KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

SCHOOL OF GRADUATE STUDIES

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DEPARTMENT OF CLINICAL MICROBIOLOGY

COMPARISON OF A STANDARDIZED ANTIMICROBIAL SUSCEPTIBILITY DISC DIFFUSION METHOD WITH KOMFO ANOKYE TEACHING HOSPITAL'S LOCALLY ADAPTED METHOD.

BY ERNEST ROBERT KYEREMEH

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A THESIS SUBMITTED TO THE DEPARTMENT OF CLINICAL MICROBIOLOGY IN PARTIAL FULFILMENT OF THE REQUIEMENT FOR THE AWARD OF THE MASTER OF SCIENCE DEGREE MSc. IN CLINICAL MICROBIOLOGY

BY

ERNEST ROBERT KYEREMEH

APRIL, 2010

DECLARATION

I hereby declare that this submission is my own work towards the Msc. Clinical Microbiology and that, to the best of my knowledge; it contains no material previously published by another person or material which has been accepted for the award of any other Degree o the University, except where due acknowledgement has been made in the text.

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HEAD OF DEPARTMENT

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ABBREVIATIONS

AMK	……...……	Amikacin
AMP	……...……	Ampicillin
AST	……...……	Antimicrobial susceptibility testing
ATCC	……...……	American type culture collection
BSAC	……...……	British society for antimicrobial chemotherapy
CASFM	……...……	Comité de l'antibiogramme de la société française de microbiologie
CAZ	……...……	Ceftazidime
CFU	……...……	Colony forming units
CHL	……...……	Chloramphenicol
CIP	……...	Ciprofloxacin

CLED	……..	Cysteine lactose electrolyte-deficient agar
CLSI	……..……	Clinical and laboratory standards institute
CRO	……..……	Ceftriaxone
CRG	……..……	Commissie richtlijnen gevoeligheidsbepalingen
CRX	……..……	Cefuroxime
CSF	……..……	Cerebrospinal fluid
CTX	……..……	Cefotaxime
DIN	……..……	Deutsches institute für normung
ERY	……..……	Erythromycin
ESBLs	……..……	Extended spectrum beta-lactamases
E-TEST	……..……	Epsilometer test
EUCAST	……..……	European committee on antimicrobial susceptibility testing
GEN	……..……	Gentamicin
GISA	……..……	Glycopeptides-intermediate *Staphylococcus aureus*
GNR	……..……	Gram-negative rods
GPC	……..……	Gram-positive cocci
HACEK	……..……	*Haemophilus sp, Aggregatibacter actinomycetemcomitants, Cardiobacterium hominis, Eikenella corrodens, Kingella sp.*
I	……..……	Intermediate
ISA	……..……	Iso-Sensitest agar
JSC	……..……	Japanese society for chemotherapy
KATH	……..……	Komfo Anokye teaching hospital
KIA	……..……	Kligler’s iron agar
L	……..……	Liter(s)

LEV	……..……	Levofloxacin
LRPs	……..……	Luciferase reporter mycobacteriophages
MBC	……..……	Minimum bactericidal concentration
MGIT	……..……	Mycobacteria growth indicator tube
MIC	……..……	Minimum inhibitory concentration
mg	……..……	Milligram(s)
mL	……..……	Milliliter(s)
mm	……..……	Millimeter(s)
MRSA	……..……	Methicillin-resistant *Staphylococcus aureus*
MTBC	……..……	*Mycobacterium tuberculosis* complex
NAL	……..……	Nalidixic acid
NCCLS	……..……	National committee for clinical laboratory standards
NCTC	……..……	National collection of type cultures
NIT	……..……	Nitrofurantoin
Nm	……..……	Nanometer(s)
ºC	……..……	degree celsius
OIE	……..……	World health organization for animal health
OXA	……..……	Oxacillin
PBPs	……..……	Penicillin binding proteins
pH	……..……	Hydrogen ion concentration
R	……..……	Resistant
®	……..……	Registered
S	……..……	Sensitive/Susceptible
SIR	……..……	Swedish reference group for antibiotics

TET	……..……	Tetracycline
µg	……..……	Microgram(s)
v	……..……	Volume
VISA	……..……	Vancomycin-intermediate *Staphylococcus aureus*
VRE	……..……	Vancomycin- resistant enterococci
WBCs	……..……	White blood cells
WHO	……..……	World health organization

ABSTRACT

The clinical microbiology laboratory of the Komfo Anokye Teaching Hospital employs a locally adapted method for antimicrobial susceptibility testing. This method is a variant of the British Society for Antimicrobial Chemotherapy (BSAC) standardized disc diffusion method. Antimicrobial susceptibility testing of bacteria isolated at the Komfo Anokye Teaching Hospital (KATH) was performed using the BSAC method. A total of 200 bacterial isolates were cultured from nine different types of specimens. The sites of origin were urine 33%, blood 32%, sputum, pus, wound, ear and aspirated specimens 31.5%, urethral smear 1.5% and cerebrospinal fluid 1.0%. Gram-negative bacteria accounted for 183 (91.5%) isolates; the main species were *Escherichia coli* (34.5%), *Klebsiella pneumoniae* (18.5%), *Pseudomonas aeruginosa* (9.0%), *Proteus mirabilis* (3.5%), *Salmonella typhi* (7.0%) and coliforms (19.0%). Gram-positive bacteria contributed 17 (8.5%) of isolates with *Staphylococcus aureus* 6.5% being the most predominant followed by *Streptococcus pneumoniae* 2.0%. *Escherichia coli* showed 100% and 92% resistance to ampicillin and cefuroxime respectively with 36% being susceptible to cefotaxime. Amikacin resistance in Gram-negative bacilli was 18.7%. *Staphylococcus aureus* strains showed 36% resistance to oxacillin. *Streptococcus pneumoniae* isolates showed 100% resistance to oxacillin and also to ceftriaxone. Comparison of susceptibility results of both methods showed 49.1% and 35.8% agreement in susceptible and resistant results respectively. As much as 29.9%, 2.8% and 5.1% of susceptibility results of the locally adapted method were reported as very major, major and minor errors respectively against the BSAC. The study recommends that the locally adapted method be revised in relation to current standard practices.

CHAPTER ONE

1. 0 INTRODUCTION

History and Development of Antimicrobial Susceptibility Testing Methodology

Antimicrobial susceptibility testing is a measure of whether or not a microorganism can grow when it is exposed to a variety of antimicrobials in a laboratory test (Wheat, 2001). It is performed daily on bacterial isolates in clinical laboratories and it is often used to determine the likelihood that a particular drug treatment regimen will be effective in eliminating or inhibiting the growth of the organism.

Antimicrobial susceptibility testing (AST) had long been practised by the pioneers of microbiology such as Pasteur, Koch, Leeuwenhoek and Ehrlich even before the discovery of penicillin in 1929 (Lechevalier *et al,* 1965), but it was not until Alexander Fleming had reported on the inhibitory effect of the mould *Penicillium notatum* on *Staphylococcus aureus* on an agar plate that scientists began pioneering work on chemotherapy (Fleming, 1928). Fleming in addition, made two significant contributions to the field of AST. He introduced the use of the ditch plate technique (Dufrenoy, 1947) and the development of the broth dilution method (Abraham *et al,* 1941). Schmith and Reymann also described an agar dilution method (Schmith *et al,* 1940). Other methods of AST such as the diffusion methods were developed later. The one-quarter inch (6-6.5mm) impregnated antibiotic filter paper discs that are still commonly utilized today were first described by Bondi and co-workers (Bondi *et al* 1947).

The Importance and Purpose of Susceptibility Testing

Over the years there have been rapid pattern changes and an increase in resistance of bacteria to some antibiotics. For example, *Staphylococcus aureus* to methicillin –

methicillin-resistant *Staphylococcus aureus*; MRSA (Brown, 2001), extended spectrum beta-lactamase (ESBL) producing bacteria such as *Escherichia coli, Klebsiella pneumoniae* and *Proteus spp* to aminoglycosides, trimethoprim and the quinolones (Kader *et al*, 2006). This problem has resulted mainly from the introduction of a variety of antimicrobials on to the market, their inappropriate use and their easy availability over the counter to the general public (Lalitha, 2006). Such complacency has contributed to the rise of antibiotic resistance among various common human pathogens, threatening the central purpose for which antibiotics were developed (Gould, 2000). Thus, performing AST as routine is not only evident in providing early warning signs of microbial resistance development, but also helps monitor pattern changes of multi-resistant strains of bacteria. In general, the purpose of such tests is to provide a reliable predictor of how an organism is likely to respond to antimicrobial therapy in an infected host.

Need and Advantages of Standardization of Susceptibility Test

Even in the initial stages, it became obvious that AST was affected by a host of factors among which include the pH of the agar medium, inoculum size, moisture, storage and age of antibiotic discs. Variables affecting AST methods using disc diffusion or dilution methods have been comprehensively studied by many groups (Heatley, 1949; Erlanson, 1951; Waterworth, 1951; Gould and Bowie, 1952; Anderson and Troyanosky, 1960; Ericsson and Sherris, 1971; World Health Organization, 1977).

Standardization minimizes the impact of these variables so that results will actually measure the organism's expression of resistance. The need to standardize AST therefore became very necessary. Several organizations began addressing this critical issue. In 1966, significant progress in standardization occurred when Bauer, Kirby and co-workers

published their attempt to establish the disc diffusion technique as a practical method of testing with broad application to clinical laboratories (Bauer *et al*, 1966).

In the Kirby-Bauer disk diffusion method, standardization involves all variables included in the procedure, such as the type of culture medium, the composition of Mueller-Hinton agar, pH, the thickness of the agar, inoculum suspension standardization against standard McFarland turbidity, age of the organism and the method of inoculation. Standards are also followed for selection, preparation and storage of antibiotic discs as well as conditions of incubation, measurement and interpretation of results. In 1975, this method became the basis of the National Committee for Clinical Laboratory Standards (NCCLS) now the Clinical and Laboratory Standards Institute (CLSI) disc diffusion standards (NCCLS, 1975). After the establishment of the NCCLS, a number of standards and guidelines were also developed world- wide. Amongst them include those of the British Society for Antimicrobial Chemotherapy (BSAC, UK), Comité de l'Antibiogramme de la Société française de Microbiologie (CASFM, France), Swedish Reference Group for Antibiotics (SIR, Sweden), Deutsches Institut für Normung (DIN, Germany), Japanese Society for Chemotherapy (JSC, Japan), Commissie richtlijnen gevoeligheidsbepalingen (CRG, the Netherlands).

It was soon noticeable (Gould, 1961) that, results obtained by the use of a standardized method were more accurate and easily reproducible. Problems were more likely to be recognized and rectified because of the fact that the process of quality assurance is an integral part of such standardized methods. Moreover, data from different sources could be combined and this is beneficial to the surveillance of AST.

Antimicrobial Susceptibility Testing at KATH

At the Komfo Anokye Teaching Hospital (KATH), isolation, identification and antimicrobial susceptibility testing of bacterial isolates from diagnostic specimens are critical functions of the clinical microbiology laboratory. A locally adapted method of antimicrobial susceptibility testing is employed at the KATH's laboratory. This locally adapted method is a variant of the British Society for Antimicrobial Chemotherapy (BSAC) standardized antimicrobial susceptibility disc diffusion method. Certain elements of the BSAC however, have been modified to obtain the method for in-house use at KATH laboratory. Modifications include: inoculation of agar plates by pouring of the inoculum suspension, visual observation of zone sizes and the interpretation of test results into either one of only two categories, sensitive and resistant.

1. 1 AIM

- To compare the Komfo Anokye Teaching Hospital's locally adapted disc diffusion antimicrobial susceptibility test method with the BSAC standardized method.

1. 2 OBJECTIVES

- To determine the antimicrobial susceptibility profile of microorganisms isolated at KATH according to the BSAC reference.
- To compare results obtained to that recorded routinely at the KATH laboratory.
- To make recommendations for any improvements of AST performed at the KATH laboratory.

1. 3 Justification of Study

Periodic audit of all laboratory procedures is important to ascertain if such procedures continue to conform to standards and are meeting their desired goals. The importance of such audit has far reaching benefits to the patient and Ghana in general, for treatment failures can lead to death, increase hospital stay and treatment costs. It is in this view that the study is justified.

CHAPTER TWO

2. 0 LITERATURE REVIEW

2. 1 Principles of Antimicrobial Susceptibility Testing

In 1874, William Roberts observed that liquid medium in which the mould *Penicillium glaucum* was growing could not easily be contaminated with bacteria (Poupard *et al*, 1994). Alexander Fleming in 1928 also reported on his observation of an area of growth inhibition of staphylococcal colonies adjacent to a *Penicillium* contaminant on an agar plate. These observations together with others led to the development of the ditch plate technique which later was to become the forerunner of a variety of agar diffusion methods. The Oxford Group used these methods initially to assay antibiotics contained in blood by allowing the antibiotics to diffuse out of reservoirs in the medium in containers placed on the surface (Reddish, 1929). These demonstrations collectively, did not only prove the scientific basis of antimicrobial susceptibility testing (AST) but also served as a springboard for the development and subsequent improvement of newer methods.

AST essentially is a three stage process: the inoculum is prepared, the test performed and then interpreted. It relies on the observation of antibiotics inhibiting the growth and or killing cultures of microorganisms in vitro (Atlas, 1995).

2. 1. 1 Factors that affect the results of Antimicrobial Susceptibility Tests

Among the many factors include: inoculum size, pH, media composition and depth, delay between application of the disc and incubation, temperature, atmosphere and duration of incubation, disc storage, antibiotic concentration of the disc and the method of reading zone size (Hedges, 1999). These are considered to significantly influence the results of tests.

2. 1. 1. 2 Size of Inoculum

In general, a denser inoculum will result in reduced zones of inhibition and a lighter inoculum will have the opposite effect. Large bacterial populations are less promptly and completely inhibited than small ones. In addition, a resistant mutant is much more likely to emerge in large populations (Brooks *et al*, 2001).

2. 1. 2. 3 pH

If the pH is too low, certain drugs will appear to lose potency (e.g., aminoglycosides, quinolones, and macrolides), while other agents such as tetracyclines may appear to have excessive activity. If the pH is too high, the opposite effects can be expected (Konig *et al*, 1993). The pH of the medium should be between 7.2 and 7.4 at room temperature after gelling (NCCLS, 2002).

2. 1. 2. 4 Media Composition

Media containing excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides and trimethoprim (Koch *et al*, 1971), thus giving smaller and less distinct zones, or even no zone at all, which may result in false-resistance reports (Stokes *et al*, 1978). The addition of other components for example, NaCl to the medium also influences the test by enhancing the detection of methicillin/oxacillin resistance in *Staphylococcus aureus* (Brooks *et al*, 2001).

2. 1. 2. 5 Depth of Agar

Variation in depth will affect the zone sizes – if the agar is too thin, larger zones will appear since the volume is decreased, and the effective antibiotic concentration increased.

If the agar is too thick, smaller zones will appear since the effective antibiotic concentration has been decreased. If the agar is intentionally thin, then small modifications to other factors will have a disproportionate effect (Barry *et al*, 1973). For sensitivity testing the depth of the agar is usually recommended to be 4mm in the centre of the plate (approximately 25ml in a 90mm plate) (Murray *et al*, 1983).

2. 1. 2. 6 Duration of Incubation and Conditions

In many instances, microorganisms are not killed but only inhibited upon short exposure to antimicrobial agents. The longer, incubation continues, the greater the chance for resistant mutants to emerge or for the least susceptible members of the antimicrobial population to begin multiplying as the drug deteriorates (Brooks *et al*, 2001).

The atmospheric conditions under which an organism is incubated will also affect the zone sizes. For example a *Streptococcus pneumoniae* or *Haemophilus influenzae* incubated aerobically will not thrive, and so will appear more sensitive to the antibiotics under test. However, incubating these organisms in CO_2 will cause a drop in the pH which will affect the zones sizes achieved to different degrees (Jorgensen, 2004).

Once the discs have been applied to the agar, the plates need to be incubated as soon as possible. If the plates are left at room temperature after discs have been applied, pre-diffusion may result in erroneously large zones of inhibition (EUCAST, 2009). Plates incubated in large stacks will have a poor transfer of heat to the middle of the stack, thus the antibiotics will diffuse before the test gets to temperature potentially resulting in larger zone sizes (Bridson, 1998).

2. 1. 2. 7 Disc Storage, Antibiotic Concentration of the Disc or Stability of Drug

The inadequate handling and the improper storage of antibiotic discs brings about loss of potency of the antibiotics. This loss of potency results in reduced zones of inhibition.

The antibiotic discs must be therefore stored according to manufacturer's instructions i.e. between -20°C and + 8°C in a sealed, desiccated environment. Cartridges not in use should be stored unopened in their original packaging in order to prevent moisture ingress. This is extremely important since it is well known that moisture is a major cause of antibiotic degradation. Opened cartridges must be refrigerated and sealed in the disc dispenser or other suitable container when not in use.

It should also be noted that knowledge about the stability of the drug is very key in susceptibility testing. For example at incubator temperature, Chlortetracycline is inactivated rapidly whereas aminoglycosides, chloramphenicol, and ciprofloxacin are quite stable for long periods (Brooks *et al*, 2001).

2. 1. 2. 8 Incubation Temperature

Incubation temperatures are normally set at 35 – 37°C and this is optimal for most human pathogens. If the temperature is lower, then the rate of growth will be reduced resulting in an increased time to reach the critical mass. An increased time to reach the critical mass will allow a greater diffusion of the antibiotics and a larger zone size. An increased incubation temperature will also result in smaller zones, since the organisms may be compromised at elevated temperatures and the antibiotics will diffuse easier due to viscosity changes within the agar (Bridson, 1998).

2. 2 Antimicrobial Susceptibility Testing Methods

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. Three basic methods are presently in use to assess the susceptibility of bacteria to antimicrobial drugs in vitro: antibiotic titration or dilution methods, breakpoint susceptibility testing, and disc diffusion methods. In addition, the commercially available 'Etest' provides a hybrid diffusion/dilution method, and automated methods are starting to be applied. Choice of method for routine use is based on the type of organism, required accuracy, technical simplicity, applicability to working practices in the individual laboratory and cost (National Standards Method BSOP 45i2, 2006)

2. 2. 1 Disc Diffusion

The disc diffusion method is the most widely used technique because it is convenient, flexible, and easy to perform (Jones, 1992). It does not require expensive automated equipment and is suitable for testing rapidly growing and certain fastidious bacterial pathogens. It however can only be used with those organisms for which interpretive criteria have been established, and it gives only a qualitative result. The Kirby-Bauer and Stokes methods are the two agar disc diffusion methods commonly used in laboratories.

In the Kirby-Bauer agar disc diffusion method, the surface of an agar plate is inoculated with a standardized inoculum of the test organism. Absorbent paper discs containing antibiotic at appropriate concentrations are applied to the surface of agar plates seeded with the test organisms. The agent diffuses into the medium and produces a concentration gradient with a high concentration close to the disc and a reducing concentration moving

away from the disc. Zones of inhibition are established, after overnight incubation, and are interpreted as categories of susceptibility (Bauer *et al*, 1966).

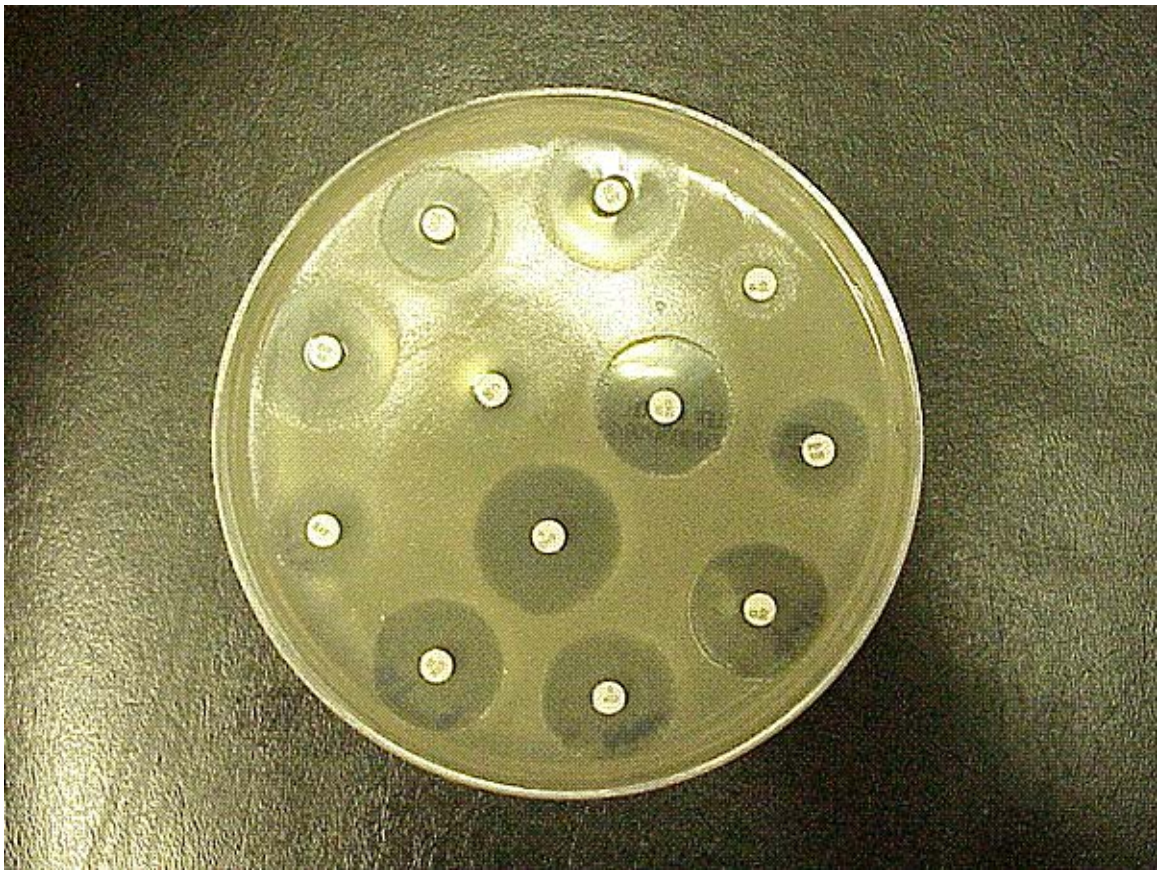
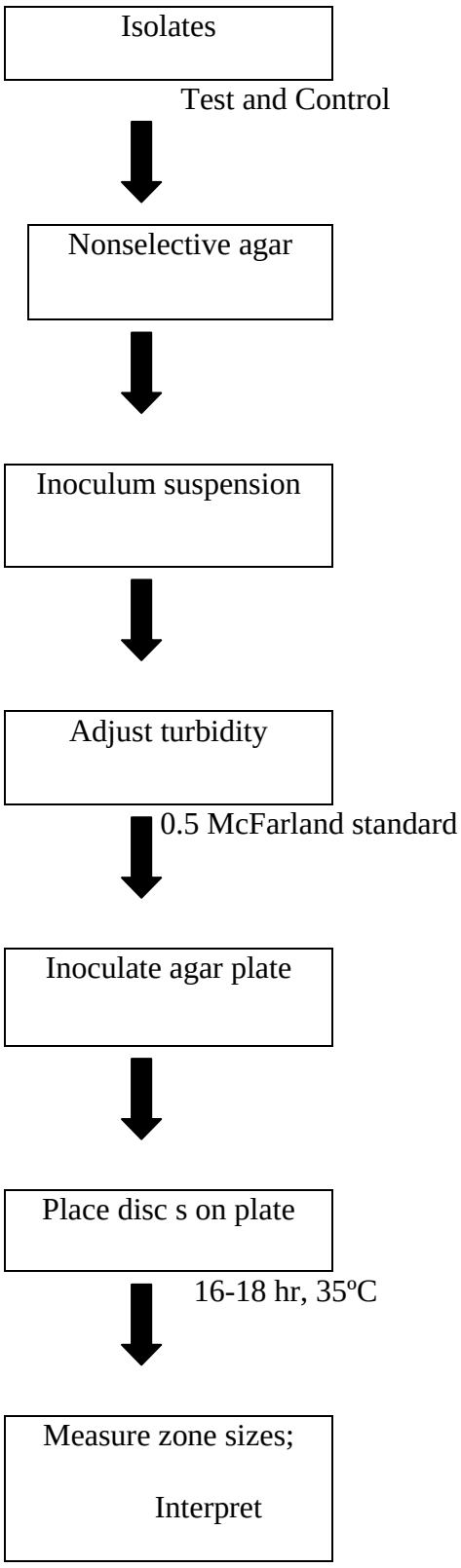


Plate1. Zones of inhibition in Kirby-Bauer disc diffusion test

(Courtesy: James V.B *et al*, 1973. *Antimicrobial Agents and Chemotherapy*)

Figure1. Procedure for disc diffusion antimicrobial susceptibility testing



In the Stokes method, control and test organism are tested in parallel on the same plate with the same disc, so that all conditions of the test are identical. Susceptibility or resistance is judged according to differences in size of the control and test zone diameters (Stokes *et al*, 1993).

2. 2. 1. 2 A move away from Stokes Method

Arguments raised over the years have questioned the usefulness of the Stokes method and have called for a move away from it. Thus, laboratories that employ it have been encouraged to change to more effective methods. These large criticisms have come from the fact that the Stokes method has not been systematically updated for some of the new, highly active antimicrobial agents or particular resistance mechanisms. There are limitations to the effectiveness of control by this method (Brown, 1990). Results are arbitrary and it is not known how they relate to the MIC which in turn could be correlated to concentrations of an antibiotic which can be attained in the blood or other body fluids of patients requiring chemotherapy (Gosden *et al*, 1998). Variations on the original Stokes method are also common and diverse (Wise, 1995).

2. 2. 2 Dilution Methods

In these tests, serial, usually twofold, dilutions of antibiotic are prepared and inoculated with a fixed inoculum of bacteria e.g. 1μg/mL, 2μg/mL, 4μg/mL, 8μg/mL, or 16μg/mL (Woods, 1995). After incubation, the presence or absence of growth is assessed and the minimum inhibitory concentration (MIC) of the agent is defined as the lowest concentration inhibiting visible growth. The test can be carried out in broth or on an agar-containing solid medium. In the broth version, the antibiotic is serially diluted in 1ml

macrodilution) or 0.1ml (microdilution) volumes. Already prepared microtitration trays containing appropriate concentrations of antibiotic facilitate the microdilution test. In the agar dilution method, appropriate concentrations of antibiotic are incorporated in agar plates and bacteria are spot-inoculated on the surface with a multipoint inoculation device, allowing many isolates of bacteria to be tested at one time. Advantages include the ability to test a large number of isolates simultaneously and the fact that results can be reported either quantitatively (MIC) or qualitatively. The minimum concentration of drug required to kill an organism known as the minimum bactericidal concentration (MBC) which comes as a second measure can be used in the management of hospital in-patients (Collier, 1995). The MBC is determined from broth dilution MIC tests (often 0.1ml of inoculum) by subculturing to agar media without antibiotics. The MBC is the first dilution at which no growth is observed. An agent is usually regarded as bactericidal if the MBC is no more than four times the MIC (French, 2006).

2. 2. 3 Breakpoint Methods

This method of susceptibility testing is essentially a truncated version of the agar dilution method, in which bacteria are exposed to a single concentration of each antibiotic corresponding to an agreed 'breakpoint' of susceptibility. If the organism is able to grow at the breakpoint it is regarded as resistant. Sometimes a second, higher breakpoint is included in order to establish a category of 'intermediate susceptibility', implying that therapy might be successful if a higher dosage is used, or if a high concentration of drug is anticipated at the site of infection for example, in the urine in cystitis (Turnidge *et al*, 2007). The chief advantage of the breakpoint method is that many organisms can be tested on a few plates, so that it is very economical. It is suitable for mechanization of

inoculation and reading can be combined with identification tests and the results are usually very clear-cut (Faiers *et al*, 1991). These features make it particularly attractive to laboratories with large workloads. The main disadvantage is that it gives an all-or-none result and provides no information as to whether the true level of susceptibility of the organism lies close to the breakpoint, or whether it is highly susceptible or highly resistant. Moreover, organisms for which the susceptibility happens to fall at the breakpoint may be inconsistently reported as susceptible or resistant because of the vagaries of inherent biological variation (Walker, 2000).

2. 2. 4 E-Test

The E-test (AB BIODISK; Solna, Sweden) also known as the epsilometer test is a commercial product based on the principle of establishment of an antimicrobial gradient in an agar medium as a means of determining the susceptibility (Citron *et al*, 1991).

It is an MIC method and consists of a 60 mm x 5 mm plastic strip with an exponential antimicrobial gradient dried on one side and an MIC scale printed on the other. The gradient of agent covers a concentration range of 0.002 to 32mg/L, 0.016 to 256mg/L or 0.064 to 1024mg/L, depending on the agent. This range corresponds to 15 two-fold dilutions in a conventional MIC method. Tests are set up in a similar way to disc diffusion tests except that the disc is replaced with the Etest strip and inocula are heavier (Baker *et al*, 1991). After overnight incubation, the MIC is read at the point of intersection of the elliptical zone with the strip. The test is very useful in routine laboratories for confirmation of unusual resistances, checking equivocal results and for testing fastidious organisms such as *Haemophilus influenzae, Streptococcus pneumoniae* and slow growing pathogens (Jorgensen *et al*, 1991) and for organisms where a

quantitative result is desirable, such as in cases of endocarditis. It can also be used for detection of extended spectrum beta lactamases (ESBLs).

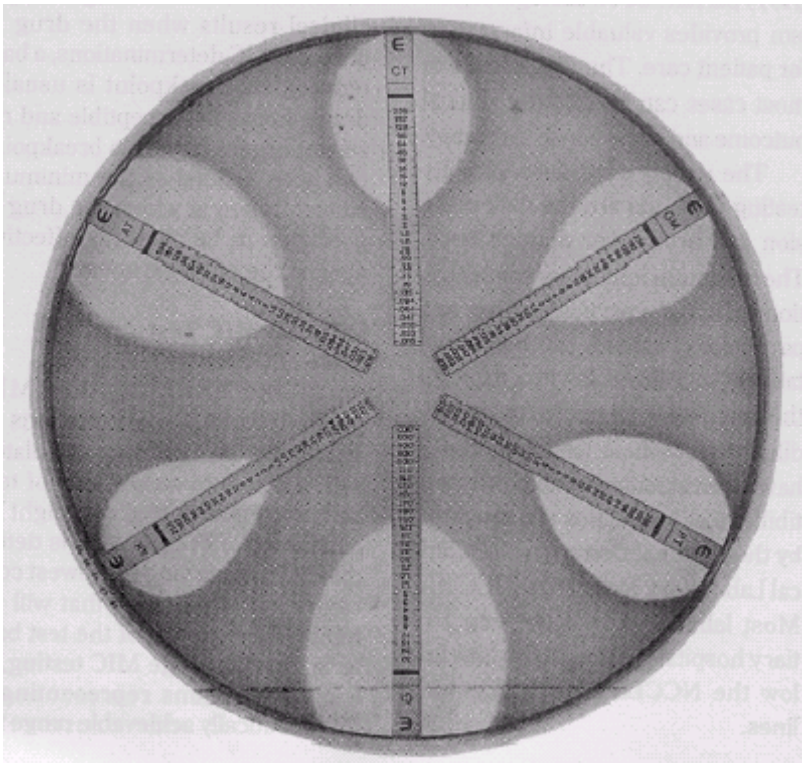


Plate 2.E-test plate AST results of an isolate of *Pseudomonas aeruginosa*

(Courtesy: Myrna T. Mendoza., 1998. *Phil J Microbiol Infect Dis*)

2. 2. 5 Automated Methods

Automated methods for the determination of antibiotic susceptibility are also currently available (Ferraro *et al*, 1999). Examples of such instruments are the MicroScan Walkaway (Dade, Microscan, West Sacramento, CA), Sensititre ARIS and the Vitek system (bioMe´rieux Vitek, Hazelwood, MO) (Felmingham *et al*, 2001). These automated systems have the advantages of standardization, the use of a wide range of agents in each test, availability of rapid results for many tests and some combine

identification with susceptibility tests as well. However, they have the disadvantages of relatively high cost and lack of versatility

2. 2. 6 Molecular Methods

Molecular test methods, including nucleic acid- probing and sequencing techniques allow for the detection of pathogens directly from human specimens. These methods are potentially useful in situations where the organism is fastidious in nature for example the HACEK group of organisms (*Haemophilus sp., Aggregatibacter actinomycetemcomitans, Cardiobacterium hominis, Eikenalla corrodens* and *Kingella sp.*) or slow growing organisms like *Mycobacterium spp. Legionella spp and Borrelia burgdorferi* (Millar *et al,* 2007). Examples of such methods are the detection of the methicillin resistance-encoding mecA gene in staphylococci (Huletsky *et al,* 2004) and a line probe assay for detecting mutations responsible for rifampicin resistance in *Mycobacterium tuberculosis* (XU *et al,* 2005). However, molecular assays for the detection of resistance have a number of limitations. New resistance mechanisms may be missed, and in some cases the number of different genes makes generating an assay too costly to compete with phenotypic assays. In addition, proper quality control for molecular assays poses a problem for many laboratories, and this results in questionable results at best (Fluit *et al*, 2001).

If molecular detection of resistance is to achieve wide acceptance, manufacturers must broaden the repertoires of their technologies, develop more off-the-shelf applications with in-built quality control, and make them suitable for laboratory personnel with no specialist expertise in molecular biology (Woodford *et al*, 2005).

2.2.7 Detection of mechanisms of resistance

Some organisms present problems in susceptibility testing because of their growth requirements or because results are particularly affected by test conditions (King, 2001). Certain of these are fastidious organisms that require enriched media and modified growth conditions for reliable susceptibility testing (Jorgensen *et al*, 2000). It is therefore difficult for clinical laboratories to rely on a single method for susceptibility testing of such fastidious organisms and as such methods as to the detection of resistance are more reliable (Jorgensen *et al*, 2000). Direct detection of resistance mechanisms is generally limited to β-lactamase testing of some fastidious organisms (National Standards Method BSOP 45i2, 2006). Nitrocefin-based tests are reliable for detecting β-lactamases in *H. influenza*, *N. gonorrhoeae* and *M. catarrhalis* (Livermore *et al*, 2001). Chloramphenicol acetyl transferase (CAT) can also be used to detect chloramphenicol resistance in *H. influenza* and *S. pneumoniae* (Swenson *et al*, 1999).

There are however, certain organisms that have difficult-to-detect resistance mechanisms. Difficult-to-detect resistance mechanisms can be found in methicillin/oxacillin resistance in staphylococci (MRSA), diminished vancomycin susceptibility in staphylococci known as vancomycin-Intermediate *S. aureus* (VISA) or glycopeptides-intermediate *S. aureus* (GISA), vancomycin resistance in enterococci (VRE) and also resistance to later-generation penicillins, cephalosporins and aztreonam in some enterobacteriaceae for example *Klebsiella pneumoniae* or *Escherichia coli* known as extended-spectrum β-lactamases (ESBLs) (Jorgensen *et al*, 2000). Methods of detection of such organisms include the testing of colonies of methicillin resistant *S. aureus* for the presence of penicillin-binding protein (PBP2a) by a latex agglutination method (Brown *et al*, 2001), and also by the oxacillin-salt agar screening procedure which appears to be the most

practical and reliable (NCCLS, 2000). The vancomycin screening agar test which is the simplest and most sensitive test for recognition of vancomycin resistance in enterococi (Wiley *et al*, 1992) also seems to offer a very simple and inexpensive way to screen for vancomycin- intermediate *Staphylococcus aureus* (Tenover *et al*, 1998). For the detection of ESBLs, the double disc synergy test is practical for routine use (Sirot, 1996).

2.2.8 Tests for Anaerobes

While antibiotic resistance among anaerobes continues to increase, (Cuchural *et al*, 1990), consensus as to the procedure and interpretation in this area has not been achieved (Wexler, 1991). This is because anaerobic infections are often mixed and detailed bacteriology of the organisms involved may take some time. Also, economic realities and prudent use of resources mandate that careful consideration be given to the necessity of routine susceptibility testing of anaerobic bacteria (Wexler *et al*, 1998). Susceptibility of anaerobic bacteria is by using data from surveillance studies or by performing in vitro susceptibility tests (Olsson-Liljequist *et al*, 1994). The agar dilution method (Wadsworth) is the reference method and is well suited for surveillance studies (NCCLS, 1997). The broth microdilution method is recommended for routine susceptibility testing of anaerobes but is currently limited to testing of *Bacteroides fragilis* group of organisms and selected antibiotics (CLSI, 2007). In view of the limited number of tests set up routinely on anaerobes, for those combinations where disc diffusion cannot be used, the Etest MIC method as described by the manufacturer is recommended (Citron *et al*, 1991).

2.2.9 Tests for Mycobacteria

Mycobacterial susceptibility testing is important for the management of patients with tuberculosis and those with diseases caused by certain nontuberculosis mycobacteria (Woods, 2000). The proportion method detailed by Vestal (Vestal, 1975) which is a modified agar dilution susceptibility test is considered as the standard method of *M. tuberculosis* complex (MTBC) susceptibility testing (Baron *et al*, 1994). There are also rapid detection methods like the radiometric BACTEC 460TB system (Morgan *et al*, 1987), the Mycobacteria Growth Indicator Tube (MGIT) (Rüsch-Gerdes *et al*, 1999) and Luciferase reporter mycobacteriophages (LRPs) for susceptibility testing of *M. tuberculosis* complex (Riska *et al*, 1997).

2. 3 Standardization and Harmonization of Antimicrobial Susceptibility Tests

Environmental factors such as bacterial inoculum size, growth medium, incubation conditions, and antimicrobial concentrations greatly impact susceptibility test results. To minimize the impact of these variables so that test results will more accurately measure the organism's expression of resistance, standardization of these conditions is paramount. Standardization helps in optimizing growth conditions so that test results cannot be attributed to limitations of nutrients, temperature, or atmosphere. It optimizes antimicrobial integrity and activity so that resistance cannot be attributed to environmental drug deactivation and also maintains inter-laboratory reproducibility and consistency of results. Important requirements of this process include control of the bacterial population of the inoculum (comparison with the 0.5 McFarland standard), the use of quality control strains with known and defined susceptibilities, and also about the choice of antimicrobial agents. As a guide to the selection of antimicrobial agents, the

following considerations could be noted: (World Organization for Animal Health (OIE), 2008)

(I) Antimicrobials in the same class may have similar in-vitro activities against select bacterial pathogens. In these cases, a representative antimicrobial should be selected that predicts susceptibility to other members of the same class. It however should be noted that the results are not as precise when agents are tested individually.

(II) Certain microorganisms can be intrinsically resistant to particular antimicrobial classes; therefore it is unnecessary and misleading to test certain agents for activity in vitro. The type of intrinsic resistance has to be determined for these organisms via either the scientific literature or through testing.

(III) The number of antimicrobials to be tested should be limited in order to ensure the relevance and practicality of AST.

(IV) The knowledge of in vitro synergism and antagonism.

In the interest of international standardization of susceptibility testing, there is currently work being undertaken by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) in an attempt to harmonize antimicrobial breakpoints including previously established breakpoints (Kahlmeter *et al,* 2003). The implication of such harmonization is that over time some MIC breakpoints will change slightly and these changes will be reflected, where necessary, in corresponding changes to zone diameter breakpoints of the various participating national committees, a development they are willing to welcome. Such international agreement is important if results achieved with different methods are to be compared and if there is to be an effective monitoring of the emergence and growth of antimicrobial resistance (WHO, 1997).

2. 4 Quality Assurance of Antimicrobial Susceptibility Tests

Quality assurance is the overall process by which the quality of the test results can be guaranteed (Brown, 2000). A major part of this process is the internal quality control; the basis of which is the inclusion of control strains to detect abnormal performance of the test (NCCLS, 2000). Quality control is routinely undertaken to monitor the precision and accuracy of the test procedure, the performance of reagents used in the test and the performance of the persons carrying out the tests. However, there are additional aspects that contribute to quality assurance, including participation in external quality assessment schemes (Snell, 2000), internal quality assessment and the validation process, in which atypical or contradictory results can be detected (Farrington, 2000)

2. 5 The British Society for Antimicrobial Chemotherapy Disc Diffusion Method

The BSAC Working Party on Susceptibility testing has developed a standardized method of disc susceptibility testing. The method employs a semi-defined medium, a semi-confluent inoculum and relates zones of inhibition with BSAC-specified MIC breakpoints to interpret susceptibility. Standards are followed for all procedures including the preparation of medium, selection of control organisms to monitor tests performance, comparison with the 0.5 McFarland standard, inoculation of agar plates, application of discs, incubation, as well as measurement of zones and interpretation of susceptibility (Andrews, 2001).

2. 5. 1 Preparation of Plates

Agar plates are prepared with Iso-Sensitest agar (ISA) or media shown to have the same performance as ISA, according to the manufacturer's instructions. Media is supplemented

for fastidious organisms with 5% defibrinated horse blood or 5% defibrinated horse blood and 20 mg/L β-nicotinamide adenine dinucleotide (NAD). For methicillin/oxacillin susceptibility testing, Mueller-Hinton or Columbia agar with 2% NaCl should be used. Sufficient molten agar is poured into Petri dishes to give a depth 4mm ±0.5mm (25mL in 90mm diameter Petri dishes). The surface of the agar should be dried to remove excess moisture before use. Plates should be stored in vented bags at 8-10°C or 4-8°C in sealed plastic bags prior to use (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

2. 5. 2 Selection of Control Organisms

The performance of the tests should be monitored by the use of appropriate control strains. The control strains include susceptible strains that have been chosen to monitor test performance and resistant strains that can be used to confirm that the method will detect a mechanism of resistance. Examples include *Escherichia coli* ATCC 25922/NCTC 12241, *Staphylococcus aureus* ATCC 25923/NCTC 12981, *Pseudomonas aeruginosa* ATCC 27853/NCTC 12934 which are susceptible or *Neisseria gonorrhoeae* ATCC 49226/NCTC12700 that has a low-level resistance to penicillin (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

2. 5. 3 Preparation of Inoculum

The inoculum should give semi-confluent growth of colonies after overnight incubation. Use of an inoculum that yields semi-confluent growth has the advantage that an incorrect inoculum can easily be observed. A denser inoculum will result in reduced zones of inhibition and a lighter inoculum will have the opposite effect (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

2. 5. 3. 1 Growth Method

For non-fastidious organisms for example Enterobacteriaceae, *Pseudomonas spp* and staphylococci, the growth method is used where at least four morphologically similar colonies are touched with a sterile loop and growth transferred into Iso-Sensitest broth or an equivalent. The broth is incubated with shaking at 35-37°C until the visible turbidity is equal to or greater than that of a 0.5 McFarland standard (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

2. 5. 3. 2 Direct Colony Suspension Method

In the direct colony suspension method, colonies are taken directly from the plate into Iso-Sensitest broth (or equivalent) or sterile distilled water. The density of the suspension should match or exceed that of a 0.5 McFarland standard. This is the method of choice for fastidious organisms (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

2. 5. 4 Comparison with the 0.5 McFarland Standard

The bacterial suspension should be compared to the 0.5McFarland standard. This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn. If the bacterial suspension does not appear to be the same density as the 0.5 McFarland, the turbidity can be reduced by adding sterile saline or broth or increased by adding more bacterial growth.

Commercial standards can be purchased for use or could be prepared by adding 0.5mL of 0.048 M $BaCl_2$ to 99.5mL of 0.18 M H_2SO_4 with constant stirring. The absorbance is then measured in a spectrophotometer at a wavelength of 625nm with an acceptable range of 0.08-0.13 and then distributed into screw-cap tubes and sealed. The turbidity

standard should be agitated on a vortex mixer immediately prior to use (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

2. 5. 5 Inoculation of Agar Plate

The adjusted suspension should be used within 15 minutes to inoculate plates by dipping a sterile cotton-wool swab into the suspension and removing excess liquid by turning the swab against the side of the container. The inoculum is spread over the entire surface of the plate by swabbing in three directions. Plates are allowed to dry before applying discs. It should however be noted that, if the plates are left at room temperature after discs have been applied, pre-diffusion may result in erroneously large zones of inhibition (EUCAST, 2009). Discs should therefore be applied to the surface of the agar within 15 minutes of inoculation (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

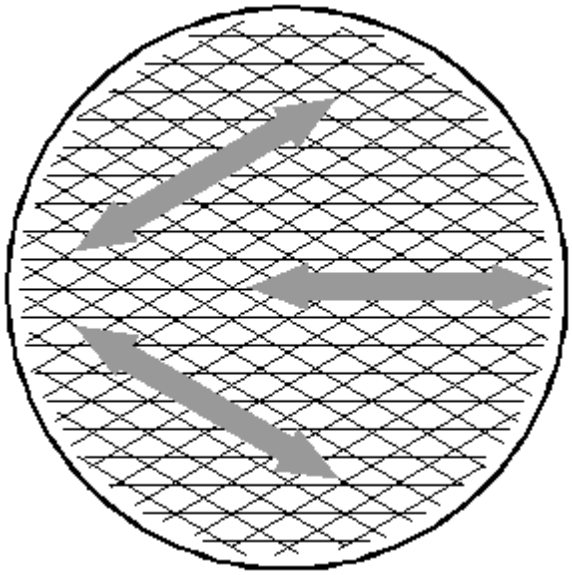


Plate 3. The agar plate should be swabbed over the entire surface in three directions

(Courtesy: Microbiology Techniques Manual, 2002)

2. 5. 6 Application of Discs

Storage and handling of discs should be proper so that there will be no loss of potency as a result. Discs should be firmly applied to the dry surface of the inoculated susceptibility plate. The contact with the agar should be even. A 90 mm plate will accommodate six discs without unacceptable overlapping of zones (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

2. 5. 7 Incubation

If plates are left for extended times at room temperature after discs are applied, the antibiotics will diffuse out before the organism starts to grow and will result in larger zones of inhibition compared with zones produced when plates are incubated immediately (Andrews, 2004). Plates should therefore be incubated within 15 minutes of disc application. For most organisms incubation conditions of 35-37°C in air for 18-20 hours is required. It is essential that plates are not stacked too high in the incubator as this may affect results owing to uneven heating of plates. It has been shown that a single plate on a metal shelf will take 1 hour to warm to within 1°C of the incubator temperature; however plates stacked five deep will take up to four hours for the centre plate to reach the same temperature (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

2. 5. 8 Measuring Zones and Interpretation of Susceptibility

The diameters of zones of inhibition are measured to the nearest millimeter with a ruler, calipers or an automated zone reader. The zone edge is taken as the point of inhibition as judged by the naked eye. Zone diameters are then subsequently categorized as sensitive /susceptible (S), intermediate (I), or resistant (R) in accordance with the interpretative

criteria. A template may also be used for interpreting zone diameters. The test plate is placed over the template and the zones of inhibition are examined in relationship to the template zones. If the zone of inhibition of the strain is within the area marked with an ‘R’, the organism is resistant. If the zone of inhibition is equal to or larger than the marked area, the organism is susceptible (BSAC Methods for Antimicrobial Susceptibility Testing, 2008).

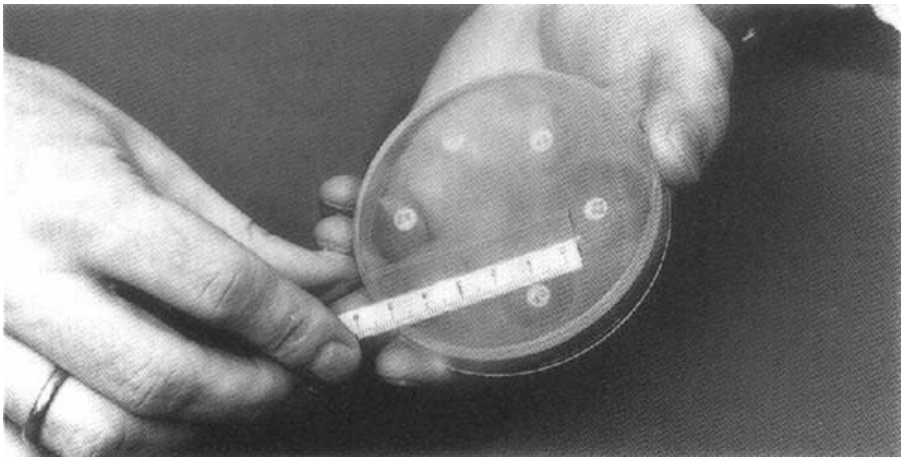


Figure 2. Measuring zones of inhibition on a disc diffusion susceptibility test plate.

(Courtesy: Cheesbrough M., 2000. District Laboratory Practice in Tropical Countries)

Table 1: BSAC MIC and zone breakpoints for Enterobacteriaceae (including *Salmonella* and *Shigella*)

	MIC breakpoint (mg/L)			Disc	Zone diameters (mm)		
Antibiotic	R>	I	S≤	Content(μg)	R≤	I	S≥
Amikacin	16	16	8	30	15	16-18	19
Ampicillin	16	16	8	10	11	12-14	15
Ceftazidime	8	2-8	1	30	17	18-29	30
Cefuroxime	1	-	1	30	24	-	25
Ciprofloxacin	1	1	0.5	1	16	17-19	20
Gentamicin	4	4	2	10	16	17-19	20
Meropenem	8	4-8	2	10	19	20-26	27

(Courtesy: BSAC Methods for Antimicrobial Susceptibility Testing, version 7.1, 2008)

2. 6 Towards a "gold standard"

If results achieved with different methods are to be compared, then comparability of results has to be shown and consensus on interpretation achieved. This was suggested as long ago as 1971 but has never been achieved (WHO, 1997). The alternative (and the ideal) is for all laboratories and networks to use the same methods and breakpoints for surveillance. Proposals in favour of the use of such a common method have in addition questioned the basis upon which the development of new standards are or should be created.

The NCCLS is commonly criticized on three fronts, namely the setting of breakpoints, its use of Mueller– Hinton medium, and, finally, its use of a light-confluent inoculum. However, breakpoints which are generally higher do not cause any significant clinical problems by reporting of false susceptibility. Results obtained by the use of a light-confluent inoculum are assumed to be satisfactory since zone sizes of control organisms are within the expected range. The use of Mueller-Hinton medium is also simple for testing most organisms. The medium is low in inhibitors and cation-adjusted Mueller-Hinton is now available. Early worries about batch variability of Mueller-Hinton no longer seem to be a problem (Gould, 2000).

While recognizing that no system of susceptibility testing is perfect, on the basis that the criticisms of the NCCLS can no longer be a concern, it certainly deserves its place as the world-wide reference method. It is not surprising therefore to see most laboratories in Europe, the North and South America, Japan and Australia use the method routinely.

2. 7 Employed Standard at the Komfo Anokye Teaching Hospital Laboratory

The BSAC standard has been employed with certain modifications for use at the KATH laboratory for over 20 years. Some elements of the BSAC method have been modified to obtain the in-house method used at KATH. These modifications include pouring of the inoculum suspension on agar plates, the visual observation of zone sizes, the pouring of molten agar into Petri dishes without considering the depth required, the visual observation of inoculum turbidity and reporting of test results as either sensitive or resistant.

The BSAC standard was chosen and modified for use at KATH because of the fact that operating procedures of the BSAC method can easily be carried out at the KATH

laboratory. Furthermore, no major problems were encountered with the isolates studied; *Escherichia* coli, *Proteus mirabilis, Klebsiella spp and Pseudomonas aeruginosa* which obviously are among the most commonly isolated pathogens at the KATH hospital. It recommends very minimal major and minor error rates in the figures of 1% and 5% respectively, and there is a good deal of similarity between the list of antibiotics described in the BSAC to those used at the KATH laboratory. Though, the BSAC continues to be revised periodically, nothing seems to have been done about the laboratory's adapted method at the Komfo Anokye Teaching Hospital.

2. 7. 1 Use of Mueller-Hinton Media other than Iso-Sensitest agar (ISA)

The recommendation of the use of media that has been shown to have the same performance as Iso-Sensitest agar (ISA) according to the manufacturer's instructions in the BSAC method clears any ambiguity about the sole use of ISA in the BSAC method. This is even more justified when in the BSAC method for detection of methicillin/oxacillin resistance in *S.aureus*, the working party clearly spells out the use of Columbia agar with added 2% NaCl or Mueller-Hinton media. Mueller-Hinton gives better discrimination between susceptible strains and MRSA than Iso-Sensitest agar (Milne *et al*, 1987). In addition, there currently exists no international standards for ISA but Mueller-Hinton media is likely to be produced according to NCCLS M6-A guidelines which tightly controls many of the variable factors in the medium.

2. 7. 2 BSAC in Comparison with NCCLS

The BSAC and NCCLS standards are the commonly used disc diffusion Kirby-Bauer techniques in laboratories. Differences in the two methods can be found in inoculum preparation, breakpoints and other procedures such as MRSA detection.

In the BSAC, draft guidelines for agar disc diffusion are based on data from 250 diverse clinical isolates (BSAC, 1998). The NCCLS (NCCLS, 1998) recommends that at least 500 isolates are tested for each antibiotic and where there are only one or two species involved, then 300 of each should be tested. The NCCLS recommends major error rates of <1.5% and minor error rates of <3%. For BSAC the corresponding figures are 1% and 5%. The NCCLS has generally higher breakpoints and uses a light confluent inoculum

Table 2: Comparison of the BSAC susceptibility testing method with the NCCLS

Points of difference	BSAC	NCCLS
Data	Preliminary	Adjusted every three years
Application	England and Wales	World-wide
Resources	Doubtful	Well documented
Inoculum	Semi-confluent	Light-confluent
Breakpoints	Conservative	High
MRSA detection	Methicillin/Oxacillin	Oxacillin
New resistance mechanisms	ESBL	Tentative recommendations
Major and minor error rates	<1.0% and <5.0%	<1.5% and <3.0%
Media	ISA/One with same performance	Mueller-Hinton

2. 7. 3 Reporting and Interpretation of Antimicrobial Susceptibility Test Results

The terms susceptible/sensitive (S), intermediate (I) and resistant (R) are normally used to report the results of an antimicrobial susceptibility test. They indicate whether a particular antimicrobial agent is likely to be therapeutically effective against a particular organism.

2. 7. 3. 1 Categories of Susceptibility

2. 7. 3. 1. 2 Susceptible

This implies that an infecting organism should be eradicated by therapy with that antibiotic at the dosage normally recommended for that type of infection and species unless otherwise contraindicated.

2. 7. 3. 1. 3 Intermediate

This indicates that a microorganism falls into a range of susceptibility for which the MIC approaches or exceeds the concentration of antibiotic that can ordinarily be achieved and for which clinical response is likely to be less than with a susceptible strain. It may mean that certain variables in the susceptibility test may not have been properly controlled and that the values have fallen into a “buffer zone” separating susceptible from resistant strains.

2. 7. 3. 1. 4 Resistant.

This implies that microorganisms are not inhibited by the concentrations of the antibiotic achieved with the dosages normally used. In such instances therapy is highly likely to fail.

2. 7. 3. 2 Susceptibility Breakpoints

Susceptibility breakpoints are the concentrations of antimicrobial agents or, in the case of disc diffusion tests, zone diameters which distinguish the different categories of susceptibility (susceptible, intermediate and resistant).

2. 8 Acceptable Accuracy and Comparability of Tests Results

A number of criteria have been proposed for defining the acceptable accuracy of susceptibility tests results. Thornsberry and colleagues used the categories very major, major and minor as a means of classification based on the impact of errors in the treatment of a patient (Thornsberry *et al*, 1980). Error rates are achieved by comparing disc diffusion which is widely used to report, with the MIC which is a reference method. Very major error, major error and minor error have been used to describe false-susceptibility or false-resistant or a response involving an intermediate result respectively. A false-susceptible result could lead to a patient being treated with a drug to which an organism is resistant. In contrast, a report of false-resistant could result in the administration of an unnecessarily expensive antibiotic instead of a less costly agent.

Antimicrobial susceptibility tests data, consisting of cumulative and ongoing summary of susceptibility patterns (antibiograms) among clinically important and surveillance microorganisms could be created, recorded and analysed periodically at regular intervals

(NCCLS, 2002). These antibiograms lend information that can be used to raise awareness of resistance problems, support the use of optimal empiric therapy, and identify opportunities to reduce inappropriate antibiotic usage and to ascertain success of such efforts (Fridkin *et al,* 2001). Results obtained also can easily be compared with different surveillance systems. The flow chart following shows the errors in reporting.

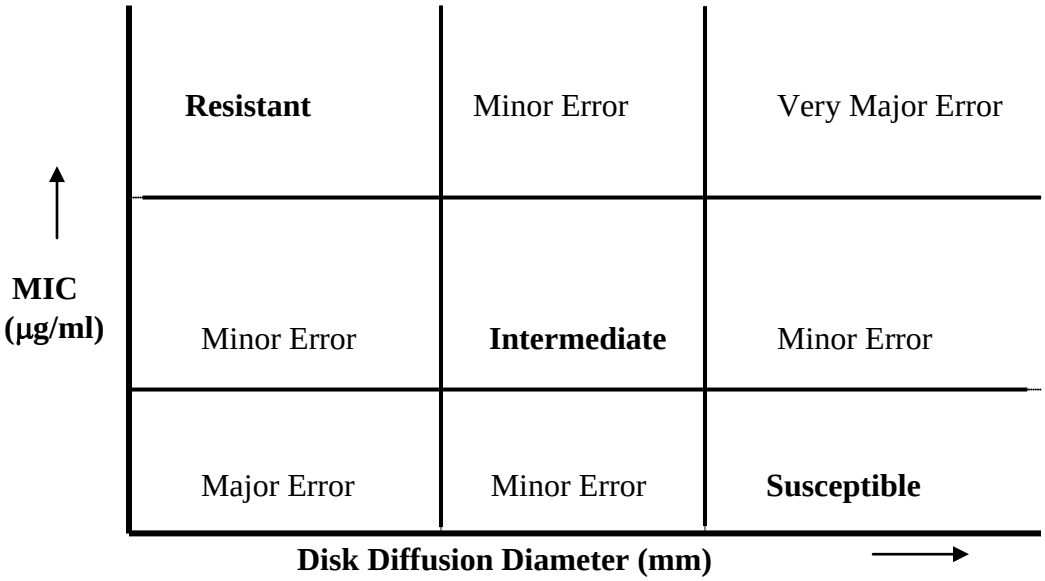


Figure 3. Flow chart showing errors in reporting susceptibility test

2. 9 Limitations of Antimicrobial Susceptibility Tests and the Way Forward

It is a common clinical experience that infected patients sometimes respond to antimicrobial therapy despite the fact that the laboratory has isolated a potential pathogen and found it to be resistant to the drug used. Conversely, patients may fail to respond to treatment with agents shown in laboratory tests to be susceptible. There are many reasons why these anomalous situations might arise: the laboratory might have gotten the answer wrong, the wrong organism (a colonizing or contaminant bacterium) might have been

tested, an inadequate or incorrect specimen might have been submitted in the first place (Gemmell, 1997). Apart from these basic errors other factors such as the microorganism itself, location of the infection in the host (Weinstein *et al*, 1968), for example the ineffectiveness of aminoglycosides (e.g. gentamicin, amikacin) against *Salmonella typhi* because these organisms enter cells that aminoglycosides cannot penetrate (Dawkins *et al*, 1967).

Host immune response and the pharmacology of the drug, e.g. synergy between penicillin and gentamicin for streptococcal infections (Rotschafer *et al*, 1992) all contribute to the reasons why laboratory tests sometimes fail to predict the outcome of treatment.

CHAPTER THREE

3. 0 Materials and Methods

3. 1 Study Site

This study was performed at the Microbiology laboratory of the Komfo Anokye Teaching Hospital (KATH) in Kumasi, a tertiary referral centre.

3. 2 Ethical Clearance

Ethical clearance for this study was obtained from the Committee on Human Research, Publications and Ethics, School of Medical Sciences/KATH, Kumasi.

3. 3 Isolates

All Enterobacteriaceae, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae* isolated within the study period were included in the research. Microorganisms not belonging to these families were not evaluated. A total of 200 non-duplicate isolates were collected, over a period of one month. The organisms were isolated from blood, urine, sputum, wound, ear, pus, aspirates, cerebrospinal fluid, urethral smear and stool from both in-patients and out-patients.

3. 4 Isolation and Identification of Microorganisms

3. 4. 1 Isolation

3. 4. 1. 1 Blood Specimens

Blood was received in the Microbiology laboratory from both in- and out-patients in a 25 ml brain-heart infusion broth. The bottle was then incubated aerobically overnight at a temperature of 35-37°C. After overnight incubation the blood samples were then subcultured on blood and MacConkey agar plates (first subculture). The plates were then incubated overnight under aerobic conditions. On the third day, the first subculture was observed for growth, and any growth identified. The samples that did not record any growth were re-incubated for another 24 hours under the same conditions. Samples that had growth present were subcultured on blood agar, chocolate agar and MacConkey agar. The blood agar and MacConkey agar plates were incubated aerobically and the chocolate agar plates in a carbon dioxide atmosphere. Up to three subcultures were performed similar to the procedure mentioned above if there was no growth from previous subcultures. A total incubation period of 7 days was generally sufficient for routine isolation. Culture samples with no growth observed in them after the 7day period were discarded. A longer incubation period should however be allowed when endocarditis is suspected and even up to 4 weeks if brucellosis is suspected.

3. 4. 1. 2 Urine Specimens

About 20ml of mid-stream-urine was collected into sterile universal bottles from patients. The appearance of the urine sample was described. About 10ml of well mixed urine was aseptically transferred to a labeled conical tube and centrifuged for about 5 minutes. The supernatant was then decanted and the sediment remixed by tapping the bottom of the

tube. A drop of the well mixed sediment was transferred to a slide and covered with cover glass. The wet preparation was then examined microscopically under the X10 and X40 objective for the presence of white blood cells (WBCs), epithelial cells, red cells, casts. A loopful of urine was inoculated onto a quarter plate of CLED agar with a 2mm diameter sterile calibrated wire loop and incubated aerobically at 35-37^{o}C overnight. The plates were then examined the following day and colonies that appear after incubation are counted to indicate the number of bacteria per milliliter. A classical criterion of greater than 10^5 colony forming units of bacteria per milliliter (CFU/ml) of urine constitutes strong evidence of active urinary tract infection (Kass, 1960).

3. 4. 1. 3 Sputum Specimens

Patients were given clean, dry, wide-neck, leak-proof containers to produce sputum by coughing. The appearance of the sputum was described. The sputum was then inoculated on chocolate, blood and MacConkey agar plates. The MacConkey plates were incubated aerobically, whiles the chocolate and blood agar plates were incubated at 35-37°C in a candle extinction jar (provides about 3-5% CO_2 content) overnight and were examined for growth on the second day. Colonies were identified.

3. 4. 1. 4 Pus, Wound, Ear and Aspirated Specimens

Specimens were collected with sterile cotton wool swabs or syringes by a medical doctor or an experienced nurse and brought to the laboratory. The specimens were macroscopically examined, described and inoculated on blood and MacConkey agar and then incubated aerobically overnight at 35-37°C. The cultured plates were examined after

24 hours. Organisms that grew on plates were identified.

3. 4. 1. 5 Cerebrospinal Fluid (CSF)

Specimens were collected aseptically by lumbar puncture by a medical officer and brought immediately to the laboratory. The specimens were examined both micro and macroscopically, described and inoculated on blood and chocolate agar. Both plates were incubated in a carbon dioxide enriched atmosphere at 35-37°C for up to 48 hours. Organisms isolated were identified.

3. 4. 1. 6 Urethral Smear

The area around the urethral opening was cleansed using a swab moistened with sterile physiological saline. Swabs were inoculated on chocolate medium in a carbon dioxide environment at 35-37°C for up to 48 hours and also on blood agar at 35-37°C aerobically overnight. Organisms isolated were identified.

3. 4. 1. 7 Stool

Patients were given a clean, dry, disinfectant-free bedpan or suitable wide-necked container in which to pass specimen directly into container. Wet preparations of the specimens were prepared and then the appearance of the specimen also described. Specimens were inoculated on MacConkey agar (Salmonella-Shigella agar was not available during the study period). Plates were incubated aerobically at 35-37°C overnight. Colonies were identified.

3. 4. 2 Identification

Isolates were identified based on their colonial morphology, Gram stain reactions and biochemical tests.

3. 4. 2. 1 Colonial Morphology

Isolates that appeared as circular, convex colonies with smooth distinct edges on blood agar, pink or yellow colonies on MacConkey or CLED agar respectively were considered to be potential *E. coli* (Cheesbrough, 2000). Isolates that appeared as large yellow mucoid colonies on Cystine-lactose-electrolyte-deficient (CLED), large grey-white colonies on blood agar and or large pink mucoid colonies on MacConkey agar were considered to be potential *Klebsiella spp.* (Cheesbrough, 2000). The appearance of 'swarming' on blood agar was considered to be potential *Proteus spp* (Cheesbrough, 2000). Isolates that appeared as smooth round colonies with a fluorescent greenish colour on MacConkey or flat with a feathered edge and β- heamolysis on blood agar were considered potential *Pseudomonas spp* (Brooks *et al*, 2001). Colonies that appeared as round, smooth, raised and glistening on either blood or MacConkey agar were considered as potential *Staphylococci spp* (Brooks *et al*, 2001). Isolates that appeared as small round colonies, at first dome-shaped and later developing a central plateau with an elevated rim and showed α- haemolysis on blood agar were considered to be potential *S. pneumoniae* (Brooks *et al*, 2001). Isolates that appeared as colourless translucent colonies on MacConkey agar were considered to be potential non lactose fermenters (Baron *et al*, 1994).

3. 4. 2. 2 Gram Stain

Gram staining was used to help identify the isolates by their Gram reaction into either Gram-negative or Gram Positive (Cheesbrough, 2000). It was also used to determine the morphology of the bacteria (e.g. cocci, rods, e.t.c), presence of yeast cells, epithelial cells and also the presence and number of pus cells.

3. 4. 2. 3 Biochemical Tests

The biochemical tests used included the following; indole test, catalase test, coagulase test, urease test, oxidase test, bile solubility test and the use of KIA medium. Bacteria belonging to the family Enterobacteriaceae that could not be identified by these tests were collectively termed as Coliforms.

Table 3. Biochemical reactions of isolated microorganisms.

Gram- negative rods (GNR)

Species	Indole	Urease	Oxidase	KIA Medium			
				Slope	Butt	H_2S	Gas
Escherichia coli	+	-	-	Y	Y	-	+
Klebsiella pneumoniae	-	+	-	Y	Y	-	+
Pseudomonas aeruginosa			+	R	R	-	-
Proteus mirabilis	-	+	-	R	Y	+	+
Salmonella typhi	-	-	-	R	Y	+ weak	-

+ = Positive, **-** = Negative, Y = Yellow (acid reaction), R = Red-pink (alkaline reaction)

Table 3. Biochemical reactions of isolated microorganisms

Gram- positive cocci (GPC)

Species	Catalase	Coagulase	Bile solubility
Staphylococcus aureus	+	+	N/A
Streptococcus pneumoniae	_	N/A	+

N/A = Biochemical test not recommended on isolate.

3. 4. 2. 3. 1 Indole Test

The indole test was done by inoculating the test organism in a bijou bottle containing sterile peptone water. Following overnight incubation, a few drops of Kovac's reagent were added. The appearance of a red surface layer confirmed the presence of *E. coli. E. coli* ATCC 25922 was used as the positive control organism.

3. 4. 2. 3. 2 Catalase Test

A small amount of pure growth was transferred with a sterile loop onto the surface of a clean, dry glass slide. A drop of 3% hydrogen peroxide (H_2O_2) was placed immediately

onto a portion of a colony on the slide. The evolution of bubbles of gas indicates a positive test and suggests the presence of Staphylococci which are catalase positive. *S. aureus* ATCC 25923 was used as the positive control organism.

3. 4. 2. 3. 3 Coagulase Test

A drop of distilled water was placed on each end of a slide. A colony of the test organism was emulsified in each of the drops. A loopful of plasma was added to only one of the suspensions. The clumping of organisms confirmed the presence of *S. aureus*. *S. aureus* ATCC 25923 was used as the positive control organism.

3. 4. 2. 3. 4 Urease Test

The test organism was inoculated in a screw –cap tube containing urea broth. This was then incubated at 35-37ºC overnight. A pink or red colour in the medium is suggestive of the presence of *Proteus spp.* or *Klebsiella spp.* A red slope, yellow butt, the presence of H_2S and the production of gas in Kligler's Iron Agar confirms the presence of *Proteus mirabilis*.

3. 4. 2. 3. 5 Oxidase Test

A piece of filter paper was placed in a clean Petri dish. Two or three drops of oxidase reagent were added. Using a slide, a colony of the test organism was picked and smeared on the filter paper. The development of a blue-purple colour within a few seconds which

indicates a positive oxidase tests confirms the presence of *Pseudomonas aeruginosa*. *P. aeruginosa* ATCC 27853 was used as the positive control organism.

3. 4. 2. 3. 6 Bile Solubility Test

Several colonies of the test organism were emulsified in a tube containing 2ml sterile physiological saline to give a turbid suspension. The organism suspension was then divided between two tubes. 2 drops of sodium deoxycholate reagent was added to one tube and mixed. 2 drops of sterile distilled water was added to the other tube. The tubes were incubated at 35-37ºC for 10-15 minutes. The clearing of turbidity suggested the presence of *S. pneumoniae*.

3. 4. 2. 3. 7 Kligler's Iron Agar (KIA)

The test organism was inoculated into KIA medium in a screw-cap test tube and incubated at 35-37ºC overnight. The appearance of a pink-red (alkaline) slope and a yellow (acid) butt and also a small amount of blackening in the medium indicated the presence of *Salmonella typhi.*

3. 5 Storage of Isolates

All isolates were stored in tubes containing 1.5 ml Brain-Heart Infusion broth with 20% v/v glycerol at -70°C until further analysis was performed.

3. 6 Subculturing

Stored frozen isolates were thawed at room temperature and subcultured on MacConkey, CLED or nutrient agar to obtain pure growth.

3. 7 Antimicrobial Susceptibility Tests (AST)

AST according to KATH procedure had been done by staff at the laboratory and results already recorded. Results were only compared with that from the BSAC.

3. 7. 1 Standard Operating Procedures of AST at KATH

- Thirty-eight grams of Mueller-Hinton agar is suspended in 1 litre of distilled water.
- Suspension is brought to boil to dissolve the medium completely.
- Medium is sterilized by autoclaving at 121°C for 15minutes.
- Sufficient molten agar is poured into sterile Petri dishes.
- The surface of the agar is dried by flaming after it has been allowed to cool down for some time.
- Plates are then stored in a refrigerator. Plates are dried prior to use.
- At least four discrete colonies of organism to be tested are touched with a sterile loop and growth transferred into a bijou bottle containing peptone water.
- Inoculum suspension is shaken and then poured onto agar plates so that it covers

the entire surface of the plates.

- Plates are allowed to dry after excess liquid is poured off them.
- Antibiotic discs are applied onto plates within 15 minutes.
- Plates are then incubated within 15 minutes of disc application at 35-37°C for 18-20 hours.
- Reading of plates is done by visually observing zones of inhibition. Any clear zone of inhibition is interpreted as sensitive while a no- zone of inhibition is interpreted as resistant. Results are then recorded in the laboratory record books.

3. 7. 2 BSAC Standardized Disc Diffusion Method (BSAC, Version 7.1)

3. 7. 2. 1 Quality Control

3. 7. 2. 2 Technique

Mueller-Hinton medium was prepared according to the manufacturer's instructions. The concentration of the inoculum suspension of control strains was adjusted to the 0.5McFarland standard.

Antimicrobial discs placed on plates had the correct disc content as those used at KATH and had not passed their expiration dates. Plates were incubated within 15 minutes of disc application at 35-37ºC.

Zone sizes were measured with a ruler to the nearest millimeter and were checked to ensure they were within the limits published by BSAC.

Control organisms used were *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *P.*

aeruginosa ATCC 27853. Daily testing was done for 20 consecutive days after which the frequency was decreased to once weekly.

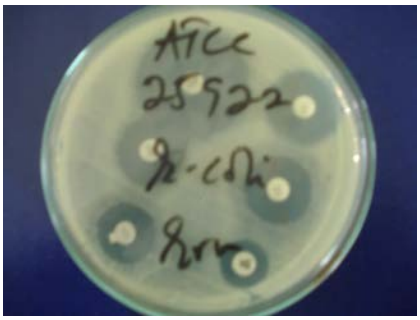


Plate 4. ATCC 25922 (*E. coli*)

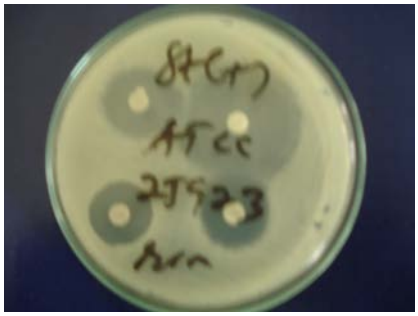


Plate 5. ATCC 25923 (*S. aureus)*

Plate 6. ATCC 27853 (*P. aeruginosa)*

3. 7. 2. 3 Preparation of Mueller-Hinton agar Plates

Mueller-Hinton agar (Oxoid Ltd, England) was prepared according to the manufacturer's instructions. Plates were prepared by pouring sufficient amount of molten agar into sterile Petri dishes to a depth of 4±0.5mm. The molten media was then allowed to solidify. The surface of the agar was dried to remove excess moisture before use. Plates that were not used immediately were stored at 4-8°C.

3. 7. 2. 4 Inoculum Preparation

At least four morphologically similar colonies were touched with a sterile bacteriological loop. The growth was then transferred into a bijou bottle containing distilled water. The bottle was then shaken to uniformly mix the inoculum. The inoculum was compared and made up to a prepared 0.5 McFarland standard. The suspension was used within 15 minutes of preparation.

3. 7. 2. 5 Plate Inoculation

A sterile cotton-wool swab was dipped in the suspension and the excess liquid removed by turning the swab stick against the side of the bottle. The inoculum was spread evenly on the Mueller-Hinton agar plate by swabbing in three directions. The plates were allowed to dry before applying discs.

3. 7. 2. 6 Disc Application

Antibiotic discs were placed individually on the agar plate within 15 minutes of inoculation using sterile forceps. The antibiotic agents used for Enterobacteriaceae including *Salmonella* and *Shigella* were as follows: Amikacin (30µg), Ampicillin (10µg), Ampicillin (25µg only in urine), Cefotaxime (30µg), Ceftazidime (30µg), Cefuroxime (30µg), Gentamicin (10µg), Nalidixic acid (30 µg) and Nitrofurantion (200 µg). Agents used for *Pseudomonas aeruginosa* were: Amikacin (30µg), Cefotaxime (30µg), Ciprofloxacin (5µg), Levofloxacin (5µg) and Gentamicin (10µg). Antimicrobial agents used for *Staphylococcus aureus* were Erythromycin (5µg), Gentamicin (10µg), Oxacillin (1µg) and Tetracycline (10µg). Antimicrobial agents used for *Streptococcus pneumoniae*

were as follows: Ceftriaxone (30µg), Chloramphenicol (10µg), Oxacillin (1µg).

Antibiotic discs used in this study had the same disc content as those used at the KATH laboratory.

3. 7. 2. 7 Incubation

Plates were incubated within 15 minutes of disc application at 35-37°C for 18-20 hours. *S. pneumoniae* plates were incubated under the same conditions but in an airtight jar with a lighted candle. For the detection of oxacillin resistance in *S. aureus*, plates were incubated for 24 hours.

3. 7. 2. 8 Measuring Zone diameters and Interpretation of Susceptibility

Sizes of the zones of inhibition of all discs were measured to the nearest millimeter with a ruler and recorded. Using the interpretative chart of the BSAC (BSAC, version 7.1, 2008), the zone sizes were categorized as sensitive/susceptible (S), intermediate (I) or resistant (R).

Rules were applied in the interpretation of results in the BSAC method. For example, *Salmonella spp*. was reported as resistant to amikacin, cefuroxime and gentamicin irrespective of susceptibility testing results, as they are inactive in-vivo (BSAC, version 7.1, 2008) and the interpretative standard of ampicillin applied only to *E. coli* and *Proteus mirabilis* (BSAC, version 7.1, 2008).

Table 4. The interpretative chart for the zone of inhibition of the antibiotics used

		Interpretation of zone diameters (mm)		
Antibiotic	Disc content (µg)	R≤	I	S≥
AMK	30	15	16-18	19
AMP[a]	10	11	12-14	15
AMP[b]	25	11	-	12
CTX[1]	30	29	-	30
CTX[2]	30	26	-	27
CAZ	30	17	18-29	30
CRO	30	23	24-27	28
CRX	30	24	-	25
CHL	10	17	-	18
CIP	5	19	20-29	30
ERY	5	19	-	20
GEN[1]	10	16	17-19	20
GEN[2]	10	17	-	18
GEN[3]	10	19	-	20

The interpretative chart for the zone of inhibition of the antibiotics used

		Interpretation of zone diameters (mm)		
Antibiotic	Disc content (μg)	R≤	I	S≥
LEV	5	16	17-21	22
NAL	30	17	-	18
NIT	200	19	-	20
OXA[4]	1	19	-	20
OXA[3]	1	14	-	15
TET	10	19	-	20

[a]= Applies to blood, stool and miscellaneous isolates [b]= Applies only to urine isolates [1]=Interpretation for Enterobacteriaceae including *Salmonella typhi* [2]= Interpretation for *P. aeruginosa* [3]= Interpretation for *S. aureus* [4]= Interpretation for *S. pneumoniae*.

AMK=Amikacin, AMP=Ampicillin, CTX=Cefotaxime, CAZ=Ceftazidime, CRO=Ceftriaxone, CRX= Cefuroxime, CHL=Chloramphenicol, CIP=Ciprofloxacin, ERY=Erythromycin, GEN=Gentamicin, LEV=Levofloxacin, NAL=Nalidixic acid, NIT= Nitrofurantoin, OXA= Oxacillin, TET=Tetracycline.

(Courtesy: BSAC Methods for Antimicrobial Susceptibility Testing, version 7.1, 2008)

3. 8 Data Analysis

SPSS 14 Evolution (SPSS® Inc, USA) software was used to analyze the overall agreement of the two methods for each isolate in terms of the interpretative categories (Susceptible, Intermediate and Resistant) and was also used to generate;

1 Distribution of isolates used in the study.

2 Distribution of specimens.

3 Antimicrobial susceptibility profile of microorganisms at KATH according to the BSAC method.

4 Error types in the susceptibility patterns of both methods.

5 Differences in resistance patterns of all isolates tested in both methods.

CHAPTER FOUR

4. 0 RESULTS

4. 1 Bacterial Isolates

A total of 200 non-duplicate isolates were collected during the study period. Ninety-one-point-five percent (n=183) and 8.5% (n=17) of all microorganisms isolated were Gram-negative and Gram-positive respectively. *Escherichia coli* were the most predominantly isolated bacteria accounting for 34.5% (n=69) of the isolates. Thirty-two percent of isolates were obtained from blood, 33% from urine, 31.5% from miscellaneous samples (i.e. sputum, pus, wound, ear and aspirated Specimens), 1.5% from urethral smear and 1.0% from cerebrospinal fluid. Table 5 shows the bacteria and specimen types from which they were isolated.

Table 5. Number and Percentage (%) of bacterial isolates in specimen types

Organism	Specimen						
	Blood	Urine	Stool	Miscellaneous	Urethral smear	CSF	Total
E. coli	11(17.19)	44(66.67)	2(100)	12(19.05)	0(0)	0(0)	69(34.50)
K. pneumoniae	4(6.25)	17(25.76)	0(0)	16(25.40)	0(0)	0(0)	37(18.50)
S. aureus	11(17.19)	0(0)	0(0)	0(0)	2(66.67)	0(0)	13(6.50)
P. aeruginosa	0(0)	1(1.52)	0(0)	16(25.40)	1(33.33)	0(0)	18(9.00)
P. mirabilis	0(0)	0(0)	0(0)	7(11.11)	0(0)	0(0)	7(3.50)
S. typhi	14(21.88)	0(0)	0(0)	0(0)	0(0)	0(0)	14(7.00)
S. pneumoniae	2(3.13)	0(0)	0(0)	0(0)	0(0)	2(100)	4(2.00)
Coliforms	22(34.38)	4(6.06)	0(0)	12(19.05)	0(0)	0(0)	38(19.00)
Total	64(32.00)	66(33.00)	2(1.00)	63(31.50)	3(1.50)	2(1.00)	200(100)

4. 2 Agreement of Results of Susceptibility Tests by both Methods.

A total of 669 zone sizes were measured in (mm) and interpreted according to the BSAC standard. At KATH, zone sizes were visually observed and interpreted as either sensitive or resistant. The agreement in (%) of both methods (i.e. KATH and BSAC) in terms of the interpretative categories, Susceptible (S) or Resistant (R) is shown in the table below.

Table 6. Agreement in (%) of the BSAC and KATH's methods for all tested antibiotics

Antibiotic	Number of tests	BSAC (S)	KATH (S)	Agreement (%)	BSAC (R)	KATH (R)	Agreement (%)
Amikacin	92	64	88	67.4	16	4	2.2
Ampicillin (10µg)	31	0	0	100	31	31	100
Ampicillin (25µg)	44	0	0	100	44	44	100
Cefotaxime	101	37	73	32.7	64	28	23. 0
Ceftazidime	3	0	2	0	3	1	33.3
Ceftriaxone	3	0	3	0	3	0	0
Cefuroxime	101	10	58	5.9	91	43	38.6
Chloramphenicol	4	4	3	75.0	0	1	0
Ciprofloxacin	14	11	13	78.6	2	1	7.1
Erythromycin	10	7	8	70.0	3	2	20.0
Gentamicin	127	26	94	18.1	80	33	22.8
Levofloxacin	3	3	3	100	0	0	100
Nalidixic acid	65	15	24	20.0	50	41	60.0
Nitrofurantoin	44	25	40	54.5	19	4	6.8
Oxacillin	15	7	15	46.7	8	0	0
Tetracycline	12	4	3	16.7	8	9	58.3

4. 3 Error in Reporting of Susceptibility Results.

Table 7. Error types in susceptibility patterns of tested antibiotics for the two methods.

Antibiotic	No. of tests	Very Major	Major	Minor
Amikacin	92	14 (15.2%)	2 (2.2%)	12 (13.0%)
Ampicillin (10µg)	32	0 (0)	0 (0)	0 (0)
Ampicillin (25µg)	44	0 (0)	0 (0)	0 (0)
Cefotaxime	101	40 (39.6%)	4 (3.9%)	0 (0)
Ceftazidime	3	2 (66.7%)	0 (0)	0 (0)
Ceftriaxone	3	3 (100%)	0 (0)	0 (0)
Cefuroxime	101	52 (51.5%)	4 (4.0%)	0 (0)
Chloramphenicol	4	0 (0)	1 (25%)	0 (0)
Ciprofloxacin	14	1 (7.1%)	0 (0)	1 (7.1%)
Erythromycin	10	1 (10%)	0 (0)	0 (0)
Gentamicin	127	51 (40.2%)	3 (2.4%)	21 (16.5%)
Levofloxacin	3	0 (0)	0 (0)	0 (0)
Nalidixic acid	65	11 (17.0%)	2 (3.0%)	0 (0)
Nitrofurantoin	44	16 (36.4%)	1 (2.3%)	0 (0)
Oxacillin	15	8 (53.3%)	0 (0)	0 (0)
Tetracycline	12	1 (1.8%)	2 (16.7%)	0 (0)
Total	669	29.9%	2.8%	5.1%

Amp (10µg) used on blood, stool and miscellaneous isolates. Amp (25µg), on urine only.

A very major error is defined as “resistant” by the BSAC standardized method and “susceptible” by the Komfo Anokye locally adapted method. A major error is defined as a “susceptible” result by the BSAC method and a “resistant” result by the KATH method. A minor error is defined as any change involving an “intermediate” result.

4. 4 Antimicrobial Susceptibility Profile of Isolated Bacteria according to BSAC

Both blood and urine isolates of *E. coli* showed 100% resistance to ampicillin.

Urinary isolates of *K. pneumoniae* tested showed 76% resistance to nalidixic acid. *Salmonella typhi* showed 100% resistance each to amikacin, gentamicin and cefuroxime. Approximately 5.1% (n=34) of all antibiotic-organism test results were recorded as intermediate (I) against the Enterobacteriaceae (excluding *Salmonella*) with amikacin, gentamicin and ciprofloxacin, the antibiotics involved. Among isolates of the coliform, resistance to various antibiotics was cefuroxime (85%), cefotaxime (78%), gentamicin (38%), nalidixic acid (25%) and none was resistant to amikacin. A 100% resistance was recorded for *P. aeruginosa* against all the cephalosporins tested. Isolates of *S. pneumoniae* showed 100% resistance to oxacillin and also to ceftriaxone. *P. mirabilis* showed 43% resistance to gentamicin and no resistance were reported for amikacin. Sixty-eight percent, 85%, 77.5%, 82% and 58% respectively of all *E. coli, K. pneumoniae, S. aureus, S. typhi* and coliforms isolated in blood were resistant to antibiotics. The antimicrobial susceptibility profile of the most frequently isolated pathogenic bacteria at the Komfo Anokye Teaching Hospital according to the BSAC standard is shown in table 8.

Table 8.Antimicrobial susceptibility profile of the isolated bacteria according to BSAC

Organism Antibiotic	*E. coli*	*K. pneumoniae*	*S. aureus*	*P. aeruginosa*	*P. mirabilis*	*S. typhi*	*S. pneumoniae*	Coliforms
	S:I:R							
Amikacin	65:30:5	87.5:12.5:0	N/A	93:0:7	80:20:0	0:0:100	N/A	87:13:0
Ampicillin(10µg)	0:0:100	N/A	N/A	N/A	0:0:100	N/A	N/A	N/A
Ampicillin(25µg)	0:0:100	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cefotaxime	36:0:64	33:0:67	N/A	0:0:100	71:0:29	71:0:29	N/A	22:0:78
Ceftazidime	N/A	N/A	N/A	0:0:100	N/A	N/A	N/A	N/A
Ceftriaxone	N/A	N/A	N/A	N/A	N/A	N/A	0:0:100	N/A
Cefuroxime	8:0:92	0:0:100	N/A	0:0:100	43:0:57	0:0:100	N/A	15:0:85
Chloramphenicol	N/A	N/A	N/A	N/A	N/A	N/A	100:0:0	N/A
Ciprofloxacin	N/A	N/A	N/A	79:7:14	N/A	N/A	N/A	N/A
Erythromycin	N/A	N/A	70:0:30	N/A	N/A	N/A	N/A	N/A
Gentamicin	4:29:67	25:30:45	8:0:92	12.5:0:87.5	43:14:43	0:0:100	N/A	41:21:38
Levofloxacin	N/A	N/A	N/A	100:0:0	N/A	N/A	N/A	N/A
Nalidixic acid	18:0:82	24:0:76	N/A	N/A	N/A	N/A	N/A	75:0:25
Nitrofurantoin	57:0:43	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Oxacillin	N/A	N/A	64:0:36	N/A	N/A	N/A	0:0:100	N/A
Tetracycline	N/A	N/A	33:0:67	N/A	N/A	N/A	N/A	N/A

N/A= Antibiotic not applied on an isolate, S= Sensitive, I= Intermediate, R=Resistant.

4. 5 Antimicrobial Susceptibility Profile according to locally adapted method.

S. aureus showed 100% susceptibility to oxacillin. The Enterobacteriaceae were more resistant to cefuroxime than to the other cephalosporins. Blood culture isolates of *E.coli* and *P. mirabilis* showed a 100% resistance to ampicillin.

P. aeruginosa showed 93% sensitivity each to amikacin and ciprofloxacin. *E.coli* showed 9% resistance to nitrofurantoin. *S. pneumoniae* strains showed no resistance to oxacillin and ceftriaxone. *E. coli, K. pneumoniae* and *P. mirabilis* showed 100% sensitivity to amikacin. No intermediate categories were recorded.

The Antimicrobial susceptibility profile of the most frequently isolated pathogenic bacteria at the Komfo Anokye Teaching Hospital according to the hospital's own locally adapted method is shown in table 9. Antimicrobial susceptibility tests on these isolates had already been done by staff at the hospital using the in-house method and results were only collected from laboratory record books for comparison. Isolates used here were the same as those used in the BSAC method.

Table 9. Antimicrobial susceptibility profile according to KATH's adapted method.

Organism / Antibiotic	*E. coli*	*K. pneumoniae*	*S. aureus*	*P. aeruginosa*	*P. mirabilis*	*S. typhi*	*S. pneumoniae*	Coliforms
	S:R							
Amikacin	100:0	100:0	N/A	93:7	100:0	86:14	N/A	96:4
Ampicillin(10µg)	0:100	N/A	N/A	N/A	0:100	N/A	N/A	N/A
Ampicillin(25µg)	0:100	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cefotaxime	52:48	61:39	N/A	60:40	86:14	100:0	N/A	81:19
Ceftazidime	N/A	N/A	N/A	67:33	N/A	N/A	N/A	N/A
Ceftriaxone	N/A	N/A	N/A	N/A	N/A	N/A	100:0	N/A
Cefuroxime	36:64	45:55	N/A	0:100	58:42	100:0	N/A	65:35
Chloramphenicol	N/A	N/A	N/A	N/A	N/A	N/A	75:25	N/A
Ciprofloxacin	N/A	N/A	N/A	93:7	N/A	N/A	N/A	N/A
Erythromycin	N/A	N/A	80:20	N/A	N/A	N/A	N/A	N/A
Gentamicin	50:50	75:25	50:50	100:0	57:43	93:7	N/A	82:18
Levofloxacin	N/A	N/A	N/A	100:0	N/A	N/A	N/A	N/A
Nalidixic acid	39:61	24:76	N/A	N/A	N/A	N/A	N/A	75:25
Nitrofurantoin	91:9	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Oxacillin	N/A	N/A	100:0	N/A	N/A	N/A	100:0	N/A
Tetracycline	N/A	N/A	25:75	N/A	N/A	N/A	N/A	N/A

N/A= Antibiotic not applied on isolate, S=Sensitive R=Resistant.

4. 6 BSAC versus KATH's- Resistance patterns

Table 10 shows the overall prevalence of resistance to the antimicrobial agents.

Table 10. Prevalence of resistance patterns of all isolates tested in both methods

Organism	BSAC	KATH
Escherichia coli	72%	56%
Klebsiella pneumoniae	59%	40%
Staphylococcus aureus	73%	62%
Pseudomonas aeruginosa	52%	11%
Proteus mirabilis	48%	42%
Salmonella typhi	82%	5%
Streptococcus pneumoniae	64%	9%
Coliforms	54%	20%

Resistance patterns of urinary isolates of *K. pneumoniae* showed no difference between the two methods. Differences in resistance patterns in blood culture isolates with those of other specimen types showed the greatest difference. Also, the difference in resistance patterns amongst blood isolates was highest in *S. typhi.* Sixty-four percent of all antibiotic-organism test results were resistant in the BSAC compared with 38% in KATH's locally adapted method. Comparison of susceptibility test results showed that only 3% were recorded as resistant against an intermediate in the BSAC.

4. 7 Comparison of the BSAC method with Komfo Anokye's locally adapted

The BSAC disc diffusion method is a standardized method for antimicrobial susceptibility testing. Media types recommended in this method include Iso-Sensitest agar (ISA) or media shown to have the same performance as ISA (e.g. Mueller-Hinton).

Media preparation involves the pouring of sufficient molten agar into sterile Petri dishes to give a depth of 4mm ± 0.5mm (25mL in 90 mm diameter Petri dishes). Inoculum suspension is compared with the 0.5McFarland standard. Plates are incubated within 15 minutes of disc application at 35-37º C in air for 18-20 hrs. Zone sizes are measured with a ruler, calipers or an automated zone reader. The zone sizes are categorized into three; susceptible/sensitive (S), intermediate (I) or resistant (R). At least once weekly testing with control strains is advised in the BSAC.

At the KATH laboratory, Mueller- Hinton agar is prepared according to the manufacturer's instructions. Molten agar is then poured into sterile Petri dishes, dried and stored in a refrigerator prior to use. The surface of the agar is dried to remove excess moisture after plates have been brought out of refrigerator for use. At least four discrete colonies of organism to be tested are touched with a sterile loop and growth transferred into a bijou bottle containing peptone water. The inoculum suspension is shaken and then poured onto agar plates so that it covers the entire surface of the plates. Antibiotic discs are then applied within 15 minutes and plates subsequently incubated within 15 minutes of discs application at 35-37°C in air for 18-20 hrs. Reading of plates is done by visually observing zones of inhibition. Any clear zone of inhibition is interpreted as sensitive while a no- zone of inhibition is interpreted as resistant.

CHAPTER FIVE

5. 1 DISCUSSION

Isolating any significant microorganism from a microbiological specimen requires careful evaluation by the clinician and prompt action is usually necessary. If the results of clinical microbiological analyses are to contribute in a meaningful way to the diagnosis and management of patients with infection, then the quality of such tests should be guaranteed. The periodic audit of all laboratory procedures is important to ascertain if such procedures continue to conform to standards and are meeting their desired goals. The lack of uniform standardization and interpretative criteria causes concern, but there are indications that routine susceptibility testing data are suitable for surveillance even if obtained with different methods (Livermore *et al*, 2000).

The Komfo Anokye Teaching Hospital has adapted a method of antimicrobial susceptibility testing for routine use in the laboratory. Differences in results obtained with this adapted method and that from a standardized method could be attributed to some of the differences in procedures between these two methods for example, the reading of zone sizes.

5. 1. 1 Comparability of Results

Bacteria causing infections from various specimens including blood, urine, stool, wound, pus, sputum, aspirates, cerebrospinal fluid and urethral smear at the Komfo Anokye Teaching Hospital were identified as *Escherichia coli* (34.5%), *Klebsiella pneumoniae* (18.5%), *Pseudomonas aeruginosa* (9.0%), *Proteus mirabilis* (3.5%), *Salmonella typhi* (7.0%), coliforms (19.0%), *Staphylococcus aureus* and *Streptococcus pneumoniae* (2.0%). *Escherichia coli* and *Proteus mirabilis* each showed 100% resistance to

ampicillin in both methods. Previous work done by Ohene Adjei between 1994 and 1996 on bacterial pathogens and their antimicrobial susceptibility in Kumasi found out *Escherichia coli* showed 88% resistance to ampicillin (Ohene Adjei, 1997). Having found a 100% resistance of *Escherichia coli* to ampicillin in this study suggests that resistance of *Escherichia coli* to ampicillin has increased over the years. The overall agreement of both methods in terms of the interpretative categories, susceptible and resistant was 49.1% and 35.8% respectively. This shows that the KATH's locally adapted method was much more unreliable in the reporting of results as resistant. Twenty-nine-point-nine percent, 2.8% and 5.1% of all tests were reported as very major, major and minor errors respectively. These values are way too high compared to that suggested by Jorgensen, lower than 1.5% and 3.0% for very major and minor errors respectively (Jorgensen, 1993) for a new susceptibility method. Higher values of very major and major errors resulted from the fact zone sizes were not measured in the locally adapted method but then only visually observed; and as such any zone of inhibition formed was perceived to be a sensitive result. Moreover, in the BSAC method, there is just a 1mm diameter difference between successive categories (i.e. R, I, S) and thus with just this thin line of separation, a zone size adjudged by the human eye as sensitive may not be so after all. Since the turbidity of the inoculum suspension was not compared with the 0.5 McFarland standard in the KATH's method, the inoculum suspension obtained by the KATH's method could be denser which could result in reduced zones of inhibition or vice versa. This could respectively yield false-resistant or false-sensitive results (Jorgensen, 1999). For sensitivity testing the depth of the agar is usually recommended to be 4mm in the centre of the plate (approximately 25ml in a 90mm plate). Variation in depth could affect the zone sizes – if the agar is too thin, larger zones could appear since the volume is

decreased, and the effective antibiotic concentration increased. If the agar is too thick, smaller zones could appear since the effective antibiotic concentration has been decreased (Barry *et al*, 1973). The technique of pouring of the inoculum suspension employed at the hospital's laboratory has the problems of the inoculum not being spread evenly on the entire surface of the plate as well as the presence of the remains of excess liquid. These could have an effect on the outcome of susceptibility test results.

Very major discrepancies were observed on amikacin (15.2%) cefotaxime (39.6%), cefuroxime (51.5%), ceftazidime (66.7%), ceftriaxone (100%), ciprofloxacin (7.1%), erythromycin (10%), gentamicin (40.2%) nalidixic acid (17%), nitrofurantoin (36.4%), oxacillin (26.7%) and tetracycline (1.8%). Very major error that occurred on amikacin and gentamicin related to *Salmonella typhi.* This was because in the BSAC method, *S. typhi* was reported resistant to amikacin and or gentamicin irrespective of susceptibility tests results (BSAC, 2008). This rule however, was not applied in the locally adapted method and that there was specific consideration of results of susceptibility tests of these isolates. The disparity in very major errors between amikacin (15.2%) and gentamicin (40.2%) suggests that individual aminoglycoside agents must be tested and that susceptibility to other aminoglycosides cannot be inferred from the gentamicin result and vice versa. Ninety-seven percent of all minor errors were recorded as an intermediate in the BSAC against a sensitive in the adapted method. This shows that the locally adapted was versatile in the detection of susceptibility. Four (4) very major errors occurred in the report of susceptibility tests of *S. pneumoniae* to oxacillin. *Staphylococcus aureus* strains showed 36% resistance to oxacillin. These are methicillin-resistant *Staphylococcus aureus* (MRSA). Methicillin-resistant strains are often multiply-resistant to several other drug classes, including macrolides, clindamycin, aminoglycosides, chloramphenicol,

fluoroquinolones and trimethoprim/sulfamethoxazole (Quintiliani *et al*, 1999). *S. aureus* strains resistant to oxacillin/methicillin pose a serious clinical and public health problem, as they can be transmitted from patient to patient in hospitals as well as in community settings (Finland, 1979).

Lower prevalence of resistance had been reported in cefuroxime (27%), cefotaxime (20%) and between 6 and 10 percent in ciprofloxacin, amikacin and ceftriaxone in Ghana. (Newman *et al*, 2006). Comparison of results in this study however shows that although these drugs have been on the market for a relatively short period of time, there is a gradual increase in resistance of bacterial isolates to these antibiotics which suggests developing trends of abuse of these antibiotics.

5. 1. 2 Need for Comparison

Presently, there are numerous standard methods available in different geographical regions because of the current problem of not having a uniform antimicrobial susceptibility testing method. These standard methods however, undergo regular updates since methodology changes, breakpoint changes and the introduction of new antibiotics can have a significant impact on the interpretation of the results obtained.

It is therefore much more necessary to periodically audit all laboratory procedures involved in any adapted method for example that of the KATH laboratory to ascertain if the performance of such a method continue to conform to standards and are meeting their desired goals.

5. 1. 3 A Call for Further Research

Comparative studies though with different methods serve as a platform from which to promote focus on antimicrobial resistance issues in the hospital. It is necessary to have locally generated susceptibility results for surveillance purposes. Hospital surveillance software could be developed and adopted. This could help alert the operator if isolates with unexpected resistance patterns are entered. Unfortunately, the finding of a lot of discrepancies of results with both methods indicates that this would be more useful if a standardized method is employed at the hospital.

5. 1. 4 Limitations of the Study

- At KATH laboratory, enterobacteriaceae that still could not be identified upon the application of all procedures were collectively described as coliforms. This was likely to create biases in the analyses because the identification of enterobacteriaceae to species level is essential for the application of expert rules for the interpretation of susceptibility in the BSAC (BSAC, 2008).
- Also two of the antibiotics (Gentamicin and Tetracycline) used in the adapted method had lost their potency because they had gone past their expiry dates (Personal observation). This was likely to create errors in the report of a false-resistant result.
- There was no records of the report of MRSA in the hospital's laboratory books and so the 36% resistance to oxacillin of *S. aureus s*trains detected by the BSAC could not be compared with any values to substantiate whether there was an increase in resistance patterns of MRSA at the hospital or not.

- Mueller-Hinton agar with 5% sheep blood is recommended for disc diffusion susceptibility of *S. pneumoniae* with chloramphenicol, erythromycin, ofloxacin, tetracycline and vancomycin, in addition to oxacillin screening for susceptibility to penicillin (CLSI, 2006). Mueller-Hinton agar was not supplemented in both methods because of lack of sheep blood and this is likely to create errors in results because, when Mueller-Hinton is supplemented with blood, the zone of inhibition of oxacillin may be 2-3mm smaller than those obtained with unsupplemented agar (Woods *et al*, 1995).

5. 2 CONCLUSION AND RECOMMENDATIONS

5. 2. 1 Conclusion

The antimicrobial susceptibility profile of bacterial isolates determined by the BSAC method showed high levels of resistance to antibiotics. Comparison of the overall susceptibility data showed discrepancies between the BSAC susceptibility results and the "Komfo Anokye adaptation" results. This shows that wrong results were reported in several instances. The discrepancies in results originated from those elements of the BSAC that were modified to obtain the locally adapted method. These modifications need to be revised to conform currently to the techniques from which they were modified. Such synchronization will help improve antimicrobial susceptibility test performed at the KATH laboratory and thus results obtained with the adapted method will be accurate and reproducible. To improve antibiotic effectiveness and help increase the efficiency of AST, local guidelines should be drawn up for treating specific infections, for empirical prescribing and for in-vitro testing. There should also be consultations between clinicians and laboratory personnel in the interpretation of susceptibility test results.

5. 2. 2 RECOMMENDATIONS

- The pouring of molten agar by intuition, the pouring of inoculum suspension, the visual observation of turbidity and zone sizes and the interpretation of results in either one of only two categories which are modifications of the BSAC that influenced errors seen in the KATH in-house method should be revised in relation to current standardized practices such as measuring the depth of agar, swabbing of inoculum suspension in three directions, comparison of turbidity with the 0.5McFarland standard, measuring of zone sizes and interpretation of results using one of three categories.
- Quality control checks should be performed at least once weekly or ideally on a daily basis to monitor all aspects of susceptibility testing: media (depth, pH, etc) inoculum level, antibiotic disc integrity, incubation temperature and atmospheric conditions (BSAC, 2008). The appropriate reference microorganisms should always be used. Reference microorganisms should be obtained from a reliable source for example the American Type Culture Collection (ATCC) or the National Collection of Type Cultures (NCTC) where known results are expected.
- The adapted method in its procedures should include techniques to detect and screen for emergent resistant strains.
- Testing ampicillin on isolates of *E.coli* and *P. mirabilis* should be done only for surveillance studies.
- Amikacin, gentamicin and cefuroxime should not be tested on *S. typhi* or if tested should be reported resistant regardless of results.

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APPENDIX I. RESULTS OBTAINED IN THE STUDY

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)															
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
1	B8379	Coliform	-	-	-	0/R	0/R	-	-	-	-	-	-	20/S	-	-	-	-	-
2	B8393	*E. coli*	-	0/R	-	0/R	15/R	-	-	-	-	-	-	11/R	-	-	-	-	-
3	B8399	Coliform	-	-	-	36/S	25/R	-	-	-	-	-	-	23/S	-	-	-	-	-
4	B8454	Coliform	-	-	-	0/R	0/R	-	-	-	-	-	-	20/S	-	-	-	-	-
5	B8467	Coliform	-	-	-	10/R	0/R	-	-	-	-	-	-	20/S	-	-	-	-	-
6	U4895	Coliform	-	-	-	-	-	-	-	-	-	-	-	-	-	23/S	-	-	-
7	M/2401	*P. aeruginosa*	28/S	-	-	-	-	-	-	-	34/S	-	-	18/S	-	-	-	-	-
8	M2402	*P. aeruginosa*	28/S		-	-	-	-	-	-	35/S	-	-	18/S	-	-	-	-	-
9	U4905	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	20/S	-	-
10	U4899	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
11	U48906	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	0/R	-	-
12	M2416	*E. coli*	-	O/R	-	0/R	0/R	-	-	-	-	-	-	20/S	-	-	-	-	-
13	M2412	*P. mirabilis*	-	0/R	-	31/S	26/S	-	-	-	-	-	-	19/I	-	-	-	-	-
14	M2413	*P. aeruginosa*	-	-	-	-	-	-	-	-	37/S	-	-	13/R	-	-	-	-	-
15	F230	*E. coli*	16/I	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-

B=Blood, U=Urine, M=Miscellaneous (sputum, pus, wound, ear and aspirated specimens), F=Stool, C=Cerebrospinal fluid,

GP=Urethral smear S=Sensitive, I=Intermediate, R=Resistant.

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)															
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
16	B8398	*S. aureus*	-	-	-	-	-	-	-	-	-	-	7/R	0/R	-	-	-	0/R	24/S
17	B8345	*S. aureus*	-	-	-	-	-	-	-	-	-	-	27/S	8/R	-	-	-	15/S	0/R
18	B8376	*S. aureus*	-	-	-	-	-	-	-	-	-	-	27/S	26/S	-	-	-	0/R	8/R
19	U4923	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	18/R	-	-
20	U4920	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	20/S	23/S	-	-
21	U4924	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	18/S	18/R	-	-
22	B8423	Coliform	-	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
23	U4930	Coliform	-	-	-	-	-	-	-	-	-	-	-	-	-	27/S	-	-	-
24	M2421	*P. mirabilis*	-	0/R	-	12.R	0/R	-	-	-	-	-	-	11/R	-	-	-	-	-
25	M2423	*E. coli*	-	0/R	-	10/R	0/R	-	-	-	-	-	-	16/R	-	-	-	-	-
26	M2425	Coliform	21/S	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
27	B8542	Coliform	-	-	-	18/R	0/R	-	-	-	-	-	-	17/I	-	-	-	-	-
28	B8543	Coliform	-	-	-	35/S	21/R	-	-	-	-	-	-	20/S	-	-	-	-	-
29	B8472	*S. aureus*	-	-	-	-	-	-	-	-	-	-	29/S	0/R	-	-	-	15/S	25/S
30	U5121	*K.pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
31	U5122	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
32	U5111	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
33	U5110	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
34	M2517	*P. aeruginosa*	-	-	-	-	-	-	-	-	25/I	-	-	12/R	-	-	-	-	-

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)																
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
35	M2519	*P. aeruginosa*	-	-	-	-	-	-	-	-	35/S	-	-	14/R	-	-	-	-	-
36	M2524	Coliform	17/I	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
37	M2527	Coliform	21/S	-	-	-	0/R	-	-	-	-	-	-	10/R	-	-	-	-	-
38	M2541	*K. pneumoniae*	18/I	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
39	M2542	*K. pneumoniae*	-	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
40	U5124	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
41	B8486	S. typhi	R	-	-	0/R	R	-	-	-	-	-	-	R	-	-	-	-	-
42	B8914	Coliform	19/S	-	-	-	22/R	-	-	-	-	-	-	17/I	-	-	-	-	-
43	B9002	*S. typhi*	R	-	-	29/R	R	-	-	-	-	-	-	R	-	-	-	-	-
44	U5233	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	25/S	26/S	-	-
45	B8990	S. typhi	R	-	-	30/S	R	-	-	-	-	-	-	R	-	-	-	-	-
46	B8991	*K. pneumoniae*	-	-	-	-	0/R	-	-	-	-	-	-	11/R	-	-	-	-	-
47	GP426	S. aureus	-	-	-	-	-	-		-	-	-	-	0/R	-	-	-	-	0/R
48	U5231	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	15/R	-	-	-
49	B8988	Coliform	-	-	-	14/R	11/R	-	-	-	-	-	-	12/R	-	-	-	-	-
50	B9127	*S. pneumoniae*	-	-	-	-	-	-	-	-	-	22/S	-	-	-	-	-	0/R	0/R
51	U5181	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	24/S	-	-
52	M2525	Coliform	28/S	-	-	14/R	26/S	-	-	-	-	-	-	19/I	-	-	-	-	-
53	M2540	Coliform	19/S	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)																
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
54	M2545	*K. pneumoniae*	-	-	-	30/S	24/R	-	-	-	-	-	-	17/I	-	-	-	-	-
55	B9024	*S. typhi*	R	-	-	31/S	R	-	-	-	-	-	-	R	-	-	-	-	-
56	F245	*E. coli*	18I	0/R	-	30/S	24/R	-	-	-	-	-	-	16/R	-	-	-	-	-
57	B9021	*K. pneumoniae*	-	-	-	28/R	19/R	-	-	-	-	-	-	16/R	-	-	-	-	-
58	B8913	*E. coli*	21/S	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
59	M2566	*E. coli*	20/S	-	-	30/S	20/R	-	-	-	-	-	-	16/R	-	-	-	-	-
60	U5230	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	19/R	-	-
61	U5234	*P. aeruginosa*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
62	M2567	*P. aeruginosa*	21/S	-	-	0/R	-	-	-	-	32/S	-	-	12/R	-	-	-	-	-
63	M2553	*P. aeruginosa*	13/R	-	-	-	-	-		-	0/R	-	-	0/R	-	-	-	-	-
64	M2578	*P. aeruginosa*	25/S	-	-	0/R	-	-	-	-	35/S	-	-	12/R	-	-	-		-
65	GP416	*P. aeruginosa*	22/S	-	-	0/R	-	-	-	-	35/S		-	13/R	-	-	-	-	-
66	M2551	*P. aeruginosa*	21/S	-	-	0/R	-	-	-	-	32/S	-	-	0/R	-	-	-	-	-
67	M2564	*p. mirabilis*	16/I	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
68	M2563	*K. pneumoniae*	18/I	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
69	B9009	*E. coli*	-	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
70	B9118	*E. coli*	20/S	0/R	-	31/S	24/R	-	-	-	-	-	-	17/I	-	-	-	-	-
71	U5228	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
72	U5159	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)																
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
73	U5193	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	23/S	-	-
74	M2572	*K. pneumoniae*	20/S	-	-	17/R	17/R	-	-	-	-	-	-	18/I	-	-	-	-	-
75	U5220	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	0/R	-	-
76	B9088	*E. coli*	12/R	0/R	-	33/S	24/R	-	-	-	-	-	-	12/R	-	-	-	-	-
77	B9051	*S. typhi*	R	-	-	28/R	R	-	-	-	-	-	-	R	-	-	-	-	-
78	M2557	*E. coli*	16/I	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
79	M2573	*K. pneumoniae*	20/S	-	-	30/S	23/R	-	-	-	-	-	-	15/R	-	-	-	-	-
80	B9119	*E. coli*	20/S	0/R		35/S	25/S	-	-	-	-	-	-	17/I	-	-	-	-	-
81	U5247	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
82	B9125	*S. aureus*	-	-	-	-	-	-		-	-	-	25/S	17/R	-	-	-	15/S	12/R
83	B9165	*S. typhi*	R	-	-	28/R	R		-	-	-	-	-	R	-	-	-	-	-
84	U4906	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	15/R	-	-
85	M2579	*K. pneumoniae*	20/S	-	-	28/R	20/R	-	-	-	-	-	-	16/R	-	-	-	-	-
86	B9026	*S. typhi*	R	-	-	30/S	R	-	-	-	-	-	-	R	-	-	-	-	-
87	U5198	*E.coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	25/S	25/S	-	-
88	U4899	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	18/S	-	-	-
89	B9061	Coliform	22/S	-	-	16/R	16/R	-	-	-	-	-	-	18/I	-	-	-	-	-
90	M3614	*K. pneumoniae*	21/S	-	-	26/R	22/R	-	-	-	-	-	-	20/S	-	-	-	-	-
91	U5201	Coliform	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)																
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
92	U4905	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	18/R	-	-
93	B9086	*E. coli*	19/S	0/R	-	31/S	24/R	-	-	-	-	-	-	17/I	-	-	-	-	-
94	M2565	*E. coli*	-	0/R	-	27/R	20/R	-	-	-	-	-	-	-	-	-	-	-	-
95	U5211	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	20/S	25/S	-	-
96	U5212	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	20/S	-	-
97	U4909	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	24/S	25/S	-	-
98	U5129	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	10/R	-	-
99	U5165	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	18/R	-	-
100	U5225	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	20/S	11/R	-	-
101	U5172	*E. coli*			0/R	-	-	-	-	-	-	-	-	-	-	22/S	27/S	-	-
102	U5242	Coliform	-	-	-	-	-	-	-	-	-	-	-	-	-	23/S	-	-	-
103	B9084	*S, aureus*	-	-	-	-	-	-	-	-	-	-	0/R	7/R	-	-	-	-	-
104	M2562	*P. mirabilis*	23/S	23/S	-	30/S	20/R	-	-	-	34/S	-	-	10/R	-	-	-	-	-
105	M2582	*P. aeruginosa*	23/S	23/S	-	-	-	-	-		-	-	-	-	-	-	-	-	-
106	M2583	*K. pneumoniae*	23/S	23/S	-	31/S	21/R	-	-	-	-	-	-	19/I	-	-	-	-	-
107	B9068	Coliform	21/S	21/S	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
108	M2504	*P. aeruginosa*	23/S	23/S	-	0/R	0/R	-	-	-	-	-	-	14/R	-	-	-	-	-
109	M2503	*K. pneumoniae*	22/S	22/S	-	-	11/R	-	-	-	-	-	-	20/S	-	-	-	-	-
110	M2513	*P. aeruginosa*	22/S	22/S	-	-	-	-	-	-	30/S	-	-	12/R	-		-	-	-

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)																
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
111	M2508	Coliform	21/S	-	-	13/R	16/R	-	-	-	-	-	-	20/S	-	-	-	-	-
112	B8796	*S. typhi*	R	-	-	30/S	R	-	-	-	-	-	-	R	-	-	-	-	-
113	U5042	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	20/S	-	-
114	B8869	*S. typhi*	R	-	-	32/S	R	-	-	-	-	-	-	R	-	-	-	-	-
115	B8857	Coliform	26/S	-	-	19/R	16/R	-	-	-	-	-	-	23/S	-	-	-	-	-
116	M2490	*E. coli*	18/I	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
117	B/8680	Coliform	-	-	-	0/R	0/R	-	-	-	-	-	-	-	-	-	-	-	-
118	B8878	Coliform	21/S	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
119	U5056	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	10/R	-	-
120	U5063	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	20/S	-	-
121	B8728	*S. aureus*	-	-	-	-	-	-	-	-	-	-	25/S	0/R	-	-	-	16S	21/S
122	M2476	*K. pneumoniae*	26/S	-	-	10/R	14/R	-	-	-	-		-	21/S	-	-	-	-	-
123	U5098	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	21S	-	-	-
124	B8670	Coliform	-	-	-	17/R	25/S	-	-	-	-	-	-	28/S	-	-	-	-	-
125	M2510	*K. pneumoniae*	21/S			30/S	17/R							20/S					
126	B8671	*S. aureus*	-	-	-	-	-	-	-	-	-	-	26/S	20/S	-	-	-	15S	24/S
127	B87566	Coliform	16/I	-	-	16/R	21/R	-	-	-	-	-	-	18/I	-	-	-	-	-
128	B8591	*E. coli*	21/S	0/R	-	18/R	26/S	-	-	-	-	-	-	0/R	-	-	-	-	-
129	M2449	*E. coli*	22/S	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)																
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
130	B8773	*S.aureus*	-	-	-	-	-	-	-	-	-	-	0/R	16/R	-	-	-	0/R	0/R
131	B8713	Coliform	22/S	-	-	0/R	0/R	-	-	-	-	-	-	17/I	-	-	-	-	-
132	B8757	Coliform	22/S	-	-	12/R	17/R	-	-	-	-	-	-	15/R	-	-	-	-	-
133	U5305	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	20/S	-	-
134	U4959	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	22/S	-	-
135	B8885	Coliform	22/S	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
136	B8772	*K. pneumoniae*	20/S	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
137	B8714	Coliform	-	-	-	20/R	23/R	-	-	-	-	-	-	26/S	-	-	-	-	-
138	M2484	*P. aeruginosa*	23/S	-	-	-	-	-	-	-	32/S	-	-	16/R	-	-	-	-	-
139	U5053	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	14/R	-	-
140	M2439	Coliform	20/S	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
141	M2431	Coliform	23/S	-	-	33/S	25/S	-	-	-	-	-	-	20/S	-	-	-	-	-
142	M2507	Coliform	23/S	-	-	30/S	15/R	-	-	-	-	-	-	20/S	-	-	-	-	-
143	U4956	*K. pneumoniae*	-	0/R	-	-	-	-	-	-	-	-	-	-	-	21/S	-	-	-
144	M2431	*P. mirabilis*	24/S	-	-	31/S	25/S	-	-	-	-	-	-	20/S	-	-	-	-	-
145	M2475	Coliform	30/S	-	-	15/R	16/R	-	-	-	-	-	-	25/S	-	-	-	-	-
146	M2509	Coliform	21/S	-	-	10/R	11/R	-	-	-	-	-	-	19/I	-	-	-	-	-
147	B9862	*S. typhi*	R	-	-	31/S	R	-	-	-	-	-	-	R	-	-	-	-	-
148	M2502	*E. coli*	21/S	0/R	-	30/S	23/R	-	-	-	-	-	-	17/I	-	-	-	-	-

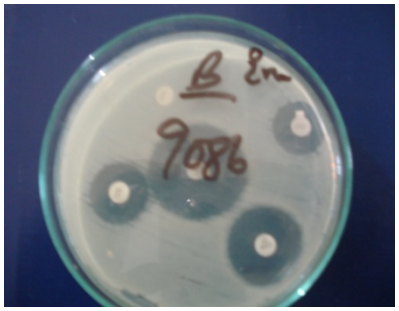
No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)															
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
149	B9882	Coliform	19/S	-	-	30/S	21/R	-	-	-	-	-	-	16/R	-	-	-	-	-
150	M3791	*K. pneumoniae*	21/S	-	-	31/S	23/R	-	-	-	-	-	-	17/I	-	-	-	-	-
151	U5645	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	26/S	-	-
152	M3793	Coliform	21/S	-	-	34/S	29/S	-	-	-	-	-	-	16/R	-	-	-	-	-
153	M3793	*E. coli*	18/I	0/R	-	30/S	23/R	-	-	-	-		-	17/I	-	-	-	-	-
154	B9842	*K. pneumoniae*	19/S	-	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
155	U5610	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	10/R	-	-
156	U5611	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	0/R	-	-
157	M3786	*P. aeruginosa*	20/S	-	-	-	-	-	0/R	-	18/R	-	-	10/R	25/S	-	-	-	-
158	M2472	*E. coli*	20/S	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
159	M2447	*E. coli*	17/I	0/R	-	30/S	21/R	-	-	-	-	-	-	17/I	-	-	-	-	-
160	U4948	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	21/S	-	-
161	B8764	Coliform	18/I	-	-	30/S	23/R	-	-	-	-	-	-	18/I	-	-	-	-	-
162	M3862	*P. aeruginosa*	25/S	-	-	-	-	-	0/R	-	-	-	-	16/R	30/S	-	-	-	-
163	M3868	*K. pneumoniae*	21/S	-	-	0/R	0/R	-	-	-	-		-	20./S	-	-	-	-	-
164	C1320	*S. pneumoniae*	-	-	-	-	-	-	-	15/R		20/S	-	-	-	-	-	0/R	-
165	U5696	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	25S	-	-	-
166	U5702	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	24/S	-	-
167	M3895	*P. aeruginosa*	25/S	-	-	-	-	-	0/R	-	-	-	-	17/R	25/S	-	-	-	-

No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)																
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
168	U5751	*E. coli*	-	-	0/R	-	-	-	-		-	-	-	-	-	0/R	21/S	-	-
169	U5752	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	21/S	-	-
170	B8782	*E. coli*	19/S	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
171	B8783	*E. coli*	19/S	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
172	U5104	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
173	U5108	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	-	-	-
174	U5089	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/R	12/R	-	-
175	U5091	*E. coli*	-	-	0/R	-	-	-	-		-	-	-	-	-	0/R	-	-	-
176	M2506	*E. coli*	21/S	0/R	-	0/R	0/R	-	-	-	-	-	-	19/I	-	-	21/S	-	-
177	C1501	*S. pneumoniae*	-	-	-	-	-	-	-	23/R	-	19/S	-	-	-	-	-	0/R	-
178	B10019	*S. typhi*	R	-	-	30/S	30/S	-	-	-	-	-	-	R	-	-	-	-	-
179	B10039	*S. typhi*	R	-	-	30/S	30/S	-	-	-	-	-	-	R	-	-	-	-	-
180	B10054	*S. pneumoniae*	-	-	-	-	-	-	-		-	20/S	-	-	-	-	-	0/R	-
181	B10118	*S. aureus*	-	-	-	-	-		-	22/R	-	-	-	0/R	-	-	-	0/R	-
182	B10131	*S. typhi*	R	-	-	32/S	32/S	-	-	-	-	-	-	R	-	-	-	-	-
183	B10263	*S. typhi*	R	-	-	30/S	30/S	-	-	-	-	-	-	R	-	-	-	-	-
184	B10131	*E. coli*	20/S	0/R	-	0/R	0/R	-	-	-	-	-	-	0/R	-	-	-	-	-
185	B10387	*S. aureus*	-	-	-	-	-	-	-	-	-	-	-	18/R	-	-	-	15/S	0/R
186	U5110	*K. pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	12/R	-	-	-

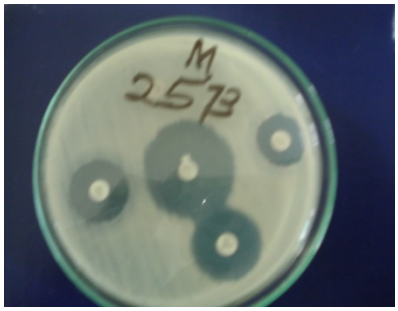
No.	ID	Isolate	Antibiotic Zone diameter (mm); Interpretation (S, I, R)															
			Amk	Amp 10µg	Amp 25µg	Ctx	Crx	Ctr	Caz	Cro	Cip	Chl	Ery	Gen	Lev	Nal	Nit	Oxa	Tet
187	U5111	*E. coli*	-	-	0/R	-	-	-	-		-	-	-	-	-	0/R	0/R	-	-
188	U5147	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	15/R	23/S	-	-
189	U5156	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	25/S	-	-
190	U5172	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	15/R	-	-
191	U5181	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	20/S	-	-
192	U5193	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	0/R	-	-
193	U5211	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	16/R	25/S	-	-
194	U5230	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	-	20/S	-	-
195	U5233	*E. coli*	-	-	0/R	-	-	-	-	-	-	-	-	-	-	0/R	0/R	-	-
196	M2589	*K. pneumoniae*	20/S	-	-	30/S	20/R	-	-	-	-	-	-	20/S	-	-	-	-	-
197	M3613	*P. mirabilis*	20/S	0/R	-	32/S	25/S	-	-	-	-	-	-	20/S	-	-	-	-	-
198	M3607	*P. mirabilis*	21/S	0/R	-	30/S	20/R	-	-	-	-	-	-	20/S	-	-	-	-	-
199	GP440	*S. aureus*	-	-	-	-	-	-	-	-	-	-	25/S	18/R	-	-	-	17/S	0/R
200	M3614	*K. pneumoniae*	20/S	-	-	0/R	0/R	-	-	-	-	-	-	18/I	-	-	-	-	-

APPENDIX II

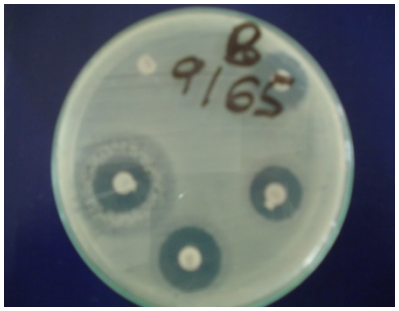
PICTURE SHOWING ZONE DIAMETERS OBTAINED USING THE BASIC STANDARDIZED METHOD OF ANTIMICROBIAL SUSCEPTIBILITY TESTING



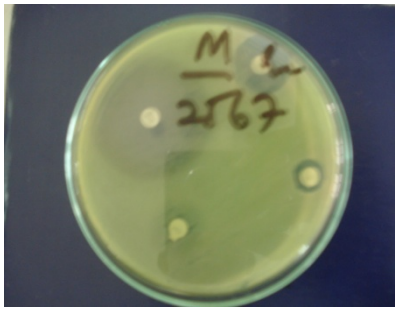
K. pneumoniae



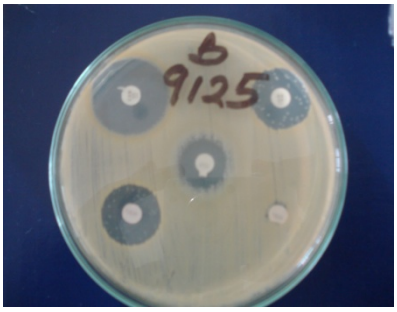
E. coli



P. aeruginosa

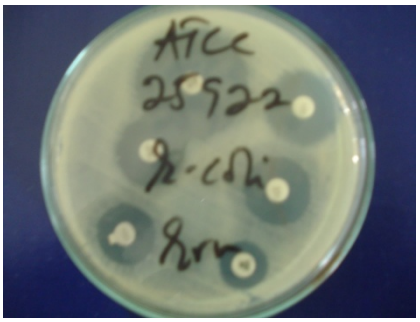


S. typhi

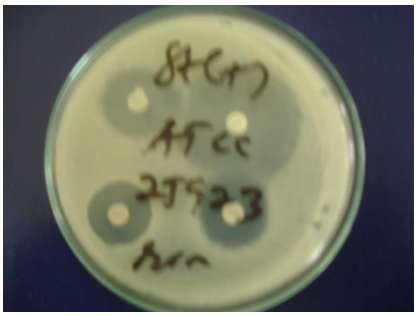


S. aureus

PICTURE SHOWNG ZONE DIAMETERS OF CONTROL STRAINS



ATCC 25922 (*E. coli)*



ATCC 25923 (*S. aureus)*

ATCC 27853 (*P. aeruginosa*)

APPENDIX III

Liquid media

A. Peptone water

Peptone water may be used as a growth medium or as the basis of carbohydrate fermentation media, whilst pure culture peptone water is a convenient inoculum for a series of fermentation tubes or other diagnostic media.

Composition

Peptone water contains the following in grams per liter according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	g/l
Peptone	10.0
Sodium chloride	5.0
pH 7.0-7.4	

Preparation

The medium was prepared by dissolving 15 grams in 1 liter of distilled water. It was mixed and distributed into bijou bottles of 5ml volumes and then sterilized by autoclaving at 121ºC.

B. Kovac's reagent

This reagent is used for the detection of indole which is released as a result of the breakdown of the amino acid tryptophan. It is prepared by dissolving 10 grams of 4-dimethylamino-benzaldehyde in 150ml of iso-amyl alcohol. After dissolution, 50ml of concentrated hydrochloric acid is added to it. It is then stored in a refrigerator in an amber bottle.

C. Brain heart infusion broth

This medium is recommended for the cultivation of streptococci, pneumococci, meningococci and other fastidious organisms. It is suitable for blood culture work.

Composition

The Brain Heart Infusion Broth contains the following in grams per liter according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	g/l
Calf brain infusion solids	2.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0

Sodium chloride	5.0
Disodium phosphate	2.5
pH 7.2-7.6	

Preparation

37 grams was dissolved in 1 liter of distilled water. It was mixed thoroughly and distributed into final containers and then sterilized by autoclaving at 121ºC.

D. Oxidase reagent

The reagent is used to assist in the identification of pseudomonas, neisseria, vibrio, brucella and pasteurella species, all of which produce the enzyme cytochrome oxidase. 10ml of the reagent is prepared by dissolving 0.1g Tetramethyl-p-phenylenediamine dihydrochloride in 10ml of distilled water. The chemical is dissolved in water. The reagent is not stable and it is therefore best prepared immediately before use.

E. Sodium deoxycholate reagent

This reagent clears turbidity after *S. pneumoniae* has been emulsified in physiological saline. It is prepared by dissolving 2g of sodium deoxycholate in 20ml of sodium chloride (8.5g/l). It is then

transferred into a clean bottle and then sterilized by autoclaving at 121ºC for 15 minutes. It is then stored at 2-8ºC.

F. Stuart transport medium

This is a transport medium for fastidious pathogenic organisms.

Composition

Stuart transport medium contains the following in grams per liter according to Oxoid Ltd. Basingstoke, Hampshire, England:

Composition	g/l
Sodium glycerophosphate	10.0
Sodium thioglycollate	0.5
Cysteine hydrochloride	0.5
Calcium chloride	0.1
Methylene blue	0.001
Agar	5.0

pH 7.2-7.6

Preparation

16 grams of the powder was dissolved in 1 liter of distilled water. It was then boiled shortly to dissolve the medium completely and then sterilized at 121ºC for 15 minutes.

G. Physiological saline

Preparation

8.5 g of Sodium chloride was added to 1 liter of distilled water. The suspension was then mixed until the salt was fully dissolved. It was then transferred into a bottle and stored at room temperature for use.

H. Kligler's iron agar

This is used in the primary identification of Enterobacteriaceae and other enteric organisms.

Composition

Peptone, 'Lab-Lemco' powder, yeast extract, sodium chloride, lactose, glucose (dextrose), ferric citrate, sodium thiosulphate, phenol red, agar.

Preparation

The medium was prepared by dissolving 5.5 g of powder in 100ml distilled water and then sterilized at 121ºC for 15 minutes. Medium was allowed to solidify in a sloped position and stored at a cool dark place for use.

I. Turbidity standard solution (0.5 McFarland)

This is the standard against which the turbidity of the test and control inocula can be compared.

Preparation

One percent solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of distilled water. Also, 1% solution of barium chloride was prepared by dissolving 0.5 grams of dehydrated barium chloride ($Bacl_2.2H_2O$) in 50ml of distilled water. Zero-point-six milliliters of the barium chloride solution was added to 99.5ml of the sulphuric acid solution and mixed. It was then distributed into screw-cap tubes of the same size and volume as those used in preparing the test.

J. Glycerol broth

Glycerol broth was used to store the samples.

Preparation

The broth was prepared by weighing 20% of brain heart infusion broth. Distilled water and glycerol was added in the ratio of 4:1 respectively. The mixture was stirred until a uniform solution was obtained. A micropipette was used to pipette 1ml of the solution into Eppendorf tubes. The broth was then sterilized at 121ºC for 15 minutes.

APPENDIX IV

Solid Media

A. MacConkey agar

This is a differential medium for the differentiation and isolation of Enterobacteriaceae. It also supports the growth of Staphylococci and Enterococci, but inhibits the growth of Streptococci and the Haemophilus. It also prevents the swarming of Proteus.

Composition

It is formulated to contain the following in grams per liter according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	g/l
Peptone	20.0
Lactose	10.0
Bile salts No. 3	1.5
Sodium chloride	5.0
Neutral red	0.003
Crystal violet	0.001
Agar	15.0

pH 6.9-7.3

Preparation

This was prepared by suspending 51.5 grams in 1 liter of distilled water. It was then boiled shortly to dissolve completely. Sterilization was done by autoclaving at 121ºC for 15 minutes.

B. Cysteine lactose electrolyte-deficient (CLED) agar

This medium is recommended for diagnostic urinary bacteriology. The medium supports the growth of all urinary potential pathogens giving good colonial differential and clear diagnostic characteristics.

Composition

CLED contains the following in grams per liter according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	g/l
Peptone	4.0
'Lab-Lemco' powder	3.0
Tryptone	3.0
Lactose	10.0

L-cystine	0.128
Bromothymol blue	0.02
Agar	15.0
pH 7.0-7.4	

Preparation

To prepare this medium 36.2 grams of the powder was suspended in 1 liter of distilled water. It was boiled shortly to dissolve completely and then sterilized by autoclaving at 121ºC for 15 minutes.

C. Blood agar

This is a non-selective general purpose medium which may be enriched with blood or serum.

Composition

Blood agar contains the following in grams per liter according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	g/l
'Lab-Lemco' powder	10.0
Peptone neutralized	10.0
Sodium chloride	5.0
Agar	15.0
pH 7.1-7.5	

Preparation

Forty grams of the powder was suspended in 1 liter of distilled water. It was boiled shortly to dissolve completely and sterilized by autoclaving at 121ºC for 15 minutes. The solution was then cooled and human blood added. It was then mixed gently and poured into Petri dishes.

D. Chocolate agar

This medium is used to culture nutritionally demanding pathogens such as *N. meningitidis* and *S. pneumoniae*.

Preparation

Medium was prepared as in that for blood agar except after adding the blood, the medium was heated for about 10-15 minutes until it became brown in colour.

E. Mueller-Hinton agar

This is used for antimicrobial susceptibility testing and it is used in internationally recognized standard procedures.

Composition

Mueller-Hinton agar contains the following in grams per liter according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	g/l
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

pH 7.2-7.4

Preparation

38 grams of the powder was dissolved in 1 liter of distilled water. It was then boiled shortly to dissolve completely and sterilized by autoclaving at 121ºC for 15 minutes.

F. Nutrient agar

This is a general purpose medium which may be enriched.

Composition

Nutrient agar contains the following in grams per liter according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	g/l
'Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

pH 7.2-7.6

Preparation

28 grams of the powder was dissolved in 1 liter of distilled water. It was then boiled shortly to dissolve completely and sterilized by autoclaving at 121ºC for 15 minutes.

Gram staining protocol

1. Make a smear of the specimen to be stained on a slide. Heat the slide for a few seconds until it becomes hot to touch so that bacteria are firmly mounted to the slide.

2. Cover the fixed smear with crystal violet stain for 30-60 seconds. Rapidly wash off the stain with clean water.

3. Add Gram's iodine for 30 seconds. This step fixes the crystal violet to the bacterial cell wall.

4. Decolorize rapidly with acetone-alcohol. Gram-positive bacteria will retain the primary stain whilst Gram-negative bacteria will lose the primary stain and will appear colourless at this stage.

5. Add the secondary stain, neutral red and wash off stain with water. Gram-positive bacteria will appear black-violet whilst Gram-negative bacteria will appear red-pink at this stage.

6. Examine the smear microscopically, first with the X40 objective to check the staining and distribution of material, and then with the oil immersion objective to report the bacteria cells.