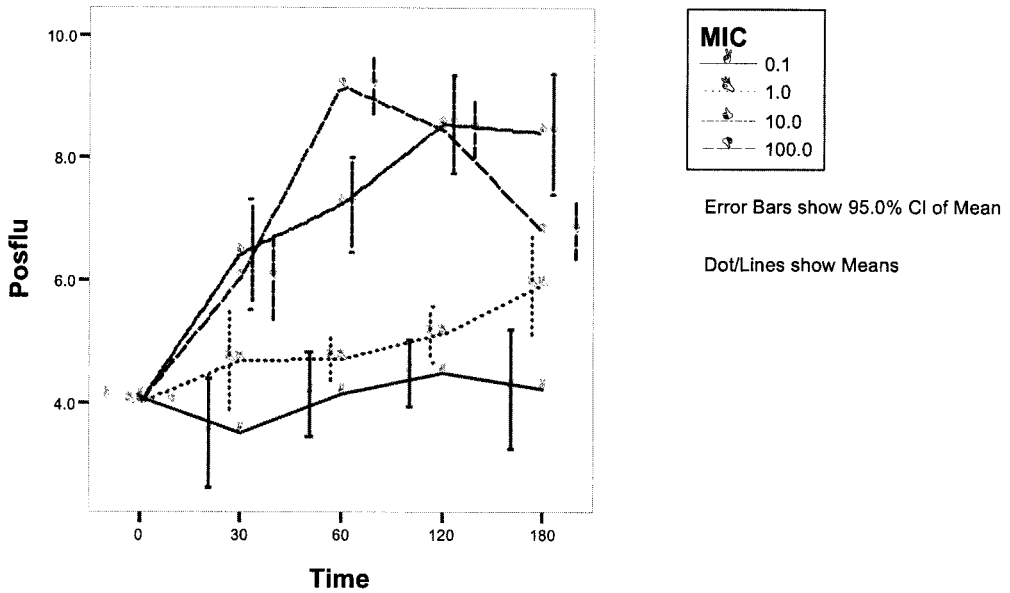


Figure 21: Effect of tetracycline on uptake of PI by a *H. influenzae* control strain at different concentrations of tetracycline and incubation times.

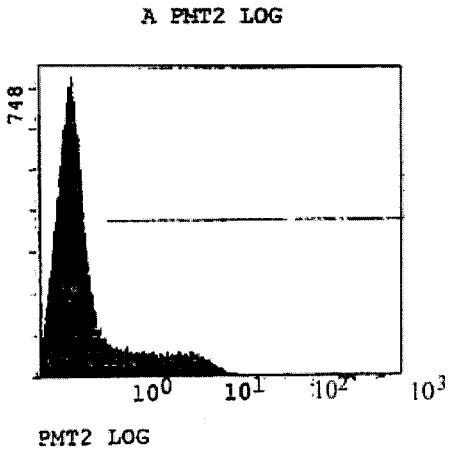


Figure 22 a(i)

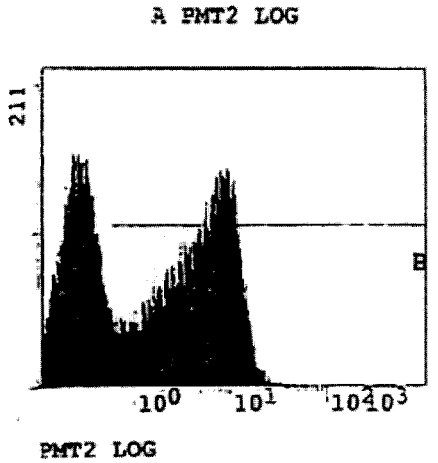


Figure 22 a(ii)

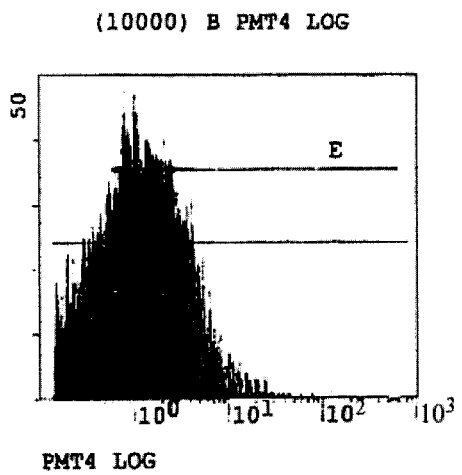


Figure 22 b(i)

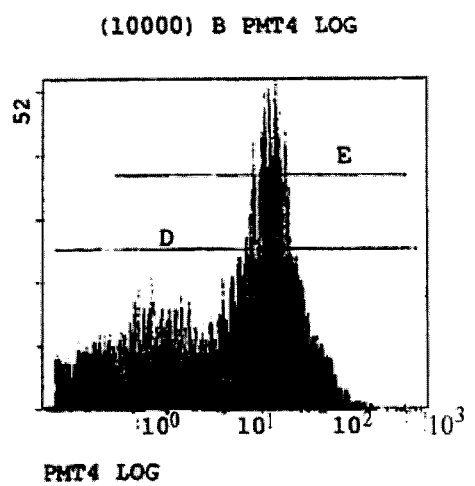


Figure 22 b(ii)

Figure 22: Single parameter histograms of $DiBAC_4(3)$–associated fluorescence (PMT2) (a(i), a(ii)) and PI–associated fluorescence (PMT4) (b(i), b(ii)) for *H. influenzae* control strain after exposure to ampicillin (i) 0 hour (ii) 3 h.

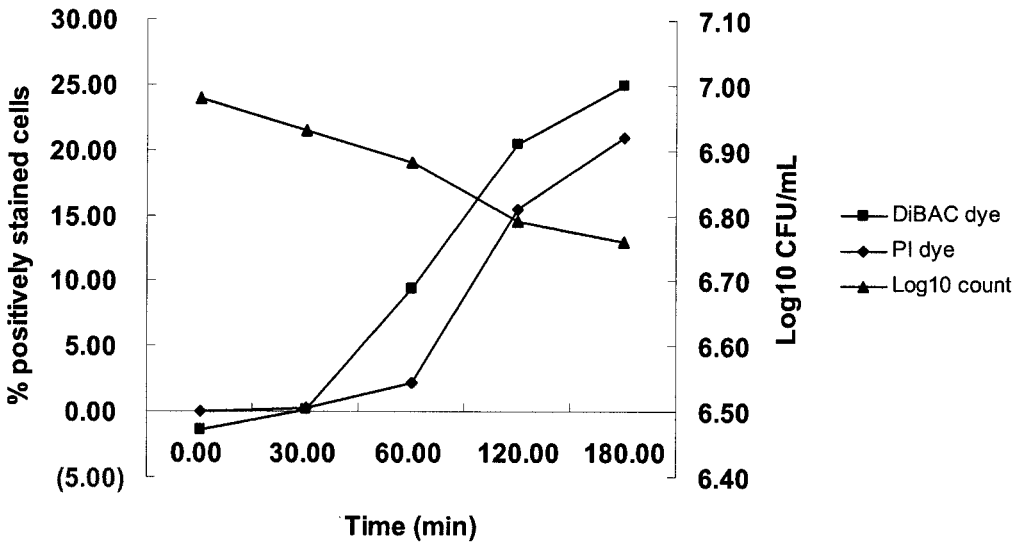


Figure 23: Graph of percentage of cell fluorescence after incubation in the presence of ampicillin at its MIC.

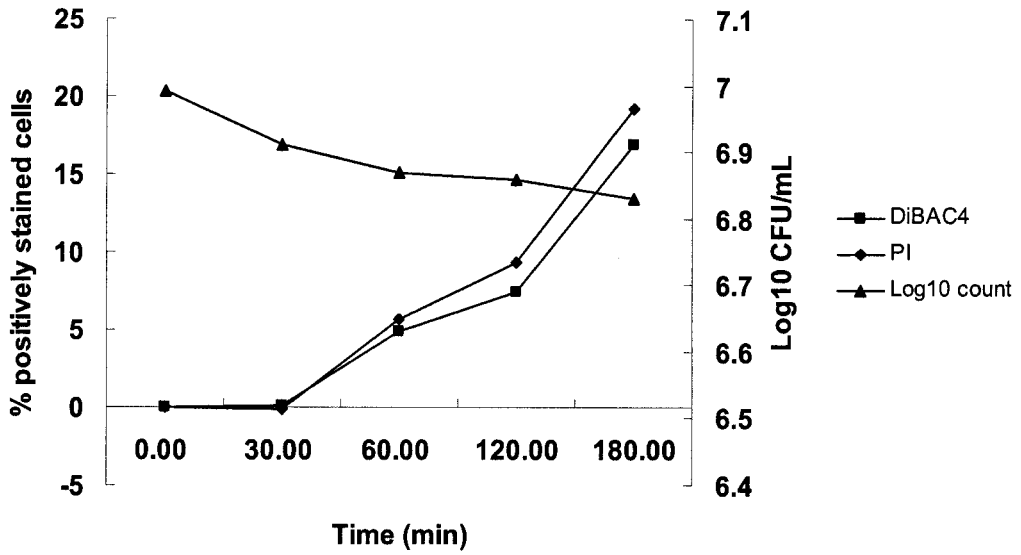


Figure 24: Graph of percentage of cell fluorescence after incubation in the presence of tetracycline at its MIC.

Chapter 8

Evaluation of Effects of Antimicrobial Agents on *Streptococcus pneumoniae* using Flow Cytometry

Introduction

Streptococcus pneumoniae is a major cause of pneumonia, otitis media, bacteremia and meningitis (Ruoff *et al*, 1999) and is a leading cause of morbidity and mortality. For many years, penicillin was the standard treatment of choice and susceptibility testing was not indicated. However, strains of *S. pneumoniae* not only resistant to penicillin but also showing resistance to other antibiotics such as erythromycin and cephalosporins are being isolated with increasing frequency in Hong Kong and elsewhere (Ho *et al* 2001; Boost *et al*, 2001).

Rapid recognition of resistant strains in the laboratory is critical for the proper selection of antimicrobial therapy. The reference methods for MIC determination of pneumococci are the conventional agar and broth dilution methods (CLSI, 2005b). These methods are labour intensive, time consuming, require high technical skills and are therefore costly. The screening test for resistance to penicillin in pneumococci is a variation of the qualitative disk diffusion test, is simple to perform and can provide results

readily interpreted by clinicians. Penicillin resistance is determined by means of testing susceptibility to a 1 ug/mL oxacillin disk rather than penicillin G, since the susceptibility categories of pneumococcus cannot be readily detected by a penicillin disk (Swenson *et al*, 1986). However, the screening method can only identify susceptible strains, but cannot distinguish between penicillin resistant (MIC>1μg/ml) and relatively resistant strains (MIC 0.12 to 1μg/ml), a distinction which is significant in both meningeal and non-meningeal foci of infection. Non-meningeal infections may be adequately treated with a high dose of penicillin in infection caused by relatively resistant strains of the pneumococcus, whereas treatment failures have been reported with high dose penicillin therapy for meningitis caused by resistant strains (Friedland and McCracken, 1994). A zone size around the oxacillin disk of less than or equal to 19mm indicates the strain may be penicillin-resistant, but this result needs to be confirmed by determination of MIC using a standard method, which increases the time required for adequate treatment, which can have a great effect on 30-day survival times in severe infections (Gleason *et al*, 1999).

Erythromycin is also recommended by the CLSI (NCCLS, 2004b; CLSI,

2005b) as a primary test and report drug for pneumococci infection. SAD and disk diffusion tests for erythromycin are incubated in CO_2, and the results obtained tend to be 1 to 2 dilutions lower than those for broth dilution tests in which incubation is performed in ambient air (Sader and Der' Alamo, 2000). The difference in results observed is thought to be caused by the use of CO_2 incubation, which acidifies the medium thereby lowering the pH. Macrolides are known to be less active at low pH (Acar and Goldstein, 1996).

All of the above mentioned susceptibility test methods require the growth (or non growth) of organisms, and therefore overnight incubation in order to determine susceptibility results. Commercially available FDA-approved panels for susceptibility testing of pneumococci include the PASCO (Difco, Wheatridge, Colo., US), MicroScan (MicroScan Inc., West Sacramento, Calif.), Sensititre (Trek Diagnostic Systems, Westlake, Ohio, US) and Vitek 2 (BioMerieux Inc.). Most of the systems have been evaluated and found to provide MIC values that are comparable to reference dilution methods (Guthrie *et al* 1999: Mohammed and Tenover, 2000). Of the commercial systems only the Vitek 2 card can claim to be a rapid susceptibility test in

that it provides results within the same day with the results available after 8 hours (Jorgensen *et al*, 2000).

The development of FCM for bacterial studies in combination with the availability of fluorochromes that can identify change in cell metabolism or cell death, and permit the assessment of individual cell viability and functional capacity (such as: membrane potential and cell wall integrity) within microbial populations. This can facilitate the evaluation of bacterial susceptibility to drugs.. FCM has proved to be very useful for studying the physiological effects of antimicrobial agents on bacterial cells due to their effects on certain metabolic parameters. FCM may be a reliable approach for susceptibility testing, offering rapid results in a few hours for bactericidal or bacteriostatic effects (Martinez *et al*, 1982; Gant *et al*, 1993; Pore, 1994; Braga *et al*, 2003). However, no work has been performed to examine the effects of antimicrobial agents on fastidious organisms, such as *S. pneumoniae* by FCM.

The results of the previous study on *H. influenzae* reported in the previous chapter suggested that the method may also be suitable for *S. pneumoniae*.

The present study analysed the dose dependent and time dependent responses of penicillin and erythromycin of *S. pneumoniae* cells using a FCM. The FCM method developed allowed the determination of the susceptibility categories of clinical isolates of *S. pneumoniae* strains for these two drugs and results were compared to conventional dilution methods.

Materials and Methods

Bacterial Strains, Media and Growth Conditions

A control strain of *S. pneumoniae* (ATCC 49619) was used for optimization of FCM conditions. Thirty five clinical isolates of *S. pneumoniae* obtained from several district hospitals in Hong Kong were used for susceptibility test studies. The strains were maintained in Protect and were subcultured at least twice on blood agar before use.

Four colonies of overnight cultures grown on horse blood agar were added to filtered MH broth supplemented with 3% LHB. Cultures were than incubated at 35°C on an orbital shaker (250 rpm) for 2 h to reach exponential phase (OD_{600} of 0.04) before the addition of antibiotics.

Antibiotics

Stock solutions of Penicillin G and Erythromycin (Sigma Chemical Co, St Louis, MO, USA) were prepared at appropriate dilutions as described by NCCLS.

Buffers and Staining Media Used

Several media were tested for the study on antibiotic-treated cells; (i) Tris-HCL (5 mM, pH 7.5), (ii) Phosphate buffered saline (PBS, pH 7.4), (iii) original MH broth, and (iv) distilled water. All buffers and diluents were filtered three times through a 0.2 um membrane filter (Corning Ltd. USA) before use.

Working concentrations of PI at 10μg/ml was prepared with distilled water, and of bis-oxonol at 5μg/ml was prepared using 70% alcohol. as previously described Valinomycin was reconstituted with filtered Milli-Q water and stored at 25 μg/ml final concentration at -20℃.

FCM Calibration

A Beckman Coulter EPICS Elite ESP Flow Cytometer (Coulter Corporation, Miami, FL, USA) was used to measure the light scattering and fluorescence of a single cell. An air-cooled argon laser lamp operated at 488nm with an

intensity of 15mw provided illumination. All settings of the machine were as described in Chapter 6. Light scattering and fluorescence were converted by photomultiplier detectors (PMTS) into equivalent electric pulses and were digitized by an analog-digital computer connected to an IBM 80486 processor microcomputer. Calibration of machine was as described previously in Chapter 6.

Optimization of the Staining Medium

The optimal staining media was determined by using MH broth, Tris-HCL (5mM, PH 7.5), PBS (PH 7.4) and distilled water. Cell staining fluorescence intensities of an alcohol fixed *S. pneumoniae* control strain in different media were compared. The medium yielding the highest fluorescence signal was used for subsequent kinetic studies and FCM-AST determination.

Reference Susceptibility Tests

The MIC values of penicillin and erythromycin were determined using SAD methods following the recommendations of CLSI (2005b).

Staining Protocols

The staining protocol for both dyes was as described in Chapter 7.

Kinetic Studies

The purpose of this part of the study was to determine the response of a

control strain of *S. pneumoniae* (ATCC 49619) to different concentrations of each of two antibiotics (penicillin G and erythromycin) at a range of incubation times.

The culture was grown to log phase as described above and then divided into five equal volumes of 10ml. Penicillin G or erythromycin at 0.1, 1, 10, and 100 times the MIC was then added. The fifth culture acted as a control. A 500µl sample from both tested and control cultures was removed after 0, 30, 60, 120, and 180 minutes. Each sample was washed three times in PBS (pH 7.4) and then centrifuged for one minute at 13000 rpm. The samples were then stained with $DiBAC_4(3)$ or PI for FCM analysis.

Flow Cytometric Susceptibility Test (FCM-AST)

Clinical isolates were grown to early exponential phase (approximately 2 h) and divided into two aliquots. Appropriate antibiotic at its breakpoint concentration (0.12 µg/ml for penicillin and 0.5µg/ml for erythromycin) was added to one aliquot (test culture). The second aliquot acted as a control. Incubation was continued (35°C at 250 rpm) for a further 3 h. Samples of the *S. pneumoniae* strains were then removed and washed three times with filtered PBS (pH7.4). The washed samples of control and test cultures were

then stained with $DiBAC_4(3)$ and PI at concentrations of 5μg/ml and 10μg/ml respectively for 15 minutes in the dark, and submitted to the FCM for analysis. The number of cells that took in one or both dyes was determined, and the fluorescence intensities of the test samples were then compared with the corresponding control samples. Before adding the dyes 20μl of test sample and control sample were withdrawn for determination of viable cell counts.

Viable Counts

CFUs were obtained by the serial dilution of liquid culture with PBS buffer (pH 7.4) and inoculated onto a blood agar. After spreading, all plates were incubated for 24 hours at 35°C in 5 % CO_2.

Bacterial Counting by FCM was performed as previously described using Flow Count Fluorospheres The bacterial concentration can be calculated as the ratio of bacteria to beads as identified by the region of interest, multiplied by the known concentration of the reference beads.

FCM cell count and standard plate count were performed on the kinetic study and FCM-AST for each strain of *S. pneumoniae* being tested.

Controls Used in this Study

Several controls were included in these experiments. Non-viable cells obtained from an alcohol fixed culture were used for the optimization studies and before each batch of FCM-AST. Valinomycin- treated samples were also included in each experiment to ensure the organism responded to staining with $DiBAC_4(3)$ after the membrane potential had dropped. These were prepared as described previously for *H. influenzae* in Chapter 6. Untreated samples of test strains (negative control) were analyzed along with antibiotic-treated samples for comparison and determination of susceptibility category of the tested organisms.

Comparison of FCM method and Standard Agar Dilution for Clinical Isolates of S. pneumoniae.

The FCM method developed was used to determine the susceptibility category of each of the 35 clinical isolates and the results compared with those determined using SAD.

Statistical Analysis

This was performed as described previously (Chapter 7). An SPSS 10.1 statistical software package was used to analyse results.

Results

Auto-fluorescence of live *S. pneumoniae* cells was found to be minimal with similar results on repeated experiments. Alcohol-fixed cells of *S. pneumoniae* were stained with PI or DiBAC4(3) in different staining media and the results are shown in Tables 39 and 40.

The Levene test for homogeneity of variance showed no significant difference for variances ($p>0.05$) in three of the four media tested using $DiBAC_4(3)$, and for all media tested using PI. However, there was a significant difference in fluorescent intensity when $DiBAC_4(3)$ or PI staining was performed in the different media (F Test < 0.0001). The results showed that PBS (pH 7.4) gave the highest fluorescent intensity for cells stained with either $DiBAC_4(3)$ or PI. Thus, PBS was used for all further experiments.

A problem that could have occurred in FCM studies of *S. pneumoniae* is clumping of cells which would have lead to exaggeration of scatter and fluorescence signals. In this study, the cells were disaggregated by vortexing

the culture at high speed for at least 2 minutes. Adequate cell separation was achieved by several vortexing steps (Figure 25). This, together with continuous shaking in an orbital shaker during incubation, further disaggregated the cell ensuring that only single cells were analysed. To check for this, viable plate counts were performed in parallel with FCM cell counts, and the results showed there was no significant different between these counts. However, the multiple steps of mechanical shaking and vortexing made the procedure labour intensive. The use of sonication may be a best alternative that is simple and less prone to variation. (Braga *et al*, 2003)

Detection of the response of S. pneumoniae control cells to penicillin exposure

Because there were subpopulations that take up PI and/or $DiBAC_4(3)$ after incubation with bacteria, to simplify interpretation, an "average value" of the fluorescence intensity of the whole cell population was measured to obtain the median fluorescence intensity.

The ability of the FCM to detect effects of penicillin on a control strain of *S. pneumoniae* at 0.1, 1, 10 and 100 times the MIC was determined. The results

are shown in Fig 26.

At 0.1 x MIC of penicillin, there was no increase in intensity of fluorescence by cells of the control strain for either dye after 180 minutes of incubation (Fig 26 a and b). This indicates that there was no loss of viability of the cells at this concentration. At 1 x MIC, there was an increase in fluorescence intensity for both dyes even after 30 minutes incubation. At this stage, 3.57% of the cell's population exhibited PI-associated fluorescence (Fig. 28) and 6.86% of cells had taken up $DiBAC_4(3)$ (Fig. 27). The fluorescence intensity for each dye continued to increase until the end of the experiment (180 minutes), when it reached its highest level (Fig. 26). At this time, the proportion of cells that took in PI and $DiBAC_4(3)$ was 5.34% and 18.99% respectively (Figs. 27 and 28) At 10 x MIC, the log of fluorescence intensity showed similar increase to 1x MIC for both dyes for the first 120 minutes, while the proportion of cells that took in PI was 5.68% and $DiBAC_4(3)$ was 26.72% (Figs. 27 and 28). Following this, the rate of increase of fluorescence intensity and percentage positively-stained cells slowed down, and then began to plateau at 180 minutes for both dyes. There was a corresponding increase in cell debris signal of 15.92% with PI (Fig. 28), and 30.72% with $DiBAC_4(3)$ (Fig. 27). For penicillin, at 100 x MIC

there was a rapid increase in log fluorescence intensity of $DiBAC_4(3)$ for the first 30 minutes, followed by a plateau, and then a decrease in fluorescence until the end of experiment (180 minutes) (Fig. 29).

A similar decrease in fluorescence intensity was observed for PI at 100 x MIC of penicillin (Fig. 30), but the response was less dramatic than that observed for $DiBAC_4(3)$. The amount of signal from PI-associated debris and $DiBAC_4(3)$ debris was 24.48% and 49.79% respectively at 180 minutes.

Detection of the response of S. pneumoniae control cells to erythromycin exposure

The pattern of response of cells to 0.1 x MIC of erythromycin on S. pneumoniae control cells was the same as that observed for penicillin. There was no change in fluorescence signals from dyes or percentage of cells that fluoresced. As with penicillin, the fluorescence signals from both dyes for cells subjected to 1 x MIC of erythromycin increased rapidly during the time of the experiment. The number of cells that took up the dyes also increased significantly. The change in fluorescence intensities by control cells treated with 10 x and 100 x MIC followed the same trends as for penicillin. The fluorescence increased rapidly for the first 30 to 120 minutes, and then the increase in intensities slow down, or even decreased until 180 minutes. The

amount of cells that took up the dyes followed the same trends and, as with penicillin, there was a corresponding marked increase in cell debris accumulation at high concentration of erythromycin (10 x and 100 x).

The results for the exposure of the cells of the control strain to increasing concentrations of erythromycin (0.1, 1, 10, 100x MIC) are shown in Figs 31 to 35 and appendix V to VIII.

Overall, the results showed that at 1 x MIC for penicillin or erythromycin. *S. pneumoniae* control strain cells responded rapidly showing increased fluorescence intensity and increased uptake of dyes. This means that if a breakpoint concentration of drugs is added to a strain, it would show a significantly positive signal if its MIC is the same as or greater than 1 $\log_2$ dilution of the susceptibility breakpoint of penicillin or erythromycin.

The results of FCM-AST for 35 clinical isolates were compared to standard susceptibility test methods and are shown in Tables 43 and 44. For penicillin and erythromycin, the setting of the F values for determination of the susceptibility categories are shown in Table 45. They were average results of two experiments. They were set by the F values result of the susceptibility

testing on the two drugs. Overall there was very good agreement between susceptibility categories determined by SAD and FCM-AST.

There were only 8 (11.4%) minor errors for the FCM-AST of the *S. pneumoniae* isolates on penicillin and erythromycin and no major errors or very major errors. For penicillin, 4 minor (11.4%) errors occurred, all being strains with intermediate results on SAD. which gave lower or higher F values. For erythromycin, there were only 4 discrepancies (11.4%), which were all minor errors that included the intermediate resistance susceptibility category.

Discussion

The optimal staining medium giving the highest fluorescence intensity for *S. pneumoniae* control cells was found to be PBS (pH 7.4) for PI; and distilled water or PBS for $DiBAC_4(3)$. Distilled water was not used because the ionic concentration difference between distilled water and sheath fluid was too high, which would lead to fluctuation of fluorescence signals for $DiBAC_4(3)$. (Shapiro, 2003a), making the results not reproducible. This was further demonstrated in the homogeneity of variance test, in which use of distilled water staining medium did not give a satisfactory variance. PBS may be

superior because it can enhance the quantum yield of the dyes.

The optimal staining medium was different from that of *H. influenzae* (Chapter 7), in which the fluorescence intensities between different staining media displayed no significant differences for PI. This may reflect the special characteristic cell walls of these fastidious organisms. The polar solvent (distilled water) may cause quenching of PI in the *S. pneumoniae* control cells, causing fluorescence intensity to be lowered.

Examination of the response of cells stained with PI showed the proportion of positive cells and the proportion of signals from cell debris to be lagging behind those stained with $DiBAC_4(3)$. This is likely to have the same explanation as for *H. influenzae* cells as discussed in Chapter 7, i.e. the stained cells would be depolarized and take in $DiBAC_4(3)$ before they became permeable and took in PI.

For accurate FCM studies, it is preferable to analyse single cells. Under normal growth conditions, *S. pneumoniae* produces a mixture of single, double, and intercalated bacterial chains of different lengths. In this study,

several vortexing steps were included, as well as the use of an orbital shaker to ensure that cells were adequately separated, and that only single cells passed through the flow cell. The results showed that adequate separation was achieved using these mechanical aids. Other studies, using *S. pyogenes*, have used more sophisticated cell separation methods, such as sonication (Braga *et al*, 2003), but our results showed that this was not necessary for separation of *S. pneumoniae* cells.

In the kinetic studies, there were no changes in signals in comparison to control strain cells when the drug concentration added was 0.1 time MIC. This means that the sensitivity of the FCM-AST was not high enough to detect such a difference. At 1 x time MIC, the fluorescence signal response was sufficient to ensure a reliable susceptibility test when the incubation time was extended to 180 minutes. However, when the antibiotic concentration was too high (10x and 100x MIC) the cells that took in dyes underwent lysis leading to a decrease in fluorescence signals and positively-stained cells. This implied that when the tested strain has a very low MIC, its cells will undergo lysis and become cell debris during the 180minute incubation period resulting in a very low fluorescent signal.

These strains could be confused with highly resistant ones due to the low fluorescence intensity observed after 180 minutes. Therefore, if FCM-AST produces low fluorescence signals, but direct observation of the culture shows a decrease in OD, the OD of the control culture and tested culture should both be measured and compared. If the ODs are similar i.e. both high, a resistant strain should be suspected. If the OD of the tested strain is lower than the control, this is a sensitive strain. Such a difficulty was noted on strain P40 for erythromycin. For that strain the MIC for erythromycin was 0.06ug/mL but the tested drug concentration was 0.5, i.e. almost 10X MIC. So, there would be considerable cell lysis in the sample during incubation compared to the control strain. The response appeared low, and a low F value was obtained. Therefore, it is essential to compare the turbidity of the control and tested cultures when a FCM-AST is performed. If the signal is low, but there is a clear test suspension, the strain should be considered to be a sensitive strain.

Comparison of AST categories between FCM-AST and SAD showed 88.6% agreement. Of the eight category discrepancies (11.4%) which occurred, five were due to not enough shifting of fluorescence channels, and insufficient

positively-stained cells leading to a lower F value. This is one of the major difficulties of FCM-AST for fastidious species like *H. influenzae* and *S. pneumoniae.* In general, the shifting of fluorescence channels and the number of cells taking up the dyes was less than that reported for non-fastidious organisms such as *E. coli* and *S. aureus* (). The amplitude of responses also differed between strains, which could lead to unpredictable results and errors. Nevertheless, overall the response amplitudes of pneumococcal strains (fluorescence intensities and percentage of cells that took in the dyes) were greater than those of *H. influenzae.*

FCM-AST is not a quantitative method and does not produce exact MIC values such as those provided by E. test. This is due to the fact that the base line of fluorescence and the amplitude of response are different between strains of the same species or different species. The response thus, cannot be exactly quantified to determine the MIC. However, it can provide a rapid qualitative result of susceptibility for both *H. influenzae* and *S. pneumoniae* (within 6 hours) at least for the drugs that have been tested. It has the potential to replace or supplement the disk diffusion method. However, the method for *S.* pneumoniae has the potential to be extended to a breakpoint

method to determine the resistance categories of tested strains. This can be affected by means of using three parallel cultures: a control culture without antibiotic, one treated with antibiotic at the breakpoint for intermediate (e.g 0.125 μg/ml penicillin), and a second exposed to antibiotic at the breakpoint for resistance (e.g 2.0 μg/ml penicillin). A sensitive strain would give high fluorescence signals for both test cultures in comparison to the control; an intermediate strain would only give a high signal for the culture containing the high penicillin concentration; and a resistant strain would not give an increased signal for any of the cultures. Such categorization would obviate the need for an MIC as resistance category would be sufficient to determine appropriate therapeutic decisions. The availability of the resistance category within one day of isolation of the strain would considerably speed up the relaying of this information to the physicians and could improve both morbidity and mortality levels in patients suffering from pneumococcal infections (Gleason *et al*, 1999; File, 2001; Metlay, 2002).

This difference in reporting speed is most appreciable in lung infection in which oxacillin testing is routinely completed before MIC determination is performed for those strains with an inhibition zone of $\geqq$19mm. If E-test is

chosen, this will take an additional day, i.e. two days in total after isolation. Even if the oxacilllin test is omitted and E-test performed directly, the result from FCM-AST could be available 19 hours earlier, which could be of critical importance in meningeal infections, as FCM-AST seems to provide an accurate susceptibility test result for penicillin in approximately 5 h. And it would also more rapid than the Vitek 2, which provide results only after an average of 8.1 hours (Jorgensen *et al*, 2000) The flexibility of using a variety of drugs or drug combinations increases the potential of the test.

The FCM-AST for fastidious organisms has not been previously evaluated. This was partly due to the fastidious nature of the organisms that require special media to support their growth. In addition, the amplitude of response of these organisms is frequently small, making it difficult to produce a discernable response. In the present studies, supplemented media were used and found to support the growth of the organisms. After a washing step and suspension in an optimal staining medium, the response of the organism was increased and could be easily detected. This together with the use of a specially derived formula, has allowed FCM-AST for *S. pneumoniae* to be evaluated. This study has demonstrated that further

studies are justified that should include more strains, other drugs and drug combinations.

Table 39: Median fluorescence intensity of alcohol fixed *S. pneumoniae* cells stained with $DiBAC_4(3)$ using different staining solutions.

Medium / Experiment no.	Tris-HCl (PH 7.5)	PBS (PH7.4)	Distilled water	Mueller Hinton Broth
1	11.6	15.8	22.1	11.8
2	11.4	17.2	23.7	11.6
3	12.2	16.3	22.4	11.6
4	12.3	16.9	19.2	11.6
5	13.2	16.4	17.3	11.4

Table 40: Median fluorescence intensity of alcohol fixed *S. pneumoniae* cells stained with PI using different staining solutions.

Medium / Experiment no.	Tris-HCl (PH 7.5)	PBS (PH7.4)	Distilled water	Mueller Hinton Broth
1	6.7	10.1	4.3	8.6
2	6.9	11.4	4.0	8.8
3	6.1	12.1	4.9	8.9
4	5.9	11.6	3.9	8.7
5	6.4	10.8	4.1	7.9

Oneway ANOVA was performed and the results are shown in Tables 41 and 42

Table 41: One-way ANOVA to compare median fluorescence intensity of $DiBAC_4(3)$ in selected media tested.

Oneway

Dye= $DiBAC_4(3)$

Descriptives

Fluorescence intensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Tris-HCL (pH7.5)	5	12.1400	.70569	.31559	11.2638	13.0162
PBS (pH 7.4)	5	16.5200	.54498	.24372	15.8433	17.1967
Mueller Hinton broth	5	11.5800	.14832	.06633	11.3958	11.7642
Total	15	13.4133	2.33662	.60331	12.1194	14.7073

Descriptives

Fluorescence intensity

	Minimum	Maximum
Tris-HCL (pH 7.5)	11.40	13.20
PBS (pH 7.4)	15.80	17.20
Mueller Hinton broth	11.40	11.80
Total	11.40	17.20

Test of Homogeneity of Variance

Fluorescence intensity

Levene Statistic	Df1	Df2	Sig
2.754	2	12	.104

ANOVA

Fluorescence intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	73.169	2	36.585	134.338	.000
Within Groups	3.268	12	.272		
Total	76.437	14			

Post Hoc Tests

Homogenous Subsets

Fluorescence intensity

Tukey B

medium	N	Subset for alpha= 0.5	
		1	2
Mueller Hinton broth	5	11.5800	
Tris-HCL (pH 7.5)	5	12.1400	
PBS (pH 7.4)	5		16.5200

Means for groups in homogeneous subsets are displayed.

Table 42: One-way ANOVA to compare median fluorescence intensity of PI in selected media tested.

Dye = PI

Descriptives

Fluorescence intensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Tris-HCL (pH 7.5)	5	6.4000	.41231	.18439	5.8880	6.9120
PBS (pH 7.4)	5	11.2000	.77136	.34496	10.2422	12.1578
Distilled Water	5	4.2400	.39749	.17776	3.7464	4.7336
Mueller Hinton broth	5	8.5800	.39623	.17720	8.0880	9.0720
Total	20	7.6050	2.69082	.60169	6.3457	8.8643

Descriptives

Fluorescence intensity

	Minimum	Maximum
Tris-HCL (pH 7.5)	5.90	6.90
PBS (pH 7.4)	10.10	12.10
Distilled Water	3.90	4.90
Mueller Hinton broth	7.90	8.90
Total	3.90	12.10

Test of Homogeneity of Variances

Fluorescence intensity

Levene Statistic	Df1	Df2	Sig.
1.564	3	16	.237

Anova

Fluorescence intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	133.249	3	44.416	164.506	.000
Within Groups	4.320	16	.270		
Total	137.569	19			

Homogeneous Subsets

Fluorescence intensity

Tukey B

medium	N	Subset for alpha =0.5			
		1	2	3	4
Distilled Water	5	4.2400			
Tris-HCL (pH 7.5)	5		6.4000		
Mueller Hinton broth	5			8.5800	
PBS (pH 7.4)	5				11.2000

Means for groups in homogenous subsets are displayed.

Table 43: Susceptibility testing of *S pneumoniae* for penicillin by FCM

Penicillin

Strain ID	Treated Strain F1+F2+F4	Treated Strain MFI DiBAC4(3)	Treated Strain MFI PI	Xγ	Control F1+F2+F4	Control MFI DiBAC4(3)	Control MFI PI	X c	F	MIC	Susceptibility Category
P40	10.3	4.3	8.5	376.5	10.9	4.1	7.0	312.8	1.20	2	R
P14	7.63	2.1	3.9	62.5	13.19	2.3	3.2	97.1	0.64	2	R
P3	29.9	10.1	5.0	1510.0	11.66	3.1	2.9	104.8	14.4	0.008	S
P103	30.33	8.8	5.4	1441.3	4.52	4.2	3.0	57.0	25.2	0.008	S
P30	6.50	1.7	2.0	22.1	3.56	1.3	2.3	10.6	2.08	0.12	I
P38	10.3	2.4	3.0	74.16	11.01	1.8	3.3	65.3	1.13	0.5	I
P46	13.5	3.6	7.1	343.8	7.51	2.5	2.2	41.3	8.3	0.016	S
P15	9.86	2.1	4.9	101.5	6.31	0.5	2.2	14.6	6.9	0.064	S
P7	5.43	0.7	3.0	11.4	6.96	1.1	3.0	20.9	0.55	2	R
P18	25.11	3.3	7.7	638.0	10.15	1.2	3.0	36.5	17.5	0.016	S
P11	4.44	1.5	2.7	18.0	4.53	1.4	2.8	17.8	1.0	2	R
P40	3.76	1.6	2.6	15.6	3.93	1.4	2.5	13.8	1.1	4	R
P41	8.30	2.1	3.0	52.3	6.03	1.3	3.0	23.5	2.3	1	I
P1	10.8	1.7	2.6	47.7	8.11	0.7	2.5	14.2	3.4	1	I
P44	6.78	0.6	2.4	9.78	3.13	0.5	2.4	3.8	2.6	0.5	I
P38	24.6	1.6	4.9	192.9	10.09	1.2	4.1	49.6	3.9	0.12	I
P42	12.5	1.5	4.2	78.5	9.79	1.6	3.0	46.0	1.7	0.5	I

Strain ID	Treated Strain F1+F2+F4	Treated Strain MFI DiBAC4(3)	Treated Strain MFI PI	Xγ	Control F1+F2+F4	Control MFI DiBAC4(3)	Control MFI PI	X c	F	MIC	Susceptibility Category
P16	30.48	5.8	4.9	866.2	10.62	1.3	4.1	56.6	15.3	0.016	S
P12	12.57	1.5	2.8	52.8	11.88	2.0	2.7	64.2	0.82	4	R
P20	7.93	0.9	2.5	17.8	8.06	0.8	2.2	14.2	1.3	4	R
P38	32.6	5.9	6.2	1192.5	14.26	2.5	4.5	160.4	7.4	0.008	S
P4	26.2	8.5	4.6	1024.4	11.44	0.4	2.8	12.8	80.0	≦0.008	S
P6	4.79	1.9	2.3	20.9	10.59	3.1	2.8	91.9	0.23	4	R
P9	9.04	1.6	2.7	39.1	12.1	2.1	2.6	66.0	0.59	2	R
P8	4.87	1.7	2.3	21.0	6.56	1.1	3.0	21.6	0.97	2	R
P25	4.28	0.8	2.9	9.9	4.55	0.9	3.3	13.5	0.73	2	R
P39	11.0	4.1	7.5	338.3	8.25	0.9	4.9	36.4	9.3	0.25	I
P20	4.55	1.1	3.9	19.5	5.89	1.5	4.0	35.3	0.55	4	R
P46	8.58	2.0	3.6	61.8	7.30	2.5	3.2	58.4	1.06	2	R
P11	8.43	1.2	1.7	17.20	7.38	1.3	1.9	18.2	0.94	0.125	I
P94	7.93	2.9	3.0	69.0	6.28	3.4	3.5	74.7	0.92	2	R
P91	38.42	5.9	6.0	1360.0	9.77	1.1	1.1	11.8	115.2	0.008	S
P96	8.23	0.9	0.9	6.7	9.76	1.3	1.3	16.5	0.59	4	R
P109	8.97	1.9	2.5	42.6	8.66	2.1	2.1	38.2	1.2	0.5	I
P23	12.19	3.5	3.5	149.3	6.53	1.4	1.4	12.8	11.7	0.06	S

11.4% minor error **F factor: R= 0-1.5, I= 1.6-4.9, S ≧5**

Table 44: Susceptibility testing of *S. pneumoniae* for erythromycin by FCM

Erythromycin

Strain ID	Treated Strain F1+F2+F4	Treated Strain MFI DiBAC4(3)	Treated Strain MFI PI	Xγ	Control F1+F2+F4	Control MFI DiBAC4(3)	Control MFI PI	X c	F	MIC	Susceptibility Category
P2	11.5	2.0	3.9	89.7	14.39	2.5	4.1	147.5	0.61	2	R
P6	15.31	4.3	6.2	408.2	15.38	4.1	5.0	315.3	1.30	8	R
P96	13.95	5.2	6.1	442.5	8.59	2.6	5.9	131.8	3.4	0.25	S
P18	8.81	2.7	4.9	116.6	7.09	3.1	5.7	125.2	0.93	0.5	I
P17	8.25	3.6	6.8	202.0	7.21	3.0	5.2	112.5	1.80	0.5	I
P14	10.19	3.6	7.3	267.8	4.06	1.7	6.3	43.5	6.16	0.125	S
P31	11.36	3.6	7.0	286.3	4.34	1.2	2.1	10.9	26.3	0.064	S
P27	11.84	1.8	5.4	115.1	11.32	1.5	5.5	93.39	1.2	2	R
P97	5.85	3.5	5.0	102.4	6.21	3.8	5.0	118.0	0.87	2	R
P35	11.34	3.0	4.5	153.1	7.88	2.2	5.3	91.9	1.7	0.5	I
P38	11.11	2.6	6.6	190.6	8.42	0.8	5.8	39.1	4.9	0.125	S
P1	9.51	1.9	6.5	117.4	6.87	1.2	5.6	46.2	2.8	0.25	S
P41	7.96	4.3	5.6	191.7	6.6	4.4	6.0	174.2	1.1	8	R
P25	8.78	0.7	5.4	33.2	11.33	0.7	5.2	41.2	0.81	8	R
P40	3.5	1.9	2.9	19.3	3.04	1.5	2.7	12.3	1.6	0.06	S
P21	13.18	3.8	7.9	395.7	6.87	2.3	6.4	101.1	3.9	0.125	S
P32	4.87	0.6	4.2	12.27	4.65	1.0	3.8	17.67	0.69	2	R

Strain ID	Treated Strain F1+F2+F4	Treated Strain MFI DiBAC4(3)	Treated Strain MFI PI	Xγ	Control F1+F2+F4	Control MFI DiBAC4(3)	Control MFI PI	X c	F	MIC	Susceptibility Category
P23	4.73	0.8	3.0	11.4	4.95	1.1	3.2	17.4	0.65	1	R
P35	6.95	0.9	1.6	10.0	5.72	0.6	1.8	6.2	1.61	0.5	I
P34	16.33	2.1	3.9	133.7	7.27	0.7	2.3	11.7	11.4	0.125	S
P30	15.34	2.5	7.8	299.1	11.22	1.4	5.7	89.5	3.3	0.25	S
P94	6.32	1.5	3.0	28.4	7.4	0.8	3.2	18.9	1.5	0.5	I
P37	12.14	3.6	7.0	305.9	6.84	1.9	5.7	74.1	4.1	0.25	S
P10	26.4	0.7	6.4	118.3	7.3	0.5	3.2	11.7	10.1	0.125	S
P18	9.78	3.5	4.6	157.5	11.36	3.1	5.2	183.1	0.86	2	R
P15	31.07	10.0	3.8	1180.7	10.78	0.7	1.2	9.1	129.7	0.06	S
P92	4.17	1.3	4.2	22.8	3.94	1.0	3.7	14.6	1.6	0.5	I
P91	12.7	3.6	4.1	187.5	10.6	2.4	4.0	101.8	1.8	0.5	I
P101	8.6	1.6	4.5	61.9	6.87	1.2	5.6	46.2	1.3	1	R
P98	8.77	1.2	4.1	43.1	6.79	0.9	4.5	27.5	1.6	2	R
P95	10.66	3.1	4.2	138.8	11.34	3.0	4.5	153.1	0.91	4	R
P97	10.21	3.8	7.5	291.0	9.85	3.5	5.0	172.4	1.7	0.5	I
P84	4.28	3.4	3.8	55.3	4.84	2.0	4.2	40.7	1.4	1	R
P99	4.27	0.6	3.5	9.0	5.19	0.5	3.2	8.30	1.1	1	R
P108	7.21	2.5	5.0	90.13	6.84	1.9	4.7	61.1	1.5	0.25	S

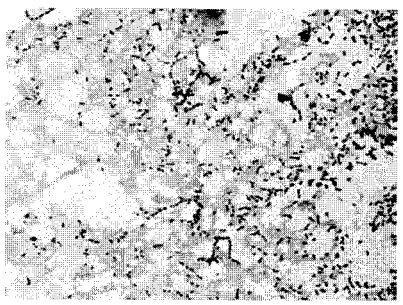
11.4% minor error **F factor: R= 0-1.4, I =1.5-2.5, S≧2.6**

Table 45: F values for determination of the susceptibility categories.

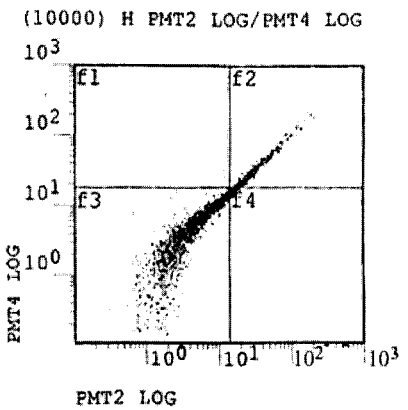
F values / Drug	F values		
	Sensitive	**Intermediate**	**Resistant**
Penicillin	≥5	1.6-4.9	0-1.5
Erythromycin	≥2.6	1.5-2.5	0-1.4

Figure 25: Clumping of *S. pneumoniae* control strain cells before vortexing (a) and the individual cells obtained following vortexing for at least 2 minutes (c). The corresponding density dot plots* of the cells before (b) and after (d) vortexing.

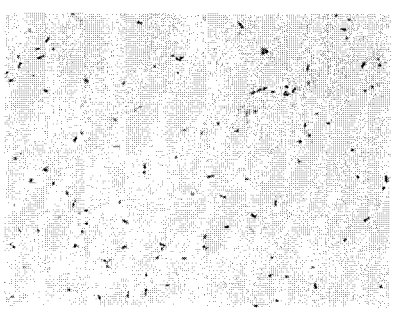
(a)



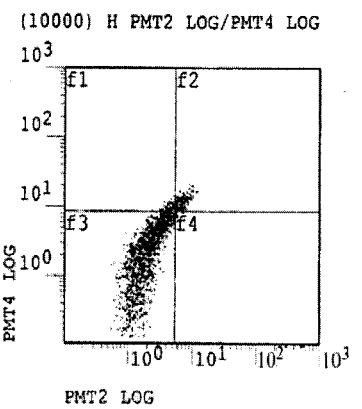
(b)



(c)



(d)



*PMT2; fluorescence from $DiBAC_4(3)$, PMT4; fluorescence from PI

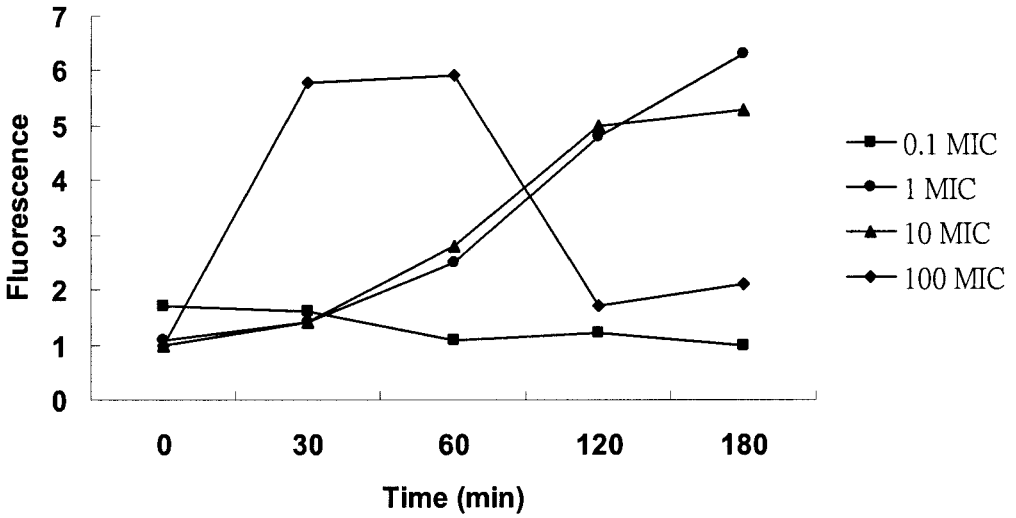


Figure 26: (a) Uptake of $DiBAC_4(3)$ by penicillin-treated *S. pneumoniae* cells at different concentrations of penicilin and incubation times.

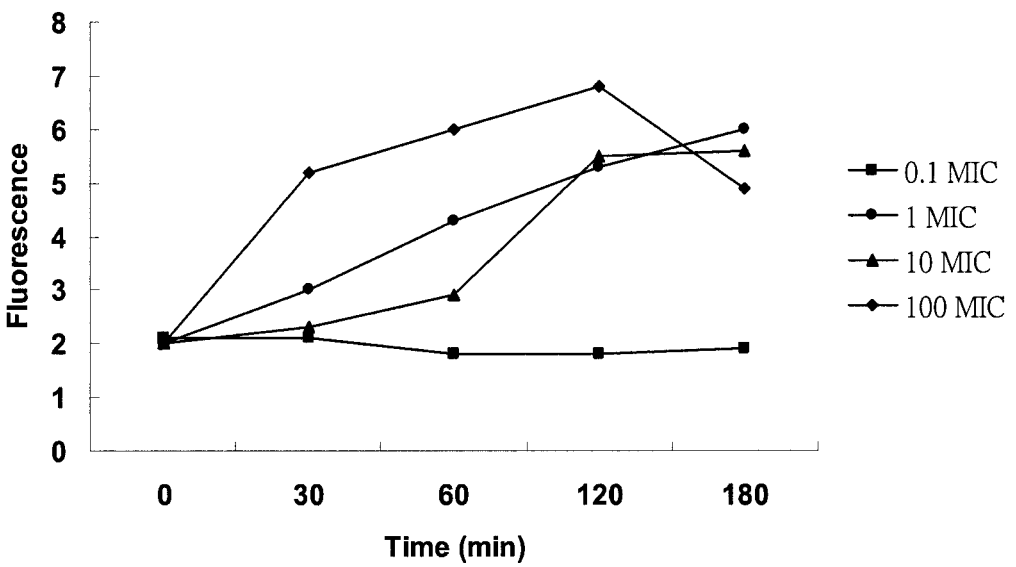


Figure 26: (b) Uptake of PI by penicillin-treated *S. pneumoniae* cells at different concentrations of penicillin and incubation times.

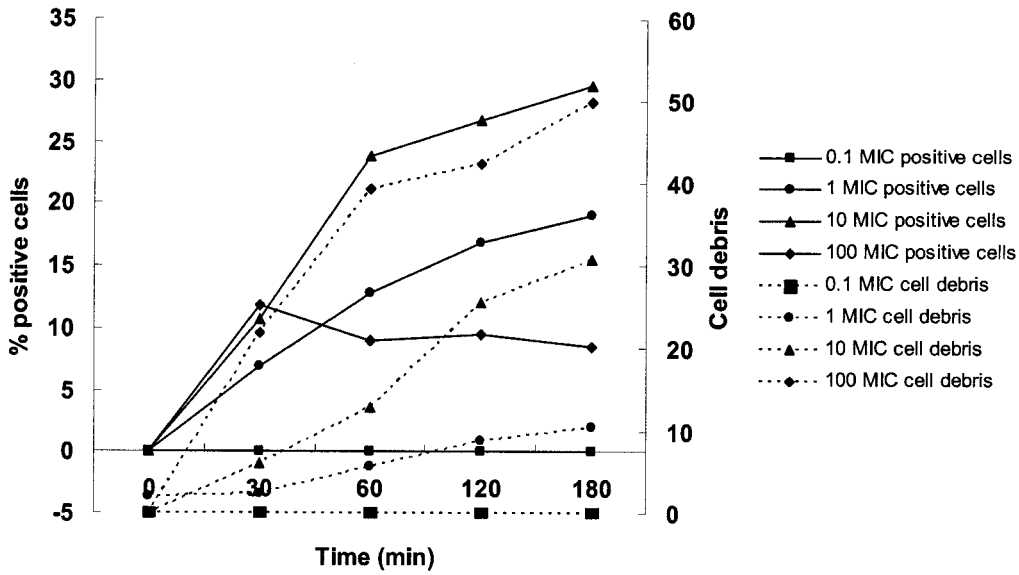


Figure 27: The percentage of cells positive for fluorescence and the corresponding increase in cell debris over time for a *S. pneumoniae* control strain following treatment with a range of penicillin concentrations and stained with $DiBAC_4(3)$.

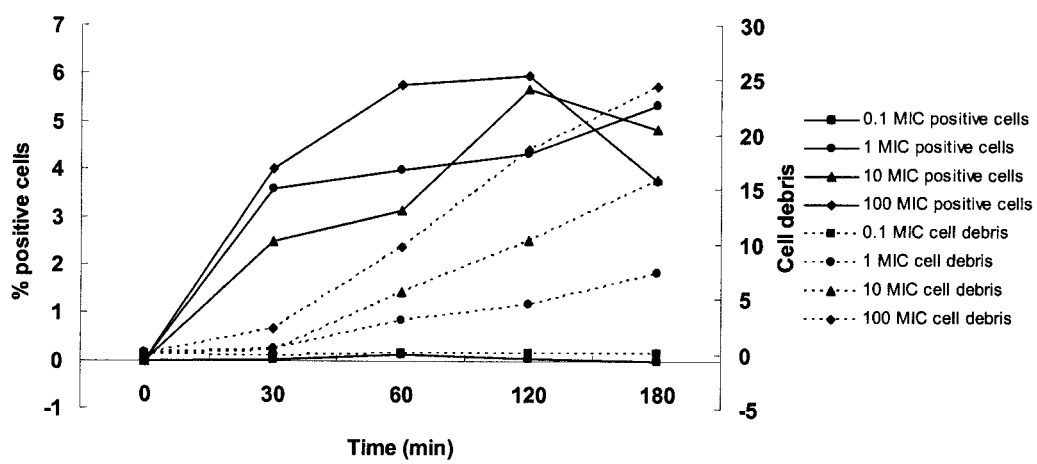


Figure 28: The percentage of cells positive for fluorescence and the corresponding increase in cell debris over time for a *S. pneumoniae* control strain following treatment with a range of penicillin concentrations and stained with PI.

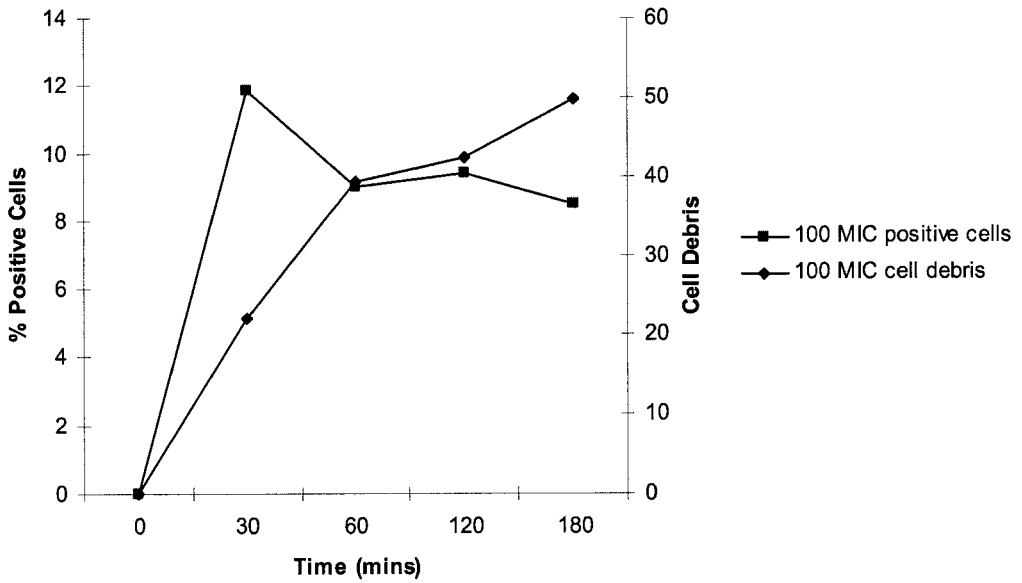


Figure 29: (a) The percentage of cells positive for fluorescence and the corresponding increase in cell debris over time for a *S. pneumoniae* control strain exposed to 100 x MIC for penicillin and stained with $DiBAC_4(3)$.

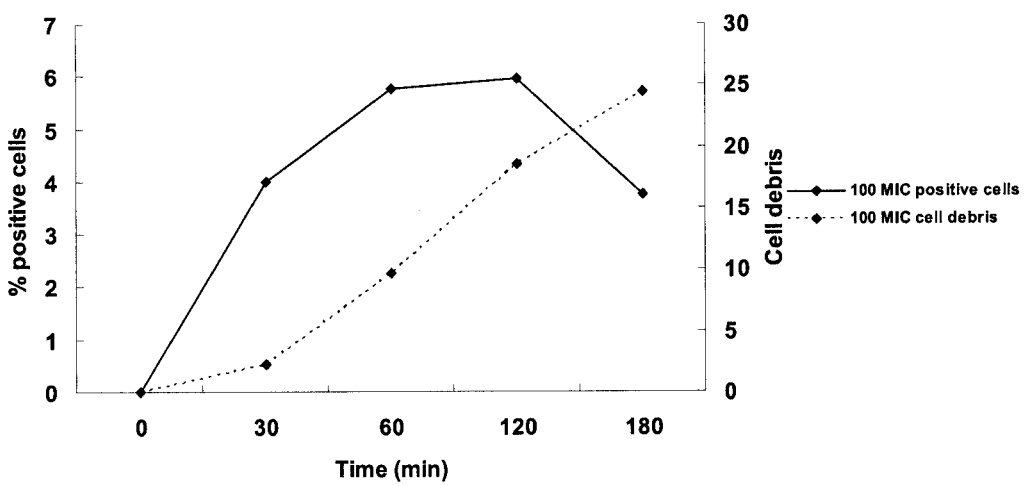


Figure 29: (b) The percentage of cells positive for fluorescence and the corresponding increase in cell debris over time for a *S. pneumoniae* control strain exposed to 100 x MIC for penicillin and stained with PI.

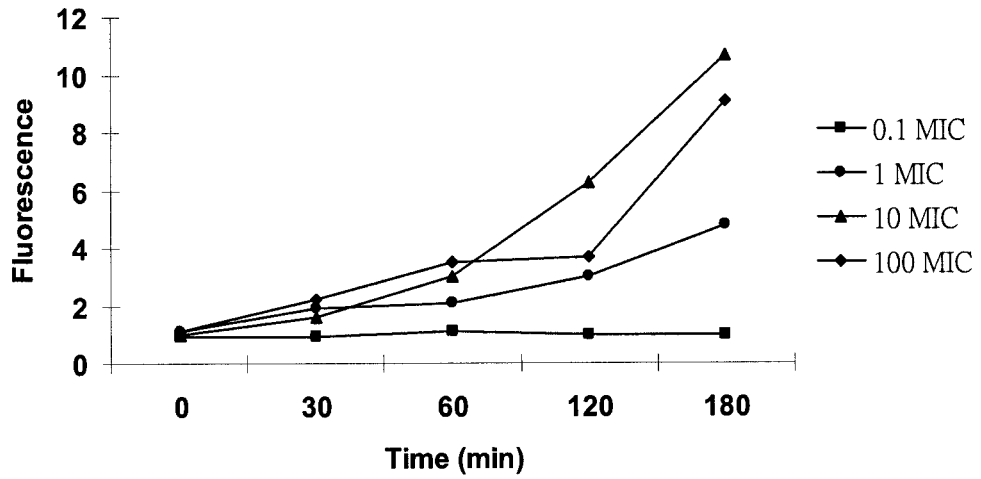


Figure 30: (a) Uptake of $DiBAC_4(3)$ by erythromycin-treated *S. pneumoniae* cells at different concentrations of erythromycin and incubation times.

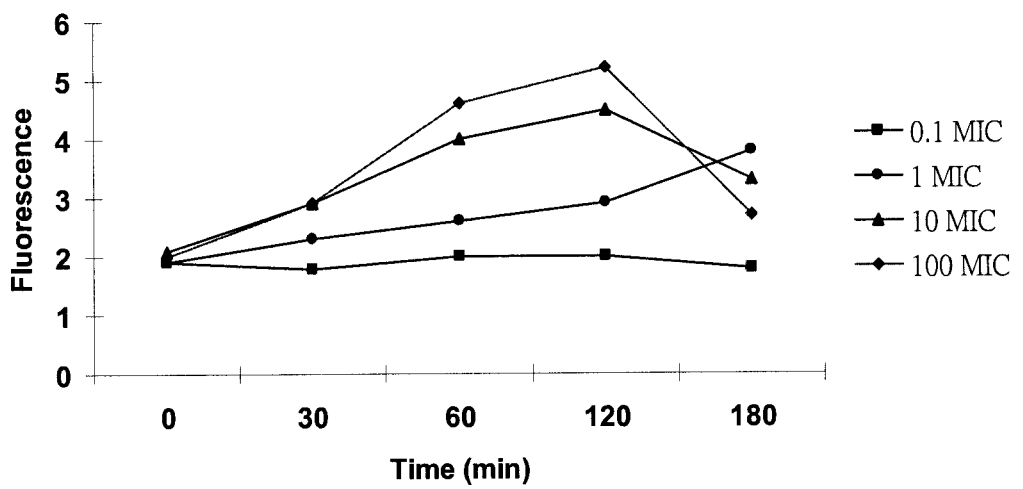


Figure 30: (b) Uptake of PI by erythromycin-treated *S. pneumoniae* cells at different concentrations of erythromycin and incubation times.

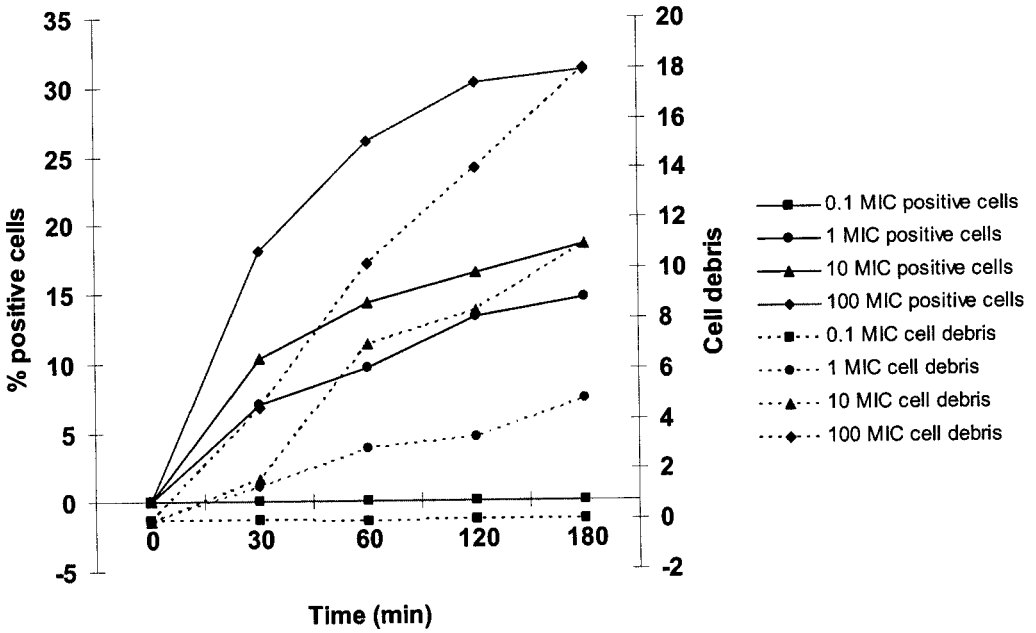


Figure 31 (a): The percentage of cells positive for fluorescence and the corresponding increase in cell debris over time for a *S. pneumoniae* control strain following treatment with a range of erythromycin concentrations and stained with $DiBAC_4(3)$.

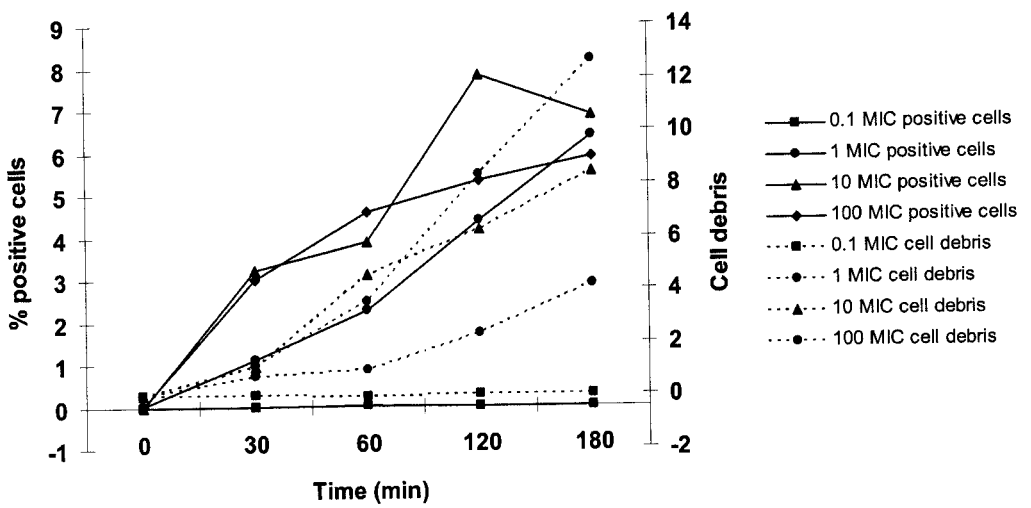


Figure 31 (b): The percentage of cells positive for fluorescence and the corresponding increase in cell debris over time for a *S. pneumoniae* control strain following treatment with a range of erythromycin concentrations and stained with PI.

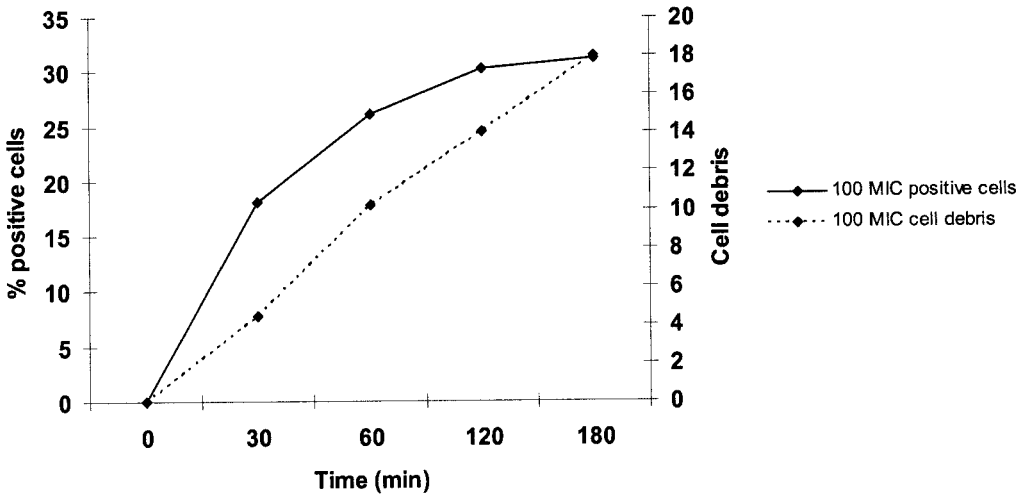


Figure 32(a): The percentage of cells positive for fluorescence and the corresponding increase in cell debris over time for a *S. pneumoniae* control strain exposed to 100 x MIC for erythromycin and stained with $DiBAC_4(3)$

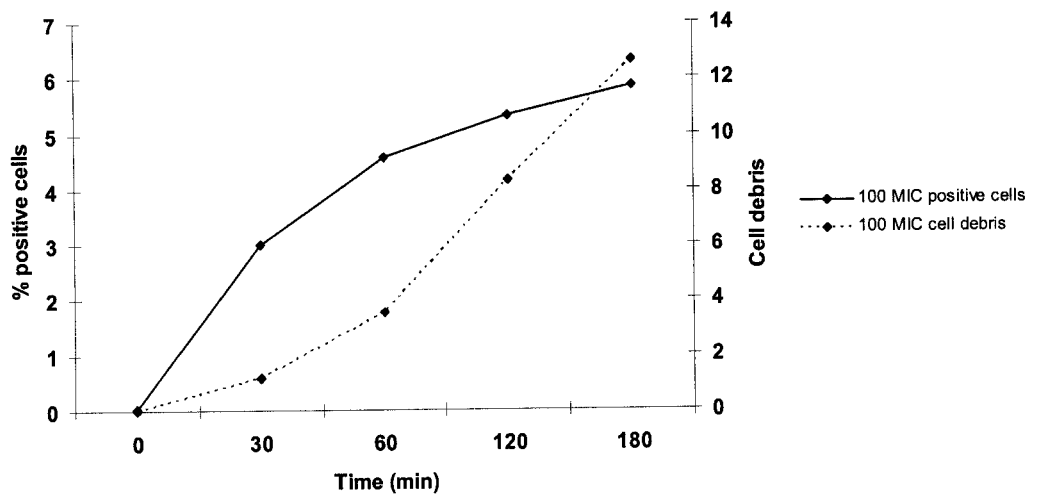


Figure 32 (b): The percentage of cells positive for fluorescence and the corresponding increase in cell debris over time for a *S. pneumoniae* control strain exposed to 100 x MIC for erythromycin and stained with PI.

Chapter 9

Summary of Major Findings

1. **To assess the reproducibility of the SGE method for AST of *S pneumoniae, H. influenzae, N. gonorrhoeae* and *M. catarrhalis*.**

   The CV of the intrabatch reproducibility of *S. pneumoniae, H. influenzae, N. gonorrhoeae* and *M. catarrhalis* using SGE was 12.1%, 6.29%, 7.17%, 14.56% respectively. The interbatch CVs of the corresponding organisms were 13%, 11.06%, 14.02% and 18.56% respectively. The overall CVs of the SGE test ranged from 7.17% to 18.56%. The CV of the standard dilution test ranged from 30.98% to 63.8% which was much higher than that of the SGE test. The results were comparable to the reproducibility reported for SGE testing of non-fastidious organisms, where a CV range of 7.05% to 14.8% was observed (Paton *et al,* 1990).

2. **To assess the effects of varying time of inoculation after antibiotic deposition, volume of agar, length of incubation time, inoculum concentration, and antibiotic deposition concentration on accuracy of SGE for AST of fastidious organisms (*H. influenzae*).**

   It was found that varying the agar depth and standing time before inoculation of

an organism after antibiotic deposition made no significant difference to the resulting MICs. The inoculum density, incubation time, and antibiotic stock concentration were the parameters that affected the results of the SGE test. The results were nearly identical to those of Paton *et al* (1990) for non-fastidious organisms..

3. **To compare and correlate the MICs of clinical isolates of *S. pneumoniae, H. influenzae,* and *N. gonorrhoeae* by SGE and standard dilution methods.**

For *S. pneumoniae*, 56% of the MIC values were the same with the SGE method. Notably, 98.1% of the SGE MICs were within ±1 Log_2 dilution of the SBD method, and 99.5% of the SGE MICs were within ±2 Log_2 dilution. Minor category changes were observed for 13 (6.3%) tests. No very major errors occurred. For *H. influenzae*, 53.8% of the MIC values of all antibiotics tested were the same for both the SGE and the reference method. Overall, 96.1% of the SGE MICs were within ±1 Log_2 dilution of the SBD method and, thus, could be considered equivalent, with 100% of the SGE MIC being within ±2 Log_2 dilution SBD method. Only 13 (6.2%) minor errors occurred. For *M. catarrhalis,* 42.3% of the SGE MICs were the same as SAD MIC. 90% of the SGE values were within ±1 Log_2 dilution and 98.2% of the SGE MICs were within ±2 Log_2

dilution of the SAD. For *N. gonorrhoeae*, 62.5% of the MICs were the same for the SGE method. 97.5% of the SGE MICs were within ±1 Log_2 dilution of the SAD method and 100% were within ±2 Log_2 dilution. Only 5% minor errors occurred.

4. **To assess advantages, disadvantages and potential application of the SGE method for AST of fastidious organisms.**

   The SGE test was found to be robust and simple to perform. The technician time required for one test is less than 5 minutes. The cost per test was less than 2 HK dollars (US$~0.25). In addition to give a more precise MIC, SGE test can be used for other tests like MBC, antibiotic tolerance and combination effect of drugs.

5. **To optimize the concentration of Propidium Iodide and $DiBAC_4(3)$ and incubation time for FCM determination for a control strain of *H. influenzae*.**

   It was found that the optimal concentration that gave the highest fluorescence for PI and $DiBAC_4(3)$ were 10 μg/ml and 5 μg/ml respectively. When the concentrations were lower, the fluorescence generated was dimmer. When the concentration was increased, quenching occurred leading to a decrease in fluorescence intensity. The optimal incubation time of dye with cells that gave steady signals for PI and $DiBAC_4(3)$ were 15 minutes and 5 minutes

respectively

6. **To determine the optimal media to give maximum fluorescence signals for FCM on fixed control strains of *H. influenzae* and *S. pneumoniae.***

The medium that gave highest fluorescence signal (significant; $P<0.05$) on *H. influenzae* fixed cells was Tris-HCl buffer (5mM, pH7.5). Other media tested were distilled water, MH broth with supplement for *H. influenzae* and PBS.

When DiBAC4(3) was used with *S. pneumoniae* cells, it was found that distilled water and PBS (pH 7.4) gave the highest fluorescence intensity. However, distilled water was not used for further experiments since it would give fluctuating signals and this was reflected in its high variance. When staining media for PI was tested, it was found that PBS (pH 7.4) gave the highest fluorescence signal.

7. **To determine the effects of concentration vs exposure time of antibiotics on *H. influenzae* (ampicillin and tetracycline) and S. pneumoniae (penicillin G and erythromycin).**

The quantitative basis of the FC-AST was improved by measuring the effects of antibiotics as functions of drug concentration and as a function of duration of exposure to drugs. The kinetics showed that the measured parameters provided

significant detection of effect of tested antibiotics at the MIC values. It was also found that PI-associated fluorescence at 1x MIC of both drugs was not as intense as that for $DiBAC_4(3)$ – associated fluorescence, only a small proportion of cells took in PI. At low concentrations of drugs (0.1 x MIC) both organisms gave a low response. However when drug concentrations were high (10 x and 100x MIC), both organisms gave a rapid response and fluorescence intensity and number of cells that took in the dyes increased. When the incubation time was longer the signals plateaud-out and even decreased due to lysis of cells. This was indicated by an increase in cell debris signals. At 1 x MIC, the resultant signal was optimal and the signals increased until the end of the experiment (180 mins). This implied that when a breakpoint concentration of drug is added to a tested strain, the strain would respond well if its MIC is below or equal to the breakpoint. Thus, a FCM-AST may be feasible.

8. **To compare numbers of fluorescence events cells (the labeled cells) and the mean fluorescent channel for the PI and $DiBAC_4(3)$ signal on *H. influenzae* and *S. pneumoniae* cells treated with antibiotics at breakpoint concentration.**

An untreated culture for the same strain was also included as a negative control and compared with the treated strain. Using a modified version of the formula suggested by Gauthier *et al* (2002) susceptibility categories of 30 strains of *H.*

influenzae and 35 strains of *S. pneumoniae* were determined by multiplication of the proportion of fluorescent cells, and PI and $DiBAC_4(3)$ mean fluorescence channel. The results were compared to standard AST methods. Of the 60 tests (2 antibiotics x 30 strains) only 1 minor error occurred for *H. influenzae*. For *S. pneumoniae*, 8 minor errors occurred in 70 tests using two drugs. The FC-AST was found to be precise and accurate.

6) To assess the cost and benefit of performing SAD test, E test, Vitek 2, SGE and FCM-AST test.

As demonstrated in Appendix ix, the SGE method was the cheapest and FCM-AST was the most expensive test. The following are in order of increasing cost: SGE, E test, Vitek 2 and FCM-AST.

SGE is convenient to perform. The storage of antibiotic powder is less problematic than that for the E tests strips, which must be keep at low temperature at all times. There is no transport problem for the SGE, so it is most suitable in rural poor village settings where budgets may be very low. In the case of Vitek 2, in addition to the high machine price, the maintence price is also expensive. The antibiotic panel may only be suitable for the USA. In other countries a specially designed panel may be required. This would be

expected to have a very high price. Moreover the machine can only test for one type of fastidious organism, *S. pneumoniae*. FCM-AST is expensive but the cost to the medical laboratory may be more than compensated for by the overall savings to the hospital since a shortening of hospital stay would be anticipated for the patient.

Chapter 10

Final Conclusions

With the emergence of resistance in fastidious organisms, AST is required to enable prescribing of narrow spectrum, cheaper antibiotics and to ensure successful therapy. Some modifications have been made to standard NCCLS disk diffusion, and MIC methods to allow reliable testing of these bacteria (NCCLS, 2000; 2004b), including supplementation of the test medium, extended incubation time, and incubation in 5% CO_2. MIC methods, which provide a more accurate estimate of susceptibility, are desirable when the disk diffusion tests give unclear results, when there is unexpected treatment failure or an unusual resistance pattern. Screening for penicillin resistance in *S. pneumoniae* using a 1 μg oxacillin disk can identify susceptible strains, but is unable to distinguish between high level and intermediate penicillin resistance, which is important especially in meningitis, and MIC determination is required (Jorgensen *et al*, 1993). Additionally, disk diffusion is not standardized for broad spectrum cephalosporin susceptibility testing, requiring MIC testing for cefotaxine or ceftriaxone (Jorgensen *et al*, 1993). As current MIC methods for pneumococci are tedious to perform, have disadvantages of large increments at higher concentrations, and high standard allowable errors (Wexler *et al*, 1990),

there is a need for a rapid and precise method.

Although the E test is a robust, simple technique, which can be used with different in vitro parameters, for testing fastidious organisms, and evaluation of its use for fastidious organisms has revealed a high level of agreement with results of reference test (Sanchez *et al*, 1992; Hughes *et al*,1993; Jorgensen *et al*, 1994a; Jorgensen *et al*, 1994b; Macias *et al*, 1994; Skulnick *et al*, 1995), the strips are relatively expensive, making this uneconomic for testing multiple drugs on each isolate and there are storage problems of the test strips.

SGE, a concentration gradient method for MIC, allows the use of enriched media and varied incubation atmosphere, and its use has shown excellent agreement with standard dilution methods for aerobic and anaerobic organisms. (James, 1990; Hill and Schalkowsky, 1990; Paton *et al*, 1990; Hill, 1991; Wexler *et al*, 1991; Wexler *et al*, 1996). There have been no reports of its use for fastidious organisms, As, initially, the SGE method gave significantly lower MICs compared to standard dilution, due to the effect of diffusion, optimization was necessary.

Intrabatch and interbatch reproducibility performed for *H. influenzae, S. pneumoniae, N. gonorrhoeae* and *M. catarrhalis* produced results superior to the standard dilution method, and were comparable to those of Paton *et al* (1990) for non-fastidious organisms, supporting the use of SGE for AST of fastidious organisms. This study attempted to reduce the effects of diffusion causing lowered MICs with SGE by varying the depth of the agar, time of inoculation, length of incubation, inoculation density, and antibiotic stock solution concentrations. Inoculation density, time of incubation, and deposition of antibiotic working concentrations were found to affect the final MICs. Following optimisation, comparison studies with standard dilution methods showed there were no significant differences between the MIC generated by SGE and standard dilution tests, with correlation coefficients ranging from 0.956 to 0.965. Only minor errors were observed and the results were clearly an improvement on those of previous studies (James, 1990; Hill and Schalkowsky, 1990; Paton *et al*, 1990; Hill, 1991; Wexler *et al*, 1991; Wexler *et al*, 1996; Pong *et al*, 1998). It may be concluded that the SGE method is suitable for AST of fastidious organisms. However, further evaluation including more clinical isolates, especially strains with an MIC around the susceptibility category cut-off points should be performed. The optimal conditions do need to be

determined for each fastidious organism and drug before the method can be routinely used. Due to time and budget constraints, only one organism and drug combination were optimized in this study. It may also be worthwhile to investigate whether the diffusion problems could be counteracted by an increase in agar concentration. Further investigation of the reproducibility is also recommended, involving a multi-centre study to determine intra-laboratory and inter-laboratory precision and accuracy.

SGE method may also represent a cost effective means for the determination of the MIC (see Appendix ix), especially if the laboratory already has a spiral plater for bacterial counting. An initial purchase of a spiral plater may also be justified, as it can also be used for other purposes, including effects of drug combinations, antibiotic tolerance studies, and determination of MBCs.

Rapid identification and AST significantly lower morbidity and mortality rates, shorten hospital stays, and reduce hospital cost (Doern *et al*, 1994; Barangfanger, 2001; Barangfanger and Short, 2001). Disk diffusion and conventional MIC dilution methods require bacterial growth to determine the endpoint, requiring overnight incubation. Two automated systems, Baxter MicroSCAN AutoSCAN

WalkAway (Baxter Diagnostic West Sacramento, CA) and Vitek System (BioMerieux Vitek, Hazehvood, MO), are capable of generating rapid (4 to 8 hours) AST results, and are approved by the FDA (Ferraro and Jorgensen, 1995). Some automated systems offer panels for AST of *S. pneumoniae* (Shanholtzen and Peterson, 1986; Krisher and Lincott, 1994; Kiska *et al*, 1995) which are approved by the FDA. The Vitek 2 system, provides a rapid method for *S. pneumoniae.* However, it still needs an average of 8.5 hours to provide a result (Jorgenson *et al*, 2000). This represents more than one work day of a microbiology laboratory. Although several semi-automated systems, were approved by FDA for AST of *S. pneumoniae*, all require overnight incubation and the initial investment for purchase of these machines is high.

Initial studies (Steen and Boye, 1981; Steen *et al*, 1982; Steen *et al*, 1986) indicated that FCM-AST could provide a reliable qualitative method for non-fastidious organisms as it provides results rapidly and can analyse morphological and physiological changes of cells in non-synchronous cultures (Kell *et al*, 1991). This study aimed to evaluate its use for fastidious organisms.

This study is one of the first to optimize FCM-AST, and the first to study

fastidious organisms which have specific growth requirements that need to be incorporated. There are no previous reports of the potential for FCM-AST on *H. influenzae and S. pneumoniae*. Optimal cell concentrations, concentrations of $DiBAC_4(3)$ and PI, and incubation times were all defined in this study. Optimal dye concentration allows the highest fluorescence intensity, without leakage of the probe into live cells (Walberg et al, 1996) which can lead to non-specific fluorescence, whilst avoiding quenching of fluorescence intensity, which can occur if the dye concentration is too high. Optimal dye concentration and time of incubation of cells with dye is essential for reproducible, sensitive and specific AST results. Optimisation of the staining medium for *H. influenzae* found that 5mM Tris-HCL buffer (pH 7.5) would increase the fluorescence intensity, leading to increased sensitivity. However, the cell wall structure of other organisms and numbers of intracellular binding sites for different fluorescent probes would be expected to be different for different species. Thus, optimization would need to be repeated for each species to be studied. This was confirmed in this study as a different staining medium, PBS (pH 7.4) was found to be optimal for *S. pneumoniae*.

This project examined two models for antibiotic activity, a bacteriocidal drug,

ampicillin, and a bacteriostatic drug, tetracycline, against *H. influenzae* as a function of dosage and exposure time. Transitions in light scatter and fluorescence intensity were observed following treatment with antibiotics at various drug concentrations and exposure times, and reductions in the numbers and viability of bacteria were observed as the antibiotic concentration and exposure times were increased. Use of $DiBAC_4(3)$ and PI to track changes in cell membrane provided a rapid, reproducible and sensitive technique to study the mechanisms of antibiotic action in bacteria. Two major considerations affected the development of the FCM-AST method: Aggregation of bacterial cells, and resolution of cells from debris. Resolution of cells from debris was achieved by triggering of the machine by side scatter, and use of specifically stained fluorescent cells to discriminate the debris. Aggregation of cells was reduced by shaking of the cultures during growth and vortexing which was shown to be effective by comparison with viable counts. The formula for interpretation of FCM-AST suggested by Gauthier *et al* (2002), was modified and results found to compare well with those of standard methods. It will be necessary to test a large number of strains to confirm these results if FCM-AST of *H. influenzae* and *S. pneumoniae* is to be adopted for routine use. These strains should include isolates with MIC close to the cut off point of

susceptibility.

Analysis of results of FCM-AST of *S. pneumoniae* found that most of the errors were due to insufficient signals from the bacterial cells, and a high percentage of intermediate resistant strains could not be accurately categorized to the correct susceptibility category. It is suggested that the method can be refined to include two breakpoint concentrations of drugs. It is hoped that all of the susceptibility categories can then be accurately identified, allowing the discrimination between intermediately susceptible strains which usually respond to high doses of beta-lactams, and highly resistance strains which may need alternative or combination therapy.

In view of the promising results obtained with *H. influenzae* and *S. pneumoniae*, optimization and evaluation of this technique should be carried out for other fastidious organisms in the future.

A number of obstacles remain to be overcome before FCM methods can be considered suitable as a standard procedure for AST. A major issue is the design and the validation of a universally applicable fluorescence probe. Membrane

potential sensitive probes, such as $DiBAC_4(3)$, offer ease of use. However, variations in uptake of dye between and even within bacterial species has been reported, and differences in dye concentration can affect the labelling properties of $DiBAC_4(3)$ (Mason and Gant, 1995). It will be essential to clearly define not only experimental conditions, but also guidelines concerning the interpretation of FCM data arising from antibiotic-induced effect, for comparison of results between laboratories. It may be possible to build a computer-based interpretation programme to determine sensitivity following FCM-AST. As stated above, the need for optimization of parameters is essential for the interpretation of results.

A second problem is the standardization of the instrument design. Many FCM models exist, with variations in optical configuration, light source and available wavelength (arc-lamp or laser), and detector sensitivity. A universal AST method and dye that can yield identical results in different laboratories or with different machines is unlikely. A purpose-designed FCM could offer a much better alternative. Such an instrument would allow standardization, be easy to use, and incorporate software for data analysis and interpretation, thus minimizing staff training.

FCM-AST offers several advantages such as short incubation time, increased precision and accuracy by decreasing the number of dilution steps, elimination of colony counting errors, and difficulties in reading turbidometric endpoints, and high speed of analysis. FCM can improve drug screening efficiency by providing information on the mode of action of the drugs. Although cost and other factors remain an issue, the potential of FCM for AST warrants further studies of its feasibility. Its use for non-fastidious organism has been successful, and this study indicates that it could be extended to fastidious organisms, proving to be equally rewarding.

Difficulties in applying standard methods of MIC testing to fastidious organisms has led to the necessity for fresh approaches to AST for these species. This study has successfully optimized two methods for this purpose, SGE and FCM. Their application may lead to improved testing of fastidious strains and improvements in the prompt and appropriate treatment of patients suffering from infections caused by these organisms. In this era of increasing resistance in respiratory pathogens, the need for improved susceptibility testing is paramount.

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Appendix I: Uptake of PI by ampicillin treated *H. influenzae* control cells

	MICtime	Time	Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	Poscell pos	Poscell debris
1	.10	.00	-.30	2.00	2.50	2.50	1.60	2.20	2.50
2	.10	30.00	-.30	2.00	2.50	2.00	1.99	2.20	3.27
3	.10	30.00	-.20	2.00	2.50	2.00	1.16	2.30	3.52
4	.10	30.00	.00	2.00	2.50	2.00	1.59	2.50	3.52
5	.10	30.00	.00	2.00	2.50	2.00	2.34	2.50	3.20
6	.10	30.00	-.30	2.00	2.50	2.00	1.57	2.20	2.62
7	.10	60.00	-.30	2.00	2.50	2.00	2.20	2.20	2.33
8	.10	60.00	.29	2.00	2.50	2.00	1.33	2.79	2.43
9	.10	60.00	-.30	2.00	2.50	2.00	1.25	2.20	2.53
10	.10	60.00	-.29	2.00	2.50	2.00	2.81	2.79	2.33
11	.10	60.00	-.30	2.00	2.50	2.00	2.20	2.20	2.23
12	.10	120.00	-.12	2.00	2.50	2.00	1.88	2.38	5.49
13	.10	120.00	.11	2.00	2.50	2.00	1.71	2.61	5.47
14	.10	120.00	-.12	2.00	2.50	2.00	2.60	2.38	5.45
15	.10	120.00	.11	2.00	2.50	2.00	2.04	2.61	5.35
16	.10	120.00	-.12	2.00	2.50	2.00	1.72	2.38	5.61
17	.10	180.00	-.28	2.00	2.50	2.50	2.50	2.22	6.50
18	.10	180.00	.10	2.00	2.50	2.00	2.11	2.60	6.84
19	.10	180.00	-.28	2.00	2.50	2.00	1.98	2.22	5.89
20	.10	180.00	.00	2.00	2.50	2.00	2.96	2.60	7.52
21	.10	180.00	-.28	2.00	2.50	2.00	2.30	2.22	5.55
22	1.00	.00	.10	2.00	2.41	.35	2.00	2.41	.35
23	1.00	30.00	1.24	2.10	1.57	.13	2.20	2.81	.27
24	1.00	30.00	-.39	2.20	1.66	.13	2.30	1.27	.32
25	1.00	30.00	-.51	2.10	1.93	.14	1.71	1.42	.43
26	1.00	30.00	-.07	2.20	1.65	.15	1.95	1.58	.28
27	1.00	30.00	.86	2.20	1.65	.16	2.24	2.51	.28
28	1.00	60.00	1.50	1.80	1.17	2.51	4.11	2.67	4.78
29	1.00	60.00	1.75	1.90	1.03	2.52	2.83	2.78	5.85
30	1.00	60.00	1.86	1.90	1.04	3.37	3.30	2.90	7.80
31	1.00	60.00	2.73	1.80	1.07	2.62	4.45	3.80	6.30
32	1.00	60.00	2.92	1.80	1.18	2.72	2.88	4.10	6.70

	MICtime	Time	Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	Poscell pos	Poscell debris
33	1.00	120.00	3.95	1.40	1.60	2.30	3.91	5.55	27.00
34	1.00	120.00	4.49	1.40	1.56	2.14	3.73	6.05	26.20
35	1.00	120.00	5.11	1.50	1.60	2.30	3.96	6.71	26.36
36	1.00	120.00	3.93	1.60	1.56	2.14	2.59	5.49	28.63
37	1.00	120.00	4.391	1.80	1.60	2.30	4.21	6.51	25.64
38	1.00	180.00	3.91	1.60	1.72	6.10	3.88	5.63	27.48
39	1.00	180.00	5.30	1.60	1.47	6.49	4.43	6.77	27.11
40	1.00	180.00	5.75	1.80	1.39	7.59	4.51	7.14	26.14
41	1.00	180.00	4.41	1.60	1.47	6.50	3.34	5.88	26.50
42	1.00	180.00	4.52	1.80	1.39	6.60	4.69	5.91	26.70
43	10.00	.00	.00	2.00	1.50	2.30	2.00	1.50	2.30
44	10.00	30.00	-.19	2.00	1.50	2.30	2.30	1.31	12.50
45	10.00	30.00	-.65	2.00	1.50	2.30	1.90	.85	12.30
46	10.00	30.00	-.65	2.00	1.50	2.30	1.81	.85	12.60
47	10.00	30.00	-.19	2.00	1.50	2.30	1.62	1.31	12.40
48	10.00	30.00	-.19	2.00	1.50	2.30	2.30	1.31	12.20
49	10.00	60.00	2.02	2.00	1.50	2.30	5.82	3.52	15.54
50	10.00	60.00	2.45	2.00	1.50	2.30	6.41	3.95	15.13
51	10.00	60.00	1.70	2.00	1.50	2.30	5.93	3.20	15.93
52	10.00	60.00	2.02	2.00	1.50	2.30	6.20	3.52	16.13
53	10.00	60.00	1.70	2.00	1.50	2.30	6.73	3.20	17.13
54	10.00	120.00	4.23	2.00	1.50	2.30	6.91	5.73	17.14
55	10.00	120.00	3.71	2.00	1.50	2.30	7.82	5.21	19.71
56	10.00	120.00	4.23	2.00	1.50	2.30	7.51	5.73	19.51
57	10.00	120.00	3.71	2.00	1.50	2.30	8.34	5.21	18.79
58	10.00	120.00	4.23	2.00	1.50	2.30	7.60	5.73	18.56
59	10.00	180.00	13.96	2.00	1.50	2.30	7.55	15.46	25.46
60	10.00	180.00	13.75	2.00	1.50	2.30	7.52	15.25	25.25
61	10.00	180.00	13.75	2.00	1.50	2.30	7.44	15.25	25.71
62	10.00	180.00	12.70	2.00	1.50	2.30	8.23	14.20	26.34
63	10.00	180.00	12.70	2.00	1.50	2.30	6.52	14.20	26.46
64	100.00	.00	.00	2.10	1.97	1.67	2.10	1.97	1.67
65	100.00	30.00	-.24	2.10	2.04	1.16	3.20	1.80	18.21
66	100.00	30.00	.97	2.20	2.31	1.29	4.20	3.28	18.12
67	100.00	30.00	-.82	2.30	2.82	2.42	4.00	2.00	17.25

	MICtime	Time	Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	Poscell pos	Poscell debris
68	100.00	30.00	-.31	2.40	2.31	2.00	4.00	2.00	17.53
69	100.00	30.00	-.31	2.50	2.31	2.00	4.00	2.00	17.10
70	100.00	60.00	5.25	1.90	1.37	3.46	4.80	6.62	39.60
71	100.00	60.00	6.40	2.00	2.54	5.00	5.40	8.94	39.77
72	100.00	60.00	4.45	2.00	2.58	5.00	6.10	7.03	38.64
73	100.00	60.00	3.56	2.30	2.00	9.99	6.00	5.56	37.34
74	100.00	60.00	5.53	2.40	2.00	9.00	6.00	7.53	37.66
75	100.00	120.00	12.43	1.20	1.00	1.74	7.90	13.43	40.21
76	100.00	120.00	17.99	1.20	1.50	2.10	7.55	19.49	44.86
77	100.00	120.00	14.48	1.20	1.50	1.91	6.20	15.98	45.28
78	100.00	120.00	14.16	1.20	1.50	2.00	8.55	15.66	46.42
79	100.00	120.00	12.63	1.20	1.50	2.00	7.59	14.13	47.21
80	100.00	180.00	16.30	1.30	1.27	6.31	6.70	17.57	55.33
81	100.00	180.00	10.84	1.40	1.73	6.42	5.96	12.57	55.00
82	100.00	180.00	12.03	1.50	1.85	7.07	7.66	13.88	52.61
83	100.00	180.00	12.35	1.60	1.80	6.00	5.84	14.15	52.17
84	100.00	180.00	12.61	1.70	1.80	6.00	6.88	14.41	53.59

Legend: MICtime = Times of MIC

Time = Times of antibiotic exposure

Diffposcel = Different in proportion of fluorescence cells before and after treatment control

Negflu = Fluorescence from negative control

Posflu = Fluorescence from treated culture

Negcellpos = Proportion of cells that take in dye before antibiotic treatment

Poscellpos = Proportion of cells take in dye on treated culture

Negcell debris = Proportion of signals from cell debris of negative control

Poscell debris = Proportion of signals from cell debris of treated culture

Appendix II: Uptake of $DiBAC_4(3)$ by ampicillin treated *H. influenzae* control cells

	MICtime	Time	Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	Poscell pos	Poscell debris
1	.10	.00	.00	6.00	2.00	.34	6.00	2.00	.34
2	.10	30.00	.30	5.50	2.00	.34	6.20	2.30	3.10
3	.10	30.00	-.10	5.50	2.00	.34	6.40	1.90	3.20
4	.10	30.00	-.10	5.50	2.00	.39	6.60	1.90	3.30
5	.10	30.00	.10	5.40	2.00	.44	6.80	2.10	3.40
6	.10	30.00	.60	5.40	2.00	.45	7.00	2.60	3.50
7	.10	60.00	-.50	6.50	5.00	.35	6.80	4.50	2.16
8	.10	60.00	-.13	6.50	5.00	.35	7.10	4.87	2.57
9	.10	60.00	.91	6.50	5.00	.35	6.75	5.91	2.62
10	.10	60.00	1.10	6.50	5.00	.35	6.53	6.10	2.18
11	.10	60.00	.80	6.50	5.00	.35	7.23	5.80	2.17
12	.10	120.00	2.00	6.50	5.00	.40	7.10	7.00	5.00
13	.10	120.00	-.49	6.50	5.00	.38	6.80	4.51	5.10
14	.10	120.00	-.10	6.50	5.00	.45	6.61	4.90	4.90
15	.10	120.00	.12	6.50	5.00	.49	7.23	5.12	4.70
16	.10	120.00	-.19	6.50	5.00	.33	6.99	4.81	5.30
17	.10	180.00	2.66	6.50	5.00	.34	6.21	7.66	5.51
18	.10	180.00	1.84	6.50	5.00	.34	6.92	6.84	6.23
19	.10	180.00	2.52	6.50	5.00	.34	6.31	7.52	6.35
20	.10	180.00	2.33	6.50	5.00	.34	6.85	7.33	7.35
21	.10	180.00	2.69	6.50	5.00	.34	6.55	7.69	7.59
22	1.00	.00	-.144	6.00	3.00	1.88	6.00	1.56	1.88
23	1.00	30.00	-.79	5.80	3.00	1.87	6.20	2.21	.94
24	1.00	30.00	.90	5.50	3.00	1.88	5.90	3.90	1.17
25	1.00	30.00	-1.00	5.10	3.00	1.88	6.20	2.00	1.74
26	1.00	30.00	.89	5.20	3.00	1.97	6.70	3.89	1.35
27	1.00	30.00	.71	5.90	3.00	1.68	6.50	3.71	2.02
28	1.00	60.00	9.65	5.70	3.00	.92	7.30	12.65	8.35
29	1.00	60.00	8.59	5.70	3.00	.94	7.80	11.59	8.24
30	1.00	60.00	9.40	5.70	3.00	.96	7.40	12.40	8.72
31	1.00	60.00	9.03	5.70	3.00	.98	8.20	12.03	8.59

	MICtime	Time	Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	Poscell pos	Poscell debris
32	1.00	60.00	10.71	5.70	3.00	1.00	8.60	13.71	8.25
33	1.00	120.00	17.71	5.50	3.00	1.01	8.01	20.71	13.66
34	1.00	120.00	19.71	5.50	3.00	1.03	8.10	22.71	14.93
35	1.00	120.00	23.14	5.50	3.00	1.09	8.40	26.14	14.96
36	1.00	120.00	17.92	5.50	3.00	1.07	7.81	20.92	13.53
37	1.00	120.00	24.23	5.50	3.00	1.05	8.21	21.23	14.13
38	1.00	180.00	22.45	5.40	3.00	1.05	8.56	25.45	11.15
39	1.00	180.00	22.86	5.40	3.00	1.05	8.50	25.86	11.23
40	1.00	180.00	23.67	5.40	3.00	1.05	8.81	26.67	11.88
41	1.00	180.00	27.00	5.40	3.00	1.05	8.01	26.03	14.45
42	1.00	180.00	28.79	5.40	3.00	1.05	9.18	28.79	14.38
43	10.00	.00	.00	5.40	2.64	1.80	6.00	2.64	1.80
44	10.00	30.00	-.85	5.40	2.64	1.80	6.55	1.79	12.20
45	10.00	30.00	.52	5.50	1.57	1.80	6.7.01	2.09	12.58
46	10.00	30.00	-.76	5.60	2.64	.94	6.92	1.88	12.31
47	10.00	30.00	1.80	5.40	1.57	.94	7.13	3.37	12.96
48	10.00	30.00	.22	5.50	2.64	1.95	6.85	2.86	12.97
49	10.00	60.00	12.44	5.00	1.91	1.71	9.30	14.35	16.11
50	10.00	60.00	14.00	5.70	1.59	1.67	8.90	15.59	15.77
51	10.00	60.00	12.67	4.50	1.59	1.71	9.30	14.26	15.22
52	10.00	60.00	13.98	5.10	1.91	1.71	8.90	15.89	15.52
53	10.00	60.00	12.09	4.80	1.59	1.70	9.60	13.68	17.31
54	10.00	120.00	16.37	5.70	1.91	1.80	8.60	18.28	18.31
55	10.00	120.00	17.66	5.70	1.91	1.80	8.90	19.57	16.08
56	10.00	120.00	16.34	5.50	1.91	1.80	8.50	18.25	16.72
57	10.00	120.00	17.02	5.50	1.91	1.80	8.80	18.93	17.93
58	10.00	120.00	18.72	5.50	1.91	1.80	9.00	20.63	16.85
59	10.00	180.00	23.19	5.80	2.58	2.31	8.50	25.77	22.48
60	10.00	180.00	24.02	5.20	2.17	1.80	8.60	26.19	23.48
61	10.00	180.00	23.92	4.80	2.58	2.18	8.90	26.50	22.69
62	10.00	180.00	24.15	5.00	2.17	2.18	7.89	26.32	21.34
63	10.00	180.00	25.25	5.00	2.58	2.18	8.10	27.83	20.55
64	100.00	.00	.00	5.00	2.28	1.75	6.00	2.28	1.75
65	100.00	30.00	2.08	5.00	2.28	1.75	7.50	4.36	24.35
66	100.00	30.00	1.71	5.00	2.28	1.75	7.80	3.99	23.50

	MICtime	Time	Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	Poscell pos	Poscell debris
67	100.00	30.00	1.88	5.00	2.28	1.75	8.20	4.16	24.78
68	100.00	30.00	2.23	5.00	2.28	1.75	7.80	4.51	24.50
69	100.00	30.00	2.45	5.00	2.28	1.75	7.80	4.73	24.50
70	100.00	60.00	4.27	5.00	2.40	1.80	9.30	7.67	29.95
71	100.00	60.00	1.76	5.00	2.40	1.80	9.71	7.16	32.86
72	100.00	60.00	2.45	5.00	2.40	1.80	9.55	7.85	33.78
73	100.00	60.00	4.15	5.00	2.40	1.80	10.01	8.55	38.10
74	100.00	60.00	3.40	5.00	2.40	1.80	10.11	9.80	32.50
75	100.00	120.00	13.35	5.00	2.30	1.90	9.21	15.65	47.26
76	100.00	120.00	12.70	5.00	2.30	1.90	8.52	15.00	43.05
77	100.00	120.00	13.52	5.00	2.30	1.90	8.91	15.82	46.63
78	100.00	120.00	13.72	5.00	2.30	1.90	8.77	16.02	41.16
79	100.00	120.00	14.26	5.00	2.30	1.90	8.54	16.56	41.02
80	100.00	180.00	12.55	5.00	2.30	1.80	8.01	14.85	51.17
81	100.00	180.00	14.25	5.00	2.30	1.80	8.88	16.55	50.03
82	100.00	180.00	13.56	5.00	2.30	1.80	7.63	15.86	51.34
83	100.00	180.00	13.35	5.00	2.30	1.80	7.84	15.65	50.89
84	100.00	180.00	14.71	5.00	2.30	1.80	8.02	17.01	52.01

Legend: MICtime = Times of MIC

Time = Times of antibiotic exposure

Diffposcel = Different in proportion of fluorescence cells before and after treatment control

Negflu = Fluorescence from negative control

Posflu = Fluorescence from treated culture

Negcellpos = Proportion of cells that take in dye before antibiotic treatment

Poscellpos = Proportion of cells take in dye on treated culture

Negcell debris = Proportion of signals from cell debris of negative control

Poscell debris = Proportion of signals from cell debris of treated culture

Appendix III: Uptake of PI by tetracycline treated *H. influenzae* control cells.

	MICtime	Time	Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	Poscell pos	Poscell debris
1	.10	.00	.00	4.10	1.36	1.16	4.10	1.36	1.16
2	.10	30.00	-.70	3.33	2.00	1.47	4.00	1.30	.31
3	.10	30.00	-.83	3.43	2.18	1.48	4.34	1.35	.38
4	.10	30.00	-.44	3.44	1.96	1.55	2.47	1.52	.39
5	.10	30.00	-.02	3.38	2.00	1.61	3.44	1.98	.36
6	.10	30.00	.13	3.43	2.00	1.38	3.40	2.13	.44
7	.10	60.00	.12	4.00	2.61	2.55	3.70	2.73	2.19
8	.10	60.00	-.23	4.11	2.75	2.56	4.70	5.52	2.29
9	.10	60.00	.12	4.16	2.93	2.57	4.78	3.05	2.28
10	.10	60.00	-.16	4.13	2.55	2.55	3.81	2.39	2.30
11	.10	60.00	-.10	4.00	2.43	2.50	3.69	2.33	2.39
12	.10	120.00	.55	4.10	1.54	1.23	4.00	2.09	1.98
13	.10	120.00	.24	4.34	1.86	1.49	4.99	2.10	1.74
14	.10	120.00	.47	4.33	2.12	1.66	4.90	2.59	2.86
15	.10	120.00	-.07	4.30	2.00	1.73	4.21	1.93	2.08
16	.10	120.00	-.10	4.21	2.11	1.80	4.38	2.01	2.24
17	.10	180.00	1.70	4.80	1.06	.57	3.50	2.76	.51
18	.10	180.00	.40	4.50	1.46	.74	3.50	1.86	.42
19	.10	180.00	-.20	4.61	1.31	.65	5.26	1.11	.40
20	.10	180.00	-.34	4.77	1.42	.88	4.79	1.08	.49
21	.10	180.00	-.10	4.23	1.29	.64	4.11	1.19	.52
22	1.00	.00	.00	4.00	4.67	4.24	4.00	4.67	4.24
23	1.00	30.00	-.34	4.70	4.95	4.38	5.61	4.61	3.80
24	1.00	30.00	-.65	4.80	4.87	4.56	4.86	4.22	4.38
25	1.00	30.00	-.17	4.70	4.76	4.52	4.70	4.59	3.98
26	1.00	30.00	.55	4.75	4.55	4.11	3.75	5.10	3.86
27	1.00	30.00	-.19	4.75	4.31	3.98	4.56	4.12	4.44
28	1.00	60.00	.56	4.40	4.39	2.35	4.49	4.95	2.18
29	1.00	60.00	2.21	4.40	3.25	2.39	4.80	5.46	1.90
30	1.00	60.00	.84	4.50	4.33	2.33	4.90	5.17	2.11
31	1.00	60.00	.31	4.50	4.68	2.35	5.13	4.99	2.38

	MICtime		Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	pos	Poscell debris
32	1.00	60.00	-.68	4.35	4.71	2.41	4.27	4.03	2.53
33	1.00	120.00	.93	4.40	4.72	3.63	5.17	5.65	5.66
34	1.00	120.00	1.70	4.20	4.18	4.51	5.40	5.88	4.72
35	1.00	120.00	1.73	4.70	4.26	3.12	5.51	5.99	5.58
36	1.00	120.00	2.01	4.50	4.55	3.33	4.62	6.56	5.87
37	1.00	120.00	2.31	4.50	4.56	4.15	4.88	6.87	5.19
38	1.00	180.00	2.80	4.70	3.62	3.20	5.50	6.42	5.03
39	1.00	180.00	4.98	4.30	2.91	1.99	6.50	7.89	5.99
40	1.00	180.00	4.62	4.90	4.00	4.08	6.80	8.62	5.11
41	1.00	180.00	3.25	4.55	3.88	4.00	5.28	7.13	5.56
42	1.00	180.00	2.72	4.79	3.56	4.53	5.55	6.28	5.87
43	10.00	.00	.00	4.00	3.96	1.00	4.00	3.96	1.00
44	10.00	30.00	4.13	4.11	6.44	.59	5.89	4.57	.43
45	10.00	30.00	5.97	4.23	6.56	.70	7.38	5.53	.50
46	10.00	30.00	4.91	4.46	6.51	.74	6.77	5.42	.56
47	10.00	30.00	4.19	4.17	6.10	.43	5.55	4.29	.68
48	10.00	30.00	4.08	3.99	6.89	.82	6.53	4.97	.67
49	10.00	60.00	14.54	4.60	3.64	.92	7.21	8.18	3.42
50	10.00	60.00	9.56	4.01	3.95	.91	7.13	7.51	4.58
51	10.00	60.00	7.62	4.00	3.81	.87	8.27	7.43	3.64
52	10.00	60.00	7.98	4.00	3.52	.96	6.88	7.50	3.69
53	10.00	60.00	6.09	4.50	3.47	1.23	6.63	9.56	4.57
54	10.00	120.00	5.37	4.60	6.83	3.35	8.66	12.20	14.75
55	10.00	120.00	4.65	3.91	7.69	3.44	8.15	12.34	14.46
56	10.00	120.00	6.81	4.05	6.31	3.15	8.29	13.12	14.13
57	10.00	120.00	4.48	4.00	7.49	3.62	7.97	11.97	15.10
58	10.00	120.00	5.69	4.27	7.44	3.64	9.61	13.13	14.89
59	10.00	180.00	8.25	4.20	3.78	1.60	7.30	12.03	24.29
60	10.00	180.00	7.98	4.20	4.19	1.59	8.90	12.17	24.75
61	10.00	180.00	9.57	4.20	3.89	1.49	9.30	13.46	25.14
62	10.00	180.00	7.26	4.36	4.33	1.64	8.48	11.59	25.22
63	10.00	180.00	7.11	4.13	4.35	1.58	7.94	11.46	24.83
64	100.00	.00	.00	4.00	4.27	1.00	4.00	4.27	1.00
65	100.00	30.00	3.29	4.81	4.38	1.35	5.95	7.67	1.92
66	100.00	30.00	2.70	4.71	4.82	1.45	6.02	7.52	1.71

	MICtime	Time	Diffposcel	Negflu	Negcell pos	Negcell debris	Posflu	Poscell pos	Poscell debris
67	100.00	30.00	2.41	4.80	4.75	1.01	6.33	7.16	1.88
68	100.00	30.00	4.45	4.80	4.16	1.55	6.69	8.61	1.76
69	100.00	30.00	3.90	4.50	4.21	1.23	5.11	8.11	1.94
70	100.00	60.00	4.54	4.50	4.70	.43	8.91	9.24	11.79
71	100.00	60.00	3.14	4.40	5.51	.34	9.12	8.65	12.51
72	100.00	60.00	2.76	4.51	5.43	.51	8.55	8.19	11.83
73	100.00	60.00	3.68	4.60	5.16	.28	9.19	8.84	12.11
74	100.00	60.00	4.31	4.44	4.88	.41	10.05	9.19	12.46
75	100.00	120.00	3.62	4.20	4.17	6.13	8.40	7.79	23.18
76	100.00	120.00	1.74	4.00	6.75	5.65	9.12	8.49	24.35
77	100.00	120.00	2.72	4.61	5.11	5.84	8.88	7.83	24.88
78	100.00	120.00	3.69	4.55	4.25	6.09	8.10	7.94	24.96
79	100.00	120.00	3.20	4.64	5.46	6.97	7.82	8.66	23.59
80	100.00	180.00	6.05	3.70	4.98	5.63	7.20	11.03	28.97
81	100.00	180.00	8.52	4.62	4.11	5.36	6.80	12.63	30.02
82	100.00	180.00	9.25	3.99	4.56	5.55	6.91	13.81	33.15
83	100.00	180.00	8.26	4.15	4.73	5.79	6.14	12.99	27.46
84	100.00	180.00	6.74	4.73	4.77	5.21	6.88	11.51	36.59

Legend: MICtime = Times of MIC

Time = Times of antibiotic exposure

Diffposcell = Different in proportion of fluorescence cells before and after treatment control

Negflu = Fluorescence from negative control

Posflu = Fluorescence from treated culture

Negcellpos = Proportion of cells

Poscellpos = Proportion of cells take in dye on treated culture

Negcell debris = Proportion of signals from cell debris of negative control

Poscell debris = Proportion of signals from cell debris of treated culture

Appendix IV: Uptake of $DiBAC_4(3)$ by tetracycline treated *H. influenzae* control cells.

	MICtime	Time	Negflu	Negcell pos	Negcell Debris	Posflu	Poscell pos	Diffposcel	Poscell Debris
1	.10	.00	2.00	4.77	3.00	4.00	4.77	.00	3.00
2	.10	30.00	4.00	4.77	3.00	4.62	5.31	.54	3.04
3	.10	30.00	4.00	4.77	3.00	4.20	4.57	-.20	2.91
4	.10	30.00	4.00	4.77	3.00	4.91	5.11	.34	2.05
5	.10	30.00	4.00	4.77	3.00	4.22	4.12	-.65	3.33
6	.10	30.00	4.00	4.77	3.00	4.11	4.21	-.56	2.89
7	.10	60.00	4.00	4.11	2.45	3.81	4.13	.02	3.15
8	.10	60.00	4.00	4.13	2.46	5.62	3.51	-.62	1.95
9	.10	60.00	4.00	4.16	2.45	4.57	3.21	-.95	1.66
10	.10	60.00	4.00	4.21	2.44	3.65	4.42	.21	2.15
11	.10	60.00	4.00	4.13	2.43	4.51	5.04	.91	3.17
12	.10	120.00	4.00	2.55	3.69	4.52	2.01	-.54	4.42
13	.10	120.00	4.00	2.59	3.69	5.36	3.15	.56	3.84
14	.10	120.00	4.00	3.00	3.67	3.49	3.27	.27	5.03
15	.10	120.00	4.00	3.04	3.66	4.59	2.11	-.93	4.39
16	.10	120.00	4.00	3.11	3.51	3.33	2.36	-.75	5.11
17	.10	180.00	4.00	4.70	1.95	3.75	4.22	-.48	2.38
18	.10	180.00	4.00	4.93	2.19	3.84	5.64	.71	3.73
19	.10	180.00	4.00	4.71	2.11	2.99	5.40	.69	1.96
20	.10	180.00	4.00	4.26	1.96	4.15	3.96	-.30	2.04
21	.10	180.00	4.00	4.55	1.93	5.28	4.19	-.36	1.84
22	1.00	.00	4.00	2.10	4.07	4.00	2.10	.00	4.07
23	1.00	30.00	4.00	4.37	4.70	4.00	4.98	.61	5.72
24	1.00	30.00	3.98	3.16	4.18	3.91	4.22	1.06	4.81
25	1.00	30.00	3.97	3.58	4.61	4.88	3.95	.37	4.78
26	1.00	30.00	3.99	4.79	3.97	3.79	3.99	-.80	5.13
27	1.00	30.00	4.00	4.52	4.77	5.13	4.01	-.51	5.14
28	1.00	60.00	4.01	4.71	4.88	5.50	9.34	4.63	5.61
29	1.00	60.00	4.12	4.53	5.26	6.40	10.41	5.88	3.75
30	1.00	60.00	3.89	4.69	5.70	5.40	9.76	5.07	4.15
31	1.00	60.00	4.00	4.50	4.96	5.88	8.97	4.47	4.15

	MICtime	Time	Negflu	Negcell pos	Negcell Debris	Posflu	Poscell pos	Diffposcel	Poscell Debris
32	1.00	60.00	4.00	4.61	5.11	6.05	9.22	4.61	4.39
33	1.00	120.00	4.11	4.88	6.19	6.30	13.91	9.03	5.53
34	1.00	120.00	4.03	4.93	5.89	7.12	12.21	7.28	5.49
35	1.00	120.00	4.00	4.89	5.49	7.63	11.58	6.69	5.08
36	1.00	120.00	3.96	4.78	6.03	7.33	12.23	7.45	5.77
37	1.00	120.00	3.89	4.99	5.88	7.64	11.99	7.00	5.61
38	1.00	180.00	4.00	4.04	6.08	9.18	21.56	17.52	6.03
39	1.00	180.00	4.00	4.92	9.97	8.52	20.74	15.82	6.31
40	1.00	180.00	4.00	3.56	10.00	8.33	22.11	18.55	6.11
41	1.00	180.00	4.12	4.13	7.13	10.39	20.03	15.90	6.79
42	1.00	180.00	3.88	4.83	6.81	8.45	21.73	16.90	5.64
43	10.00	.00	4.00	4.15	4.00	4.00	4.15	.00	4.00
44	10.00	30.00	4.00	4.26	4.00	7.32	7.54	3.28	4.88
45	10.00	30.00	4.00	4.59	4.00	7.91	8.91	4.32	4.82
46	10.00	30.00	4.00	4.00	4.00	8.16	7.56	3.56	4.11
47	10.00	30.00	4.00	4.70	4.00	6.12	7.99	3.29	5.25
48	10.00	30.00	4.00	4.60	4.00	6.16	8.24	3.64	5.37
49	10.00	60.00	4.00	4.50	4.00	9.21	17.12	12.62	4.03
50	10.00	60.00	4.00	4.80	4.00	7.89	14.56	9.76	4.15
51	10.00	60.00	4.00	4.55	4.00	8.89	19.74	15.19	3.74
52	10.00	60.00	4.00	4.67	4.00	7.77	19.25	14.58	4.43
53	10.00	60.00	4.00	4.82	4.00	8.24	18.33	13.51	4.26
54	10.00	120.00	4.00	4.55	4.00	9.11	21.63	17.08	7.72
55	10.00	120.00	4.00	4.79	4.00	9.36	16.49	11.70	7.09
56	10.00	120.00	4.00	4.56	4.00	8.60	23.33	18.77	6.56
57	10.00	120.00	4.00	4.44	4.00	9.68	21.59	17.15	5.34
58	10.00	120.00	4.00	4.46	4.00	10.03	20.49	16.03	6.22
59	10.00	180.00	4.00	4.13	4.00	9.50	25.44	21.31	18.46
60	10.00	180.00	4.00	4.44	4.00	9.11	22.37	17.93	19.38
61	10.00	180.00	4.00	4.55	4.00	9.25	29.33	24.78	16.86
62	10.00	180.00	4.00	4.58	4.00	9.69	27.25	22.67	18.75
63	10.00	180.00	4.00	4.59	4.00	8.01	29.23	24.64	20.17
64	100.00	.00	4.00	3.25	5.53	4.00	3.25	.00	5.53
65	100.00	30.00	4.90	3.25	3.23	8.81	10.17	6.92	4.41
66	100.00	30.00	4.80	3.14	3.15	8.73	9.96	6.82	4.64

	MICtime	Time	Negflu	Negcell pos	Negcell Debris	Posflu	Poscell pos	Diffposcel	Poscell Debris
67	100.00	30.00	5.00	3.84	3.62	7.91	10.23	6.39	5.78
68	100.00	30.00	4.77	3.55	4.79	7.53	9.97	6.42	4.18
69	100.00	30.00	4.89	3.68	3.55	6.72	10.49	6.81	4.72
70	100.00	60.00	4.52	4.33	4.34	9.92	8.78	4.45	15.29
71	100.00	60.00	4.44	4.13	4.78	9.83	9.67	5.54	15.89
72	100.00	60.00	4.63	4.10	4.31	9.88	10.69	6.59	15.57
73	100.00	60.00	4.28	4.56	4.52	9.13	9.97	5.41	14.33
74	100.00	60.00	4.57	4.12	4.71	8.17	10.21	6.09	14.56
75	100.00	120.00	4.50	4.10	6.14	8.80	17.96	3.86	21.24
76	100.00	120.00	4.60	4.77	7.12	8.50	18.20	3.43	21.63
77	100.00	120.00	4.40	3.65	8.39	9.56	18.11	4.46	22.39
78	100.00	120.00	4.90	4.51	7.69	9.79	19.79	5.28	22.79
79	100.00	120.00	4.70	4.29	6.54	8.11	18.27	3.98	20.83
80	100.00	180.00	4.31	3.85	7.04	7.44	17.96	4.11	34.49
81	100.00	180.00	4.50	4.12	6.99	7.56	18.06	3.94	35.26
82	100.00	180.00	4.20	3.73	7.18	5.17	18.17	4.44	38.12
83	100.00	180.00	4.19	4.57	6.57	6.28	18.66	4.09	40.44
84	100.00	180.00	4.82	4.44	7.12	6.59	17.89	3.45	42.17

Legend: MICtime = Times of MIC

Time = Times of antibiotic exposure

Diffposcel = Different in proportion of fluorescence cells before and after treatment control

Negflu = Fluorescence from negative control

Posflu = Fluorescence from treated culture

Negcellpos = Proportion of cells

Poscellpos = Proportion of cells take in dye on treated culture

Negcell debris = Proportion of signals from cell debris of negative control

Poscell debris = Proportion of signals from cell debris of treated culture

Appendix V: Uptake of $DiBAC_4(3)$ by penicillin G treated *S. pneumoniae* control cells (Results were typical of 3 experiments).

	MICtime	Time (min.)	Diffposcel	Negflu	Posflu	Diffcelldebris
1	0.1	0	0.01	1.1	1.7	0
2	0.1	30	0.02	1.2	1.6	0.01
3	0.1	60	-0.03	1.0	1.1	0.01
4	0.1	120	0.00	1.1	1.2	0.03
5	0.1	180	0.02	1.3	1.0	0.015
6	1	0	-0.02	1.0	1.1	2.0
7	1	30	6.86	1.0	1.4	2.45
8	1	60	12.81	1.1	2.5	5.63
9	1	120	16.81	1.0	4.8	8.78
10	1	180	18.99	1.0	6.3	10.42
11	10	0	0.02	1.0	1.0	0.01
12	10	30	10.73	1.1	1.4	6.01
13	10	60	23.80	0.9	2.8	12.97
14	10	120	26.72	0.9	5.0	25.66
15	10	180	29.57	1.0	5.3	30.72
16	100	0	0.00	1.0	1.0	0.00
17	100	30	11.87	1.0	5.8	21.94
18	100	60	9.02	1.0	5.9	39.26
19	100	120	9.43	1.0	1.7	42.38
20	100	180	8.52	1.1	2.1	49.79

Appendix VI: Uptake of PI by penicillin G treated *S. pneumoniae* control cells (Results were typical of 3 experiments).

	MICtime	Time (min.)	Diffposcel	Negflu	Posflu	Diffcelldebris
1	0.1	0	0.0	2.0	2.1	-0.05
2	0.1	30	0.02	2.0	2.1	-0.13
3	0.1	60	0.12	2.1	1.8	0.08
4	0.1	120	0.05	1.9	1.8	0.05
5	0.1	180	-0.02	2.0	1.9	0.12
6	1	0	0	2.2	2.0	0.13
7	1	30	3.57	2.1	3.0	0.43
8	1	60	3.98	1.9	4.3	3.05
9	1	120	4.32	2.3	5.3	4.54
10	1	180	5.34	2.0	6.0	7.35
11	10	0	0	2.0	2.0	6.14
12	10	30	2.49	2.0	2.3	0.37
13	10	60	3.13	2.0	2.9	5.68
14	10	120	5.68	2.0	5.5	10.32
15	10	180	4.83	2.0	5.6	15.92
16	100	0	0	1.9	2.0	0
17	100	30	4.00	1.9	5.2	2.3
18	100	60	5.77	2.0	6.0	9.69
19	100	120	5.96	2.1	6.8	18.60
20	100	180	3.75	2.1	4.9	24.48

Appendix VII: Uptake of $DiBAC_4(3)$ by erythromycin treated *S. pneumoniae* control cells (Results were typical of 3 experiments).

	MICtime	Time (min.)	Diffposcel	Negflu	Posflu	Diffcelldebris
1	0.1	0	0.01	1.0	0.9	0
2	0.1	30	0.01	1.1	0.9	0.02
3	0.1	60	-0.02	1.1	1.1	-0.03
4	0.1	120	0.03	1.2	1.0	0.01
5	0.1	180	0.0	0.9	1.0	0
6	1	0	0	1.0	1.1	0
7	1	30	7.02	1.0	1.9	1.36
8	1	60	9.67	0.9	2.1	2.83
9	1	120	13.33	0.9	3.0	3.31
10	1	180	14.67	0.9	4.8	4.79
11	10	0	0.03	1.1	1.0	-0.03
12	10	30	10.28	1.1	1.6	1.67
13	10	60	14.30	1.0	3.0	6.97
14	10	120	16.53	1.0	6.3	8.30
15	10	180	18.52	1.0	10.7	10.97
16	100	0	0.0	1.0	1.1	0.01
17	100	30	18.12	0.9	2.2	4.48
18	100	60	26.13	0.9	3.5	10.17
19	100	120	30.31	1.0	3.7	14.02
20	100	180	31.28	1.1	9.12	18.01

Appendix VIII: Uptake of PI by penicillin G treated *S. pneumoniae* control cells (Results were typical of 3 experiments).

	MICtime	Time (min.)	Diffposcel	Negflu	Posflu	Diffcelldebris
1	0.1	0	0	2.0	1.9	0
2	0.1	30	0	2.0	1.8	0.01
3	0.1	60	0.01	1.9	2.0	-0.02
4	0.1	120	-0.02	2.0	2.0	0.0
5	0.1	180	-0.01	2.0	1.8	0.01
6	1	0	0.02	2.0	1.9	0
7	1	30	1.1	2.1	2.3	0.76
8	1	60	2.26	1.9	2.6	0.97
9	1	120	4.38	1.9	2.9	2.3
10	1	180	6.37	2.0	3.8	4.19
11	10	0	-0.01	2.0	2.1	0.01
12	10	30	3.21	1.9	2.9	1.11
13	10	60	3.88	2.0	4.0	4.55
14	10	120	6.2	2.0	4.5	6.24
15	10	180	6.86	2.1	3.3	8.42
16	100	0	0.03	2.0	2.0	0
17	100	30	3.01	2.0	2.9	1.15
18	100	60	4.58	1.9	4.6	3.54
19	100	120	5.32	2.0	5.2	8.33
20	100	180	5.86	2.0	2.7	12.63

Legend:	MICtime	=	Times of MIC
	Time	=	Times of antibiotic exposure
	Diffposcel	=	Different in proportion of fluorescence cells before and after antibiotic treatment
	Negflu	=	Fluorescence from negative control
	Posflu	=	Fluorescence from treated culture
	Diffcelldebris	=	Different in proportion of signals from cell debris before and after antibiotics treatment

Appendix ix

Cost analysis

Introduction

In these days of worldwide fiscal constraints, a detailed cost comparison between the method studied in this project, other automated methods (Vitek 2) and conventional agar dilution methods are essential to fully determine the advantages of a novel method. Real-time cost estimates, including the cost of materials and labor need to be considered before it is possible to determine the cost effectiveness of each method.

The following items are taken into account when performing the cost analysis:

1. Labor costs
2. Plastic ware
3. Culture media

4. Antimicrobial agents

5. Fluorescent probes required

6. Special chemicals (beads)

The following assumptions have been made for the purposes of this calculation.

1. The labor skills required for each method were assumed to be the same. The salary was set at the mid-point for a Medical Laboratory Technician II with at least 5 years of routine laboratory experience. The salary of the technician was set at $49/hour and costs/100tests were calculated as processing time for 100 tests x $49.

2. The laboratory performing the tests was assumed to be a district hospital that would carry out 30 MICs or 30 rapid susceptibility tests per week (1560 test/year). Larger or smaller numbers would affect costs of the various tests differently. This is particularly the case for SAD costs in this analysis where calculations are based on the assumption that tests are performed in one batch on each day.

3. The price of equipment and maintenance costs have not been taken into account.

   For the SAD and SGE, six organisms were assumed to be tested on each occasion rather than more strains since this would more accurately represent a true picture in a medium size hospital where requests for MICs or rapid ASTs are on average 6 strains are per day (30 strains per week).

4 . For ease of calculation, the cost per 100 tests was used for comparison of methods

tested.

5. The cost for growth supplement (lysed horse blood) for *S. pneumoniae* was assumed to be negligible since it is cheap and is not needed in large amounts.
6. Penicillin was the representative of the drug used – its cost was about average for all antibiotic.

The price for 100 tests of using the various AST methods and based on one antibiotic (penicillin G) and one organism (*S. pneumonia*) are shown in the following table:

Table : Comparison of cost of tested methods for 100 tests.

Items	SAD			Vitek 2			SGE			FCM		
	Details	Unit price (HK$)	Price/ 100 tests	Details	Unit price	Price/ 100 tests	Details	Unit price	Price/ 100 tests	Details	Unit price	Price/ 100 tests
Container	Petri disk	1	1200[a]	--	--	--	14cm diameter petri disk	2.8	93.3[b]	plastic bottle (25 mL)	100/500x200	40
Growth media	Cation adjust Mueller Hinton agar	0.738/g	113.6[c]	--	--	--	Cation adjust Muller Hinton agar	0.738/g	47.3[d]	Cation adjust Muller Hinton broth	0.928/g	38.98
Antibiotic powder	Penicillin G	59.6/g	5.96[e]	Susceptibility card	675/20/test	3375	Penicillin G	59.6/g	5.96[e]	Penicillin G	59.6/g	5.96[e]
Probes	--	--	--	--	--	--	--	--	--	Propidium iodide $DiBAC_4(3)$	266/mg 266/mg	32.0 32.0

Items	SAD			Vitek 2			SGE			FCM		
	Details	Unit price	Price /100 tests	Details	Unit price	Price /100 tests	Details	Unit price	Price /100 tests	Details	Unit price	Price /100 tests
Technician time	1. prepare of plate 2. inoculation of organism 3. reading of results	13.3 x 49 1/6x100x49 0.5/6x100x49	653.3[f] 816.6[g] 408.3[h]	1. prepare of susceptibility cards 2. Reading and entry of results	5/60x100x49 5/60x100x49	408.3[i] 408.3[i]	1. prepare of plate 2. inoculation of organism 3. reading of result	100/60/2x2x49 5/60x100/6x49 5/60x100/6x49	81.7[j] 68.1[j] 68.1[j]	1. prepare of log phase organism 2. add antibiotics 4. prepare of sample for FCM 5. run sample	0.25x100x49 5/60x100x49 5/60x100x49 5/60x100x49	1225[k] 408.3[k] 408.3[k] 408.3[k]
Special chemicals	--	--	--	--	--	--	--	--	--	1. valinomycin 2. alignment beads 3. calibration beads	35.7/mg 123/mg 4048/6/100test	35.7[l] 123[m] 674.7

Items	SAD			Vitek 2			SGE			FCM		
	Details	Unit price	Price/ 100 tests	Details	Unit price	Price/ 100 tests	Details	Unit price	Price/100 tests	Details	Unit price	Price/100 tests
Acessories										1. Syringe 2. syringe filter	256/50 13.84	512 1384
Total price	3197.8			4191			364.5			5296		

a. 12 plates were required for each test; number of plates required was 12 x 100/6.

b. 2 plates were required for 6 tests, number of plates required for 100 tests were 2 x 100/6.

c. Growth medium volume required for 100 tests was 200x20/1000.x38.5x0.738 (38.5 gm were required for 1000mL)

d. Growth medium volume required for 100 tests was 100/6x2x50/1000x38.5x0.738 (50mL was required for a plate).

e. 1 mL volume of aliquot with a 1 mg/mL concentration was used for every test. Any remaining solution was discarded.

f. Agar preparation, antibiotic dilution, pouring plate was included. Plates with antibiotics were kept for one week before discarded.
4 hours was assumed to be required for each batch. On each batch, plates required for one week (30 tests) were produced. Total time : 4 x 100/30.

g. 1 hour /6 tests was required for preparation of bacteria suspension, diluted to appropriate density and inoculation of plates.

h. 0.5 hour /6 tests was required for reading and entry of results.

i. 5 minutes were assumed to be required for preparation of cards and another 5 minutes for taking and entry of results.

j. 2 hours were assumed to be used for preparation of plates, 5 mins for inoculation of 6 strains, and 5 mins for reading of 6 results. 60 plates were assumed to prepared on each batch. Time for preparation of plates: 100tests/tests performed on each batch of plates x time required for each batch of preparation.

k. 0.25 hour was required for preparation of bacterial suspension, another 0.25 hour for diluting and adding antibiotics and 5/60 hour preparing the suspension (staining) for submitting to the FCM and 5/60 hour for running the sample.

l. 1 mL of 1mg/mL stock solution was used for control of each strain.

m. 1 mL of 1mg/mL suspension was used as alignment check of each test.

E test costs

100 strips will cost $1200

Labor (assume plates already made) 20 mins/test (incl. reading result) equal :

20/60x100x49 = $1633

Plates required = 100

Price of media = 0.738x20/1000x100x38.5 = $56.8

Manpower for making the plates = 3 hours x $49 =147

Total $3037/100 tests

Comment

The highest cost was found to be the FCM method. This was due to the high price of the accessories for the tests. Cost would be further increased when the price of the machine and maintenance costs are taken into account. However, it may still be justified to use this method, since it may produce a rapid result, leading to improved patient care, shorter hospital stay, and use of cheaper drugs. Each additional day spent in a general hospital ward or ICU is very expensive. Use of FCM-AST may lead to an increase in laboratory budgets, but a large savings in hospital running costs. In fact, if a hospital stay is 1 day shorter, the amount of money saved would be $13,900, which is more than the price for 100 tests by the FCM. For the present studies, the machine was a general purpose type. It can also be used for other laboratory tests such as the immunology tests, and when taken this into account the high price for FCM-AST may be justified. If specialized machines for FCM-AST were developed in the future, the cost of the test would be cheaper.

Vitek 2 had a second high cost for susceptibility testing of *S. pneumoniae.* It is a precise method. However, it can only be used for *S. pneumoniae* and not other

fastidious organisms. Moreover, the panels are limited to preset drugs that may be suitable only for sensitivity pattern in the USA. Variations in drugs panels may be required in different geographical locations. This would be expected to have a very high price. Thus, it may not be possible to justify purchase of such a machine at high cost when it can only be used for 1 type of fastidious organism and does not allow flexibility in choice of drugs to be used without additional cost.

The SGE method cost few hundred HK$/100tests and was the cheapest method. Due to the low complexity of the SGE plater, the working life of the machine may be increased, this together with the low maintenance cost may result in further reductions in cost for this method. This means that SGE method still has a high potential to be used as a routine method in the microbiology laboratory for other fastidious organisms after it has been more intensively studied .

In a developed country such as Hong Kong, the E test may be the preferred method since it is easier to be performed. However it requires overnight incubation, so when a rapid AST is required, the flow cytometry method seems to be better. E test has a high price and the storage problem of the strips is also a disadvantage. For SGE test only antibiotics powder, which can be stored longer and easy to be kept, is required. Test can be performed with short notice. In rural areas of the world like poor villages in

mainland China, the transport of the E test strips is a big problem since the strips are very heat sensitive and must be kept at low temperature at all times. The strips cannot be delivered without deterioration since a long time of transportation is required. SGE test may be preferred in such settings.

In most emergency cases, only 1 MIC may be needed. In this situation, the standard dilution test becomes expensive. If Vitek 2 is used, there will be some waste of materials since there are many antibiotics included in one Vitek 2 AST card. At this time, SGE test may also be the most useful as only some simple procedures will be required. When the rapid AST results are required, FCM-AST should be used. And it can be used at least for *S. pneumoniae* and *H. influenzae*. When further evaluations have proven the FCM-AST to be precise enough, specially designed equipment may be produced and the cost for the FCM test will then be expected to be lowered

SAD is tedious and technically demanding and is prone to errors. A more senior technician may be required to perform the test as that of FCM-AST. This may make the price of the test higher. However due to conventional reasons, it is still the gold standard for other susceptibility tests. Nevertheless, SAD or SBD are rarely performed in the routine laboratory.

In larger hospitals, the number of strains that require MICs or rapid susceptibility categories test results may be greater. The price of the Vitek 2, SGE and FCM-AST would be lower when a large amount of strains were tested in a same batch in large-scale hospitals.