AUTOMATIC DISK DIFFUSION ASSAY

BY

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ABSTRACT

Bacterial Infections have been a life-threatening issue for humanity for thousands of years. Antibiotics were the only choices available for us to deal with it since it was discovered in 1928. However, with the report from CDC in 2019, confirming that there was a rising in antibiotic resistances in various species of bacteria. It may not show the immediate lethality like how the virus pandemic does, but it will eventually become a great threat we need to deal with in the future. Therefore, a raise in antibiotics development and production needs to be conducted. In order to support and make this process more efficient. Some improvements in each individual step of the whole procedure could be possible. One interesting procedure is the Antibiotic susceptibility test to test the efficiency of the drug produced.An automating process could be applied to it and make this process much more simple to use. Therefore, the concept of this project is to Design and create a device that is capable of automatically simulating the mentioned antibiotic susceptibility test, in this case Disc Diffusion Test and applying the concept of image processing and Artificial Neural Network. The prototype hardware and corresponding software was constructed and tested with the simple pair of antibiotic and microorganism, *E.coli* and Ampicillin. It was found from the test result that this prototype system has some potential to detect the Zone of inhibition inside the microorganism plate sample with a 100 percent of accuracy. Although there was some problem with the accuracy of the zone of inhibition size measuring function, these results are the proof of concept for automating the Disc Diffusion technique and opens the opportunity to improve this system much more in the future.

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In the period of the novel coronavirus pandemic, many workplaces, companies and agencies would be forced to shut themselves down and lock the permission to go in and out these places. Of course, our KMITL is not the exception, therefore we cannot access the facilities inside for our project.

However, with the precious help from Miss Manao Bunkum who volunteered to support us from inside the KMITL facilities. Providing us a chance to complete our work without any issues.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbols/Abbreviations	**Terms**
DDM	Disk Diffusion Method
ADDM	Automatic Disk Diffusion Method
MIC	Minimum Inhibitory Concentration
E. coli	Escherichia coli
CLSI	Clinical and Laboratory Standards Institute
BSAC	British Society for Antimicrobial Chemotherapy
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ANN	Artificial Neural Network

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CHAPTER 1

INTRODUCTION

1.1 Statement of Problem

Antibiotic resistance related diseases are one of the world's most critical problems in the medical field [1]. It is rising to dangerously high levels in all parts of the world. It is able to cause huge damage to the global public health and economic system if countermeasures, prevention, or rapid diagnostic systems are not invented. Surprisingly, threats from antibiotic resistance are comparable in the same scale as the infamous coronavirus pandemic. Comparison of Coronavirus Disease 2019 and Antimicrobial Resistance is shown in Table 9 [2] below.

Table 1: Comparison of Coronavirus Disease 2019 and Antimicrobial Resistance [2].

Table 1.
Comparison of Coronavirus Disease 2019 and Antimicrobial Resistance

	Coronavirus Disease 2019	Antimicrobial Resistance
Characteristic		
Number affected worldwide	6.28 million[a] (annually, unknown)	64.5 million annually[b]
Knowledge of problem	Developing	Established
Spread	Fast	Gradual
Mechanism	New transfer from nonhuman host	Natural selection in humans, animals, and environment
Behavior change required		
Handwashing	Continuously needed	Continuously needed
Physical distancing	Urgent, possibly recurrent	Probably recurrent
Travel restrictions	Urgent, possibly recurrent	Probably recurrent
Quarantine	Confirmed and suspected cases	Confirmed and suspected cases
Impacts		
Mortality worldwide	379 000[a] (annually, unknown)	812 000 annually[c]
Economic impact	Unknown	$400 billion[d]
Health inequity	Increased	Increased
Management needs		
Vaccine	In development	Not available for resistant microbes
Augmented testing	Real-time picture of spread	Surveillance of problem
Rapid diagnostics	In development	Some useful tests (procalcitonin, C-reactive protein)
New drugs	In development	Few in development
Stewardship	In time	Continuously and internationally

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Annually, the worldwide mortality of antimicrobial resistance exceeds over 800,000 cases whereas the worldwide mortality of coronavirus is about 379,000 cases. This shows that antibiotic resistance shows more threats to the society than the coronavirus. In terms of availability of vaccines, vaccines against coronavirus are now available. While the availability of vaccines against antimicrobial resistance microorganisms are not available. The reason why vaccines against antimicrobial resistance microorganisms are not available is because the microorganisms are resistant against antibiotics that are supposed to eliminate it.

An example of a specific antimicrobial resistance case is with *Salmonella Typhi*. This certain type of microorganism tends to increase its resistance to ciprofloxacin overtime. Reaching the high of 74 percent in 2017 as shown in Figure 1 [45] below.

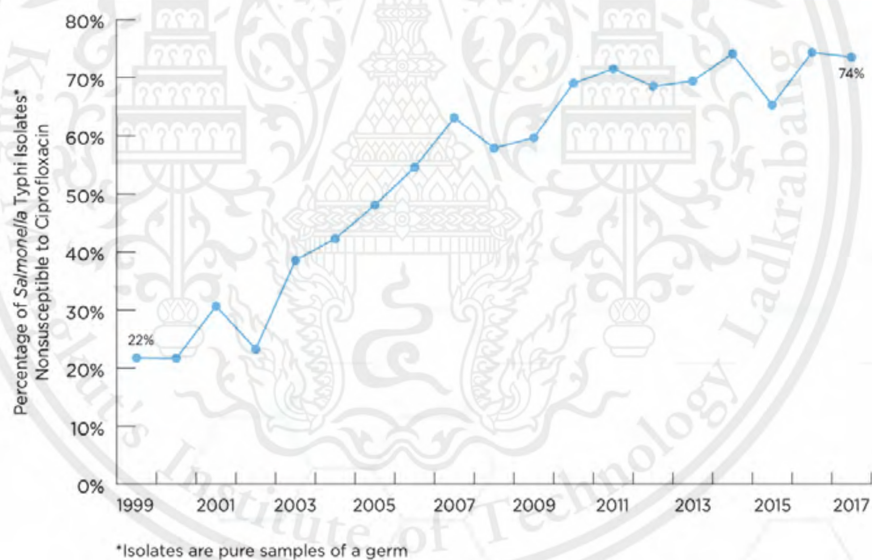

Figure 1: The percent of *Salmonella Typhi* infections non susceptible to ciprofloxacin [3].

Despite the annual launching of new top performance antibiotics, the expensiveness of these antibiotics are unreachable for poor people around the world [4]. Not to mention the group of poorest countries in the world where these expensive medicines are unreachable to them. This concludes that the expensiveness of the new antibiotics are the problems to solve [4].

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One of the few ways to solve this problem is to basically lower the cost related to giving these new high-performance antibiotics to people. To eliminate the drug resistance problems of infectious disease, prescription of the broad spectrum of antibiotics needs to be received by patients along with sending their specimens to the microbiological laboratory for antimicrobial susceptibility testing [4]. Generally speaking, the correct type of antibiotic given to the patient must be the one according to the testing report of each corresponding patient [4].

Another problem is too much time is taken for generating successful reports. Therefore, many efforts have been made to speed up the reporting process. One of those efforts is our device [4].

Nowadays, disk diffusion method (DDM) is the most common way to perform the antimicrobial susceptibility test [5]. This is because it has the ability to perform multiple combinations of bacterial specimens with its corresponding antibiotics.

However, DDM takes too much time. So, automated susceptibility tests based on disk diffusion methods (ADDM) is a must in order to provide a way to successfully solve these problems [4].

1.2 Objective

1.2.1 To design and assemble the Disc diffusion Camera Box that can be used to perform the Disc diffusion image analysis

1.2.2 To design and construct the software that is capable of detecting the presence of zone of inhibition and being able to measure its dimensions

1.2.3 To Observe and analyse the performance of the system when using the real disc diffusion microbial plates.

1.3 Research Scope

This research will focus on 2 main parts. The first part is the design and manufacturing of the Disc diffusion Camera Box using 5 main components: 2mm Acrylic plate; 220V LED lamp; plastic color filter; Blower electric fan; USB PC webcam along with the python software to detect and analyse the microbial plate image. The second part of this study is to observe the accuracy of this software in the object detection section and observe the changes in the pixel compositions in the images while the antibiotic.

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CHAPTER 2
LITERATURE REVIEW AND THEORY RELATED

This chapter reviews the automatic disk diffusion assay. Guiding on its state-of-the-art modern technologies which comprehend the convenience in performing the antimicrobial susceptibility tests that are contained in section 2.2 . Section 2.3 is about modern ADDM devices which share similar ideas with our ADDM device. Section 2.4 compares ADDM over DDM with especially focusing on ADDM convenience in terms of usage over that of DDM. Later on this chapter is about the comparison of advantages and disadvantages of ADDM against traditional DDM. Ending the chapter with the summary.

2.1 Disk Diffusion Assay/ Disk Diffusion Method

Disk diffusion method (DDM) also known as: Kirby–Bauer test, disc-diffusion antibiotic susceptibility test, disc-diffusion antibiotic sensitivity test and KB test is the most commonly used antibiotic susceptibility test in considering whether to choose the best antibiotics for use with the corresponding type of or even a species specific bacteria [23]. That particular bacteria will be culturing on the agar plate while the antibiotic inhibits the growth of the bacteria. The results of this can be graded as susceptible (sensitive), intermediate, or resistant to a particular antimicrobial by using a threshold. Thresholds are values which are categorized in standard tables depending on the combination of antibiotic and bacteria used [23]. These standard tables are published in guidelines of a reference body, such as the Clinical and Laboratory Standards Institute (CLSI), the British Society for Antimicrobial Chemotherapy (BSAC) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [30]. On the global scale, considering in terms of specific field of study, DDM is more preferable for the usage in the field of study of microbiology and in the field of hospitalization [30]. On the other hand, another purpose of DDM is also to determine the antimicrobial susceptibility of many organisms against wide-spectrum antibiotics [23]. DDM is also one of the most flexible susceptibility testing methods in terms of antimicrobial agents that can be tested [23].

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To begin with, the procedure of DDM starts by placing round paper disks which are saturated with a single type of antibiotic on colonies of bacteria, cultured on the surface of an agar medium [6]. Then, incubating that particular plate overnight. Lastly, identify the appearance of the zone if inhibitions exist [6]. If present, then we will be continuing on measuring the size of diameter [6]. However in case of absence of a zone of inhibition, countermeasures are needed to apply [6]. The presence and absence of zones of inhibition also apply [6].

The origin of the name 'Kirby-Bauer method,' is from studies conducted at the University of Washington in the mid-1960s in which it was published by Bauer and colleagues in 1966 [6]. This particular method standardized the variables of disk size, inoculum size, temperature, and time of incubation. Results are reported qualitatively as susceptible, intermediate, or resistant [6]. This particular Kirby-Bauer method (DDM) has been continually developed and distributed by the Clinical and Laboratory Standards Institute (CLSI) in the United States [6]. Several other international societies (e.gThe European Union Committee for Antimicrobial Susceptibility Testing (EUCAST), and the British Society for Antimicrobial Chemotherapy) also use this technique or any similar technique which has the same mechanism [7]. An alternative method to be shown in the next paragraph.

Alternative methods include devices that are able to measure the zones of inhibition using cameras and can definitely speed up the process of reading disk diffusion plates. These particular devices can also transform the zone diameter measured from the photograph produced by the camera into acceptable MIC values .

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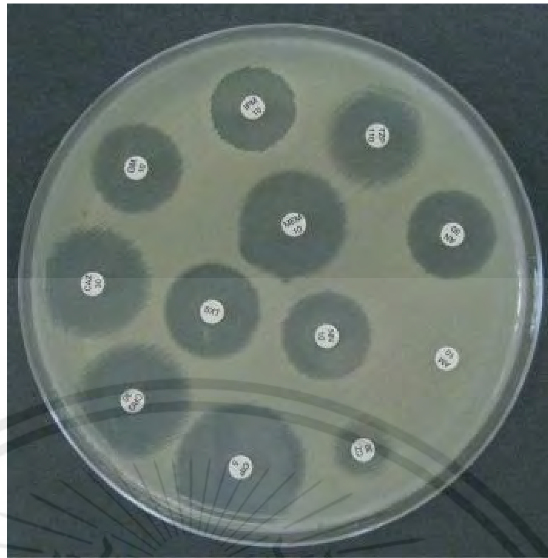

Figure 2: Photograph of a bacterial plate with tested antibiotic [8].

2.2 Procedure of DDM

Disc Diffusion Method (DDM) is a standardized referencing method which could be a regularly used technique to test the effectiveness and the efficiency of antibiotics or other substances which have effects on certain types of bacteria in the laboratory in several fields [9] . As we have mentioned, DDM has developed into a well-known, standardized version which can be used by everyone in the laboratory. Explanation of the steps that have to be taken in order to complete the Disk Diffusion Method (DDM) are described in the next paragraph [9].

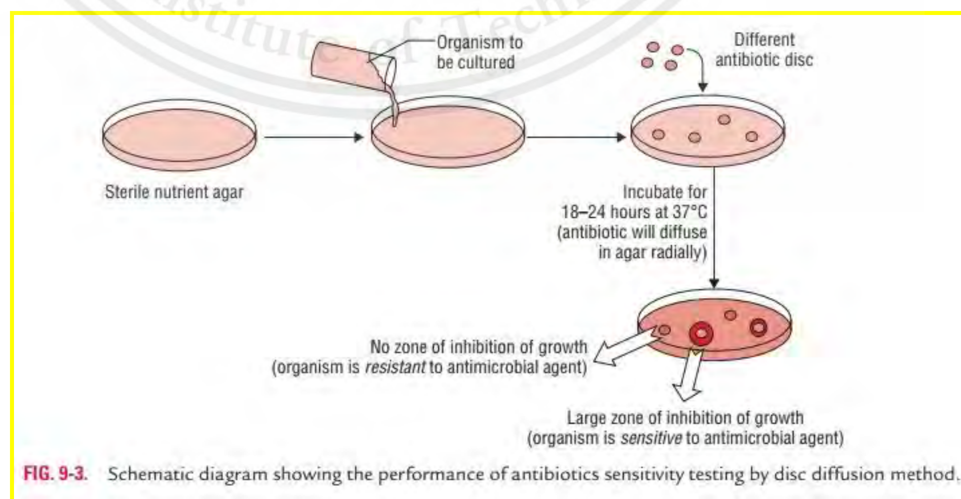

Figure 3: Explanation of steps in disc diffusion method [10].

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Prepare the bacteria from the primary culture plate by touching with a smear loop at the tops of each colony or any amount with similar visual appearance. Then take it to be tested in a tube of saline.

Compare the tube with the 0.5 McFarland turbidity standard (which will be discussed later on in this chapter) which contains cell density of 1.5 $x10^8$ CFU/ml. Then, add more bacteria or add more saline to adjust the density of the test suspension into the standard of turbidity. One thing to take into account is that adjusting the turbidity to meet its standard is very crucial in order to guarantee that the resulting lawn of growth is perfect or almost perfect.

Dipping a sterile swab into the bacterial plate. Remove the extra bacteria by pressing and rotating the swab firmly against the side of the tube above the level of the liquid. Step after this is the process of distribution of the bacteria across the bacterial plate. Now it can be done in 2 ways as follows: streak plate, and spread plate.

Streak plate

For the streak plate, start by wiping the swab across the agar plate around 3 to 4 times, rotating the plate through an angle of 60 °after reaching each imaginary corner of the plate. Please note that both the plate and the swab can be rotated in clockwise or in anticlockwise directions. However, the direction of the swab's rotation must be in the opposite direction of the direction of the plate's rotation. Lastly, rotate the swab around the edge of the agar surface, similar to Figure 3 [11] below.

Figure 4: Streak plate proper result [11].

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Spread plate

For the spread plate, wipe the swab across the agar plate in a triangular direction. Continuing on wiping the swab across the surface until the bacteria dry out. Similar to the streak plate method, both the plate and the swab can be rotated in clockwise or in anticlockwise directions. However, the direction of the swab's rotation must be in the opposite direction of the direction of the plate's rotation. The finished product must be similar to Figure 4 [12] shown below.

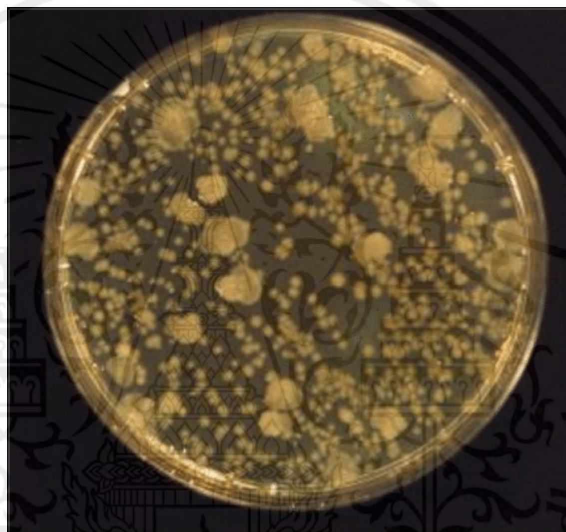

Figure 5: Spread plate proper result [12].

After finishing up on the streak/spread plate, leave the plate to dry for 3 to 5 minutes but don't exceed 15 minutes at room temperature with the lid closed. Prepare the antibiotic by buying the antibiotic. Put the antibiotic on disks. Place the antibiotic disk on the surface of the agar plate. Position the disks in a way that the minimal distance of center to center of a pair of the corresponding disks is approximately 24 mm apart. With the minimal distance from the edge of the petri dish of no closer than 10 to 15 mm.

In terms of population density, a maximum of 6 disks may be placed in a 90 mm petri dish and 12 disks on a 150 mm plate. Proper position of the disks are shown in Figure 5 [13] below.

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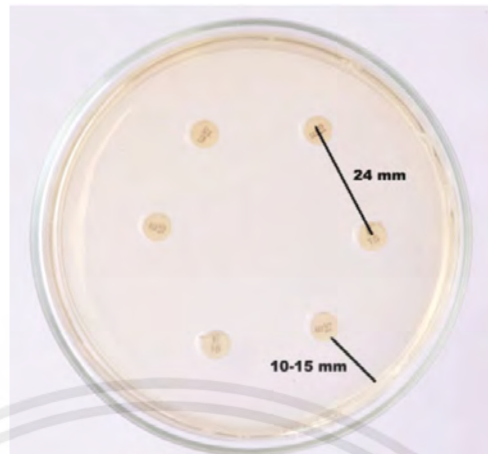

Figure 6: Disks proper positioning [13].

The discs can be placed on the prepared spread/streak plates using a pair of forceps. Always remember that the forceps must be sterilized first. The pattern of the placement of the antibiotic discs can be in any pattern, uniform pattern is preferred, because it can lead to easier measuring of the zone of inhibition. Template can also be used as a convenient tool to place the antibiotic uniformly rather than using the traditional sterile needle tip. Antibiotic disc dispensers are shown in Figure 6 [14] below.

Figure 7: Automatic disc dispenser [14].

Caution, disks should not be placed closer than 24 mm (center to center) on the plate. For both sterile tip usage and the automatic disk dispenser usage, the disks also should not be placed directly next to the edge of the plate. Furthermore, no more

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than 12 disks should be placed on a 150-mm plate or more than 5 disks on a 100-mm plate.

Another thing to remember for automatic disc dispensers, when using it, ensure that the disc is not attached to the edge. Also each disc should be gently pressed down, not too hard and not too soft. Pressing too hard will cause a hole in the agar. Pressing too soft will not cause the disc to stick, leading to no result.

The plates then should be placed in an incubator at 37 °C for 16 hours within 30 minutes of preparation. Temperatures above 37 °C invalidate results for oxacillin/methicillin. As a caution, do not incubate in an atmosphere where lots of carbon dioxide is present, because this will decrease the pH of the agar and result in errors due to incorrect pH of the media.

After 16 hours of incubation, diameters of each zone of inhibition which include the diameter of the disc should be measured and recorded in mm in which the endpoint of inhibition is judged by the naked eye at the edge where the growth starts. However, there are 3 exceptions for determining the edge of the zone of inhibition as follows. For the case where sulfonamides and co-trimoxazole are presented, the slight growth occurs within the inhibition zone; such growth should be ignored. Also, for the case where the bacteria is β-lactamase-producing staphylococci and the antibiotic is penicillin, the zones of inhibition are produced with a heaped-up, clearly defined edge; these are readily recognizable when compared with the sensitive control, and regardless of size of zone of inhibition, they should be reported as resistant. Furthermore, for the case where certain Proteus species are presented in the zone of inhibition. The layer of it should be ignored if the edge of the zone of inhibition is clearly visible,

These recorded diameters are then interpreted according to the antimicrobial susceptibility interpretation chart. This chart is shown below. Please note that the values in antimicrobial susceptibility interpretation charts vary among the differences in usage of species of bacteria and its corresponding antibiotics. To show the difference in value, 2 examples are shown below.

In this case, the bacteria is *Pseudomonas spp.* and its corresponding antibiotics used. Its antimicrobial susceptibility interpretation chart is shown in Table 2 [15] below.

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Table 2: Antimicrobial susceptibility interpretation chart of *Pseudomonas spp*. bacteria [15]

Antimicrobial agent	Symbols	Disc content (µg)	Inhibition zone diameter (mm)		
			R	I	S
Ampicillin	AM	10	≤11	12-13	≥14
Amoxacillin	AX	25	≤13	14-16	≥17
Cephradine	CE	30	≤14	15-17	≥18
Cefuroxime	CXM	30	≤14	15-17	≥18
Cefoperazone	CEP	75	≤15	16-20	≥21
Cefepime	FEP	30	≤14	15-17	≥18
Imipenem	IMP	10	≤13	14-15	≥16
Amikacin	AK	30	≤14	15-16	≥17
Gentamicin	CN	10	≤12	13-14	≥15
Ciprofloxacin	CIP	5	≤15	16-20	≥21
Levofloxacin	LEV	5	≤12	13-15	≥16

R: Resistant, I: intermediate, S: sensitive.

Another case of example where the bacteria is *E. coli* and its corresponding antibiotics (we use *E. coli* as our experimental bacteria, and we use ampicillin as its corresponding antibiotic), its Antimicrobial susceptibility interpretation chart is shown in Table 3 below.

Table 3: Antimicrobial susceptibility interpretation chart of *E. coli* and its corresponding antibiotics [16].

Antimicrobial	Susceptible	Intermediate	Resistant	Reference
Amikacin	≤16	32	≥64	CLSI M100-S22, 2012
Ampicillin	≤8	16	≥32	CLSI M100-S22, 2012
Amoxicillin-Clavulanate	≤8/4	16/8	≥32/16	CLSI M100-S22, 2012
Cefoxitin	≤8	16	≥32	CLSI M100-S22, 2012
Ceftiofur	≤2	4	≥8	CLSI M31-A4, 2013
Ceftriaxone	≤1	2	≥4	CLSI M100-S22, 2012
Chloramphenicol	≤8	16	≥32	CLSI M100-S22, 2012
Ciprofloxacin	≤1	2	≥4	CLSI M100-S21, 2011
Gentamicin	≤4	8	≥16	CLSI M100-S22, 2012
Kanamycin	≤16	32	≥64	CLSI M100-S22, 2012
Nalidixic Acid	≤16	-	≥32	CLSI M100-S22, 2012
Streptomycin	≤32	-	≥64	NARMS Executive Report 2009
Sulfisoxazole	≤256	-	≥512	CLSI M100-S22, 2012
Tetracycline	≤4	8	≥16	CLSI M100-S22, 2012
Trimethoprim-Sulfamethoxazole	≤2/38	-	≥4/76	CLSI M100-S22, 2012

CLSI = Clinical and Laboratory Standards Institute.

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The diameters of the zone of inhibitions can be measured by a ruler. To start measuring, place the ruler on the surface of the plate (on the upside down side of the plate, not on the side where bacteria and antibiotics are presented) and also keep the lid closed. Another way to measure the diameter of the zone of inhibition is to measure it with a pair of calipers, this way of measurement is eligible for measurement of opaque plates. An example of measuring the zone of inhibition by using a ruler is shown in Figure 7 [17] below. Also an example of measuring a zone of inhibition by using a pair of calipers is shown in Figure 8 [18] below.

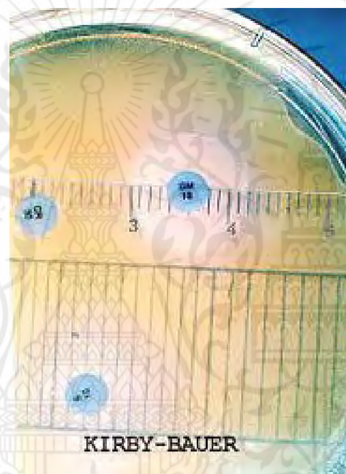

Figure 8: Using a ruler to measure the zone of inhibition [17].

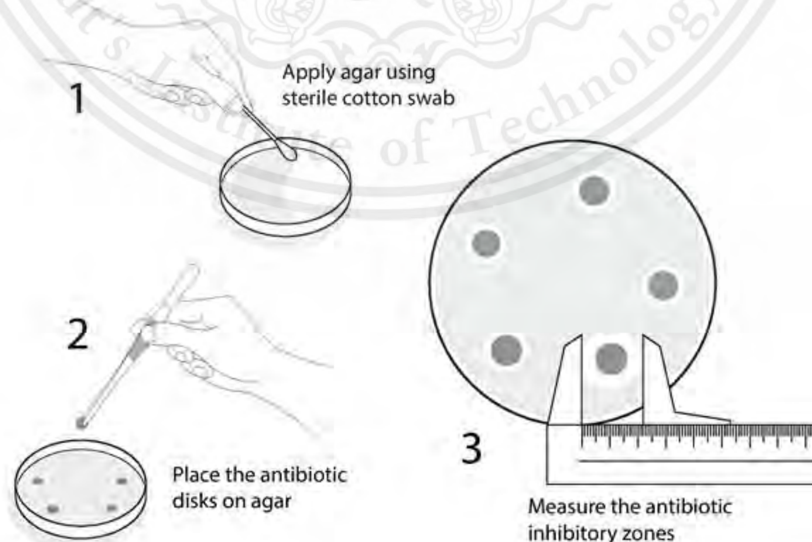

Figure 9: Using a pair of calipers to measure the zone of inhibition [18].

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After that, use a template to assess the final result of the susceptibility tests. Another way to categorize the result is to use the published Clinical and Laboratory Standards Institute (CLSI) guideline or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline, to determine whether the test is categorized as susceptibility or resistance. Take into account that there are different charts for different bacteria and its corresponding antibiotics. The flowchart summarizing antibiotic susceptibility testing is shown in Figure 9 [9] below.

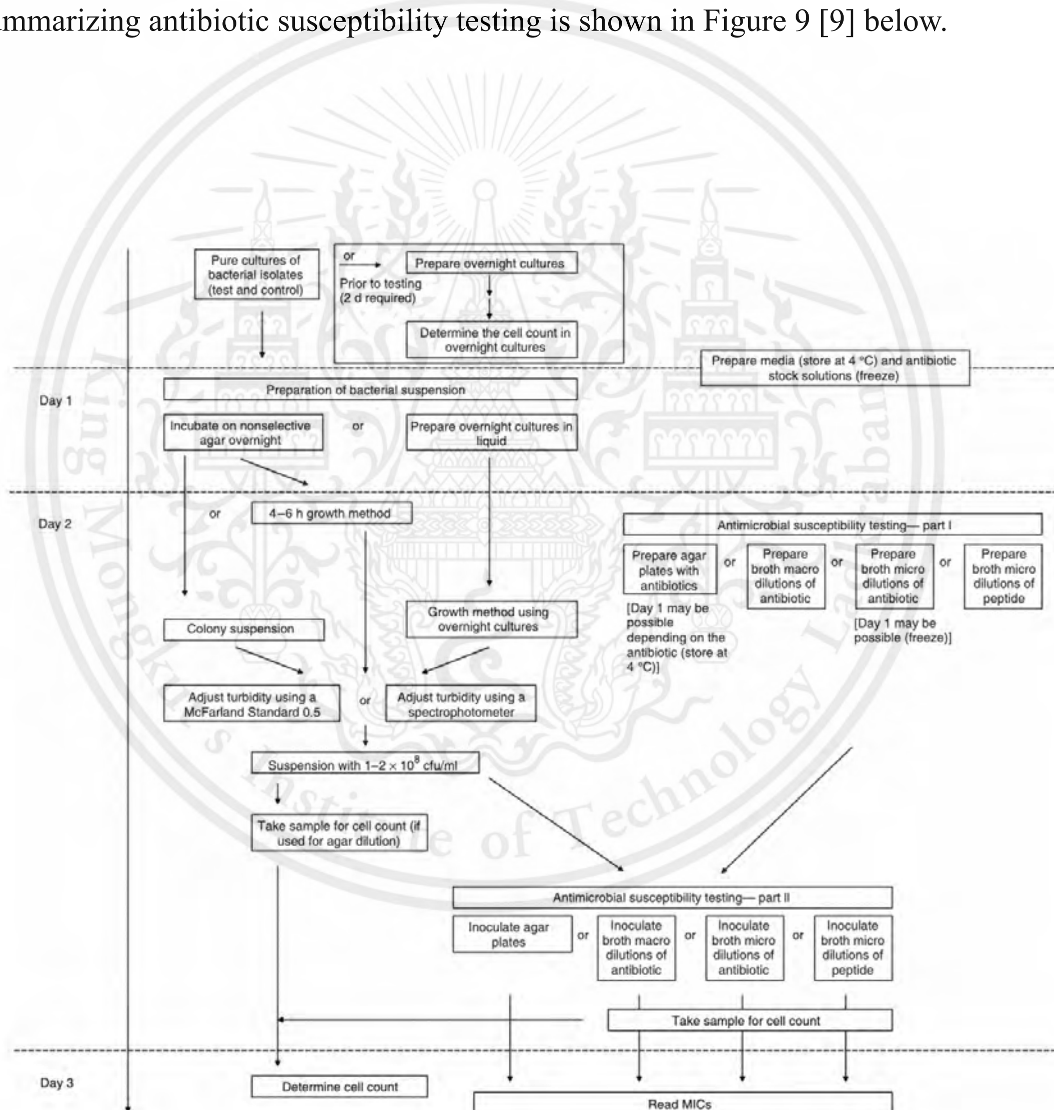

Figure 10: Flowchart summarizing antibiotic susceptibility test [19].

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2.3 Problem of DDM

There are approximately three problems in the area of disk diffusion method (DDM) which are the mentioned limitations of DDM (2 limitations), and the convenience in terms of usage. The idea of using a typical ruler and or a pair of calipers is not convenient at all. Also using a ruler or a pair of calipers required a definite set of skills of both measuring instruments, such that human errors when measuring could occur. With these mentioned counterparts in both ruler and a pair of calipers usages, a new and modernized approach to minimize the human errors upon measuring the diameter of the zone of inhibition must be invented. In response, we came up with a new way of measuring the zone of inhibition which is called "Automatic Disk Diffusion Method" (ADDM). The ADDM will be discussed later on in chapter 2 of this document.

2.3.1 Rejection Criteria

<u>Steps</u>

1. Plates with contaminants similar to what is shown in Figure 10 [20] below are rejected.

Figure 11: An example of a plate with contaminants [20].

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2. Plates with overlapped zones of inhibition are rejected.

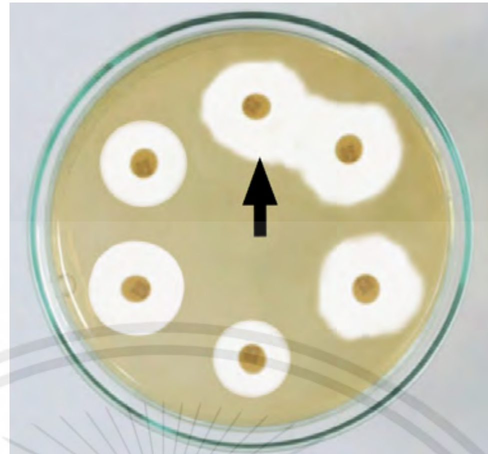

Figure 12: An example of a plate with an overlapped zone of inhibition [21].

3. Plates with irregular shape of zone of inhibition are rejected as well.

Figure 13: An example of a plate with irregular shape of zone of inhibition [22].

2.3.2 Limitations of DDM in clinical field

Limitations of DDM in the clinical field are measurement variation, human error, time consuming, and excessive laborious work [23]. Another limitation of the disk diffusion method is the determination flaws in which it cannot provide accurate information about the Minimum Inhibitory Concentration (MIC) value [24].

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2.3.2.1 MIC value and ways of determination

Minimum inhibitory concentration (MIC) is the lowest concentration of a substance (in this case, the antibiotic) which prevents visible growth of a bacteria. There are many factors which determine the MIC value, such as the type of bacteria itself, human or host status, and the antibiotic itself [24]. The unit of measurement for MIC is in micrograms per milliliter (μg/mL) or milligrams per liter (mg/L) [24].

In the clinical field, the minimum inhibitory concentration needs to be identified when a patient is in these three following situations [24]. First situation is when the patient is not responding to treatment which is supposed to be successful. Second situation is when the patient is having a relapse. The third situation is when there is immunosuppression.

For our experiment with ADDM, the lowest concentration when the isolated bacteria is completely inhibited can be determined by identifying the visible area where bacteria are absent. This is used to be documented as minimal inhibitory concentration (MIC). There are 3 dilution methods used to achieve minimal inhibitory concentration value which are broth dilution method, agar dilution method, and e-test method. The agar dilution method is indeed the disk diffusion method that we have already discussed. For the broth dilution method, it will be explained later on in this chapter. The most interesting method is the e-test method, because it is the newest dilution method in which it overcomes some disadvantages of agar dilution method and broth dilution method. The e-test method will also be discussed within this chapter later on.

Furthermore, there are 3 reasons behind the mentioned susceptibility test being chosen to test on bacteria which are suspected of causing disease or other forms of abnormality for humans. Second reason for the susceptibility tests to be taken is when a particular type of bacteria is expected to belong to a suspected species and are prone to appear to be resistant to frequently used antibiotics. In the field of epidemiological studies, the third reason for the susceptibility test is in the application of resistance surveillance, where usage of susceptibility tests are for testing the efficiency and the effectiveness of the existing agents or for the discovery of new type of antibiotics for better usage while also showing some comparisons of it in variety of terms.

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The usage of dilution methods to determine the minimum inhibitory concentrations (MIC) of antibiotics is to act as reference methods for the susceptibility test to determine whether the results are susceptible or resistant. Another usage of these methods is to compare the result of the new agent with the result of the existing agents. The reason for the comparison is to establish the susceptibility of bacteria that give uncertain results in disc tests. The other reason for tests on bacteria is for when the disc test is undependable.

The mentioned three types of dilution method are separated by the type of plate and food they used. During the dilution tests, bacterias are tested for their ability to grow on two different sets of plates which are growing in agar plates (agar dilution) and growing in broth (broth dilution) in which they contain dilutions of their antibiotics depending on their corresponding combinations. The result which contains the lowest concentration of an antibiotic in the unit of milligram per millilitre that inhibits the appearance of visible growth of the cultured bacteria is known as the MIC. Note that MIC value needs to be identified under two circumstances. One being the condition that must be defined within a period of time. Second being the condition must be in vitro.

Main applications of MIC include acting as a reference for the doctor to choose the best antibiotic for treatment, and for scientists for completing their susceptibility test in their respective laboratories. Leading to the necessary approaches for consideration of careful control and standardization for operations in both inside the laboratories and for the outside of laboratories.

<u>How is MIC reported?</u>

MIC results in 3 ways which are sensitive, resistant, or intermediate [24]. Sensitive means that the bacteria is inhibited by the antibiotic using normal dosage. Resistant is the opposite of sensitive. Lastly, intermediate means that the bacteria is inhibited by the antibiotic using normal dosage using the maximum amount of dosage possible.

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<u>When are MICs cannot be used?</u>

There are 4 situations when the MICs cannot be used which are as follows

1. Some bacteria cannot use MIC altogether.
2. When the CLSI guidelines for certain bacteria are not available.
3. The antibiotic is not effective at all in clinical practices. Usable antibiotic list is shown in Table 4 [24] below.

Table 4: Table displaying usable antibiotics for determining MIC value [24].

Antibiotic	Susceptible	Intermediate	Resistant breakpoint
Amikacin	≤4	8	≥16
Amoxicillin (skin and soft tissue)*	≤0.25	0.5	≥1
Amoxicillin (urine)*	≤8	16	≥32
Amoxicillin/clavulanic acid (skin and soft tissue)*	≤0.25	0.5	≥1
Amoxicillin/clavulanic acid (urine)*	≤8	16	≥32
Benzylpenicillin *Enterococcus*	≤8		≥16
Benzylpenicillin *Staphylococcus*	≤0.12		≥0.25
Cefovecin (skin and soft tissue)	≤0.5	1	≥2
Cefovecin (urine)	≤2	4	≥8
Cefpodoxime	≤2	4	≥8
Ceftazidime Enterobacteriaceae	≤4	8	≥16
Ceftazidime *Pseudomonas*	≤8	16	≥32
Cephalexin (skin and soft tissue)	≤2	4	≥8
Cephalexin (urine)	≤16		≥32
Chloramphenicol	≤8	16	≥32
Clindamycin gram-positive	≤0.5	1–2	≥4
Ciprofloxacin gram-negative	≤1	2	≥4
Doxycycline (respiratory, skin, and soft tissue)'	≤0.12	0.25	≥0.5
Doxycycline (urine)'	≤4	8	≥16
Enrofloxacin	≤0.5	1–2	≥4
Erythromycin	≤0.5	1–4	≥8
Florfenicol	≤2	4'	≥8
Gentamicin gram-negative	≤2	4	≥8
Gentamicin *Staphylococcus*	≤4	8	≥16
Imipenem gram-negative	≤2	4	≥8
Marbofloxacin	≤1	2	≥4
Minocycline (skin and soft tissue)	≤0.5	1	≥2
Nitrofurantoin (only reported on urine cultures)	≤32	64	≥128
Oxacillin *Staphylococcus aureus*	≤2		≥4
Oxacillin *Staphylococcus* (non-*S. aureus*)	≤0.25		≥0.5
Polymyxin B (not reported on urine cultures)	≤2	4	≥8
Pradofloxacin	≤0.25	0.5–1	≥2
Trimethoprim/sulfa	≤40		≥80

2.3.2.1.1 Broth Dilution Method

Broth dilution method (BDM) is a technique in which containers holding identical volumes of broth with antimicrobial solution in incrementally (usually geometrically) increasing concentrations are inoculated with a known number of bacteria [25]. It is generally accepted that broth minimum inhibitory concentrations tests are acceptable within one doubling dilution of the real end point. Where the endpoint is for one well or for a suitable tube amount [25].

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There are two other types of broth dilution which are the broth microdilution and broth macrodilution [26]. Broth microdilution denote denotes the performance of the broth dilution test in microdilution plates with a capacity of equally or less than 500 microlitre per well, so this dilution method uses broth volume of 1 ml in standard test tubes. Whereas the broth macrodilution uses about 0.05 to 0.1 ml total broth volume and can be performed in a microtiter plate or tray. In general, the procedure for both macrodilution and microdilution are the same except they are different in terms of their broth volume values.

Antibiotic solution can be prepared by commercially available antimicrobial powders with known potency by using this following formula [26].

1000/P * V * C = AW

<u>Where</u>

P = potency of antibiotic powder (microgram per milligram)

V = Volume required (mL)

C = Final concentration of solution (milligram per litre)

AW = Weight of antibiotics in milligram to be dissolved in V to achieve C

For the preparation of antibiotics dilution is advised to be prepared at a concentration at tenth times the highest concentration to be tested [27]. Store the solution by distributing small quantities into sterile tubes and freeze at -60 degree celsius [39]. Frozen antibiotics stock should be thought only once then discarded [27].

For the material required, antibiotic powder, microtiter plates, gestures, pipettes, cation-adjusted mueller hinton broth, sheep blood agar, mcfarland standard, slime, test bacteria, and QC bacterias [27]. Next up we make the plates [27]. For non-fosterious bacterias, cation-adjusted mueller hinton broth is necessary. The range of antibiotic dilutions in the microtiter plate should be two-fold higher than the actual working concentration [27].

Next up is antibiotic controlling, the necessary equipment for this is the antibiotic and cation-adjusted mueller hinton broth, and most importantly is the microtiter plate [27]. For the mapping of the microtiter plates is shown in Figure 13 [28] below.

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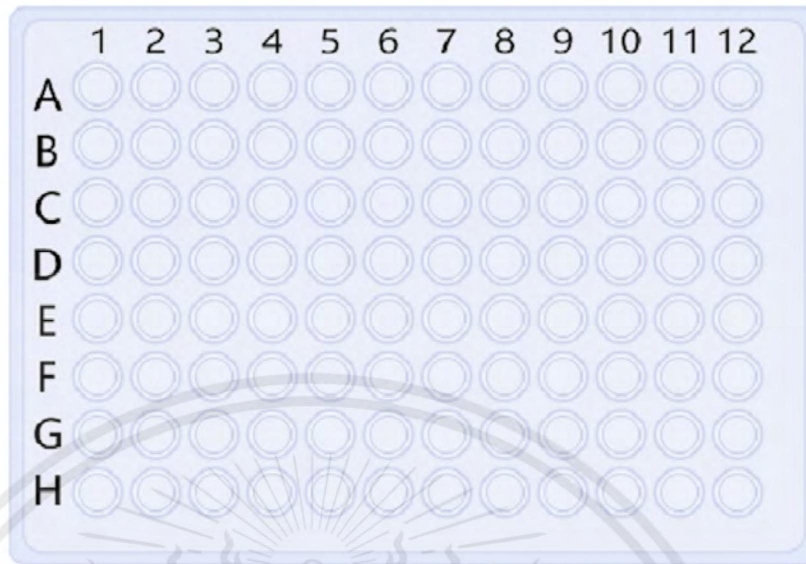

Figure 14: Map of microtiter plate [28].

For this map, advice for coordination is to firstly use the 1-column holes as the antibiotic control. So, place antibiotic and cation-adjusted mueller hinton broth. The 2-column holes for highest concentration in microgram per millilitre. 3 to 11-columns for antibiotic serial dilutions where the eleventh column is the lowest concentration in that particular serial dilution. Column twelveth is the growth control contains only cation-adjusted mueller hinton broth with bacterial suspensions [27].

For the inoculation process start off by using a sterile pipette dispense 200 microliter of antibiotic solution in the first column [27]. Bacteria suspension is diluted by adding 10 microliter of suspension in 990 microliter of saline [29]. Dispense 100 microliter of antibiotic solution and 100 microliter of cation-adjusted mueller hinton broth in column 1 of antibiotic control [27]. Moving up on to the second column toward the twelfth dispense 100 microliter of cation-adjusted mueller hinton broth with 100 microliter of antibiotic solution transferred from the first column and mix with cation-adjusted mueller hinton broth. Then, mix thoroughly and serially dilute the cation-adjusted mueller hinton broth and antibiotic solution sequently and consecutively until reaching the eleventh column. After that, discard that particular 100 microliter. For the last column which is the twelfth column, place only the cation-adjusted mueller hinton broth without any antibiotic [29].

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For the growth control, start by diluting the bacterial suspension by one by thousand. This is done by adding 100 microliter of diluted inoculum into the second column thoroughly toward the eleventh column. Next up, add 200 microliter of cation-adjusted mueller hinton broth in the sterility control column which is the twelfth column. Then, cover up the microtiter plate with a sterile lid or parafilm [29].

To check the purity of the inoculum suspension, streak a drop of bacterial dilution into sheep blood agar plate and incubate [29]. The optimal temperature of incubation is at 35 degree celsius in ambient air [29]. Upon finishing up the incubation, record the result [29]. Observe the result by identifying the growth on the purity microtiter plate [29]. Growth control column should have visible growth [29]. While the sterility control and antibiotic control column should not have any growth [29]. MIC value of controlled bacteria must be within the recommended range of CLSI standard antimicrobial susceptibility chart [29]. Test is valid if and only if the MIC value falls within the mentioned range [29].

2.3.2.1.2 Agar Dilution Method

Agar dilution method is another method for the determination of MIC value of an antibiotic. The procedure is as the following steps [29].

Firstly, begin with preparation of antibiotic stock solution. Then, prepare antibiotic dilution range. After that, prepare agar dilution plates. Then, prepare the inoculum and perform inoculation. Incubate it. Finally, read and interpret results.

Please note that the antibiotic stock solution can be prepared by commercially available antimicrobial powders in different portions which can be calculated by the following formulas shown in Figure 14 [30] below.

Formula 1: Formula to determine the amount of antimicrobial powder needed for standard solution.

Formula 2: Formula to determine the amount of diluent needed for standard solution.

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$$\text{(1)Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration (µg/mL)}}{\text{Potency (µg/mg)}}$$

or

$$\text{(2)Volume (mL)} = \frac{\text{Weight (mg)} \cdot \text{Potency (µg/mg)}}{\text{Concentration (µg/mL)}}$$

Figure 15: Formulas for determining amount of antimicrobial powder (1), diluent (2) needed for standard solution [30].

Upon using the formula for calculation, please note that there are 2 requirements. First is the concentration of antimicrobial agent stock solution is needed to be prepared in the amount of at least 1000 µg/mL [29]. The second requirement is the concentration of antimicrobial agent stock solution is needed to be prepared in the amount of at least 10 times the highest concentration to be tested [29].

For the filtration, membrane filtration is acceptable for the filtering of the solutions. To begin with, dispense small volumes of the sterile stocks solutions into sterile glass, polypropylene, polystyrene, or polyethylene vials. Then, cautiously seal and store at the temperature ranging from −60 °C to −20 °C [29]. However, filtration is not required if the solution has been separately prepared. This is because microbial contamination is very rare [29]. Some new and useful applications of agar dilution include the ability to assess the antimicrobial activity of natural plant material [31]. These include essential oils and plant extracts [31]. Antimicrobial effects of these plant materials can also be evaluated by the method [31].

<u>Preparation of antibiotic dilution range</u>

- For the test tube use sterile 13- x 100-mm test tubes. For later use, please freeze it.
- Use plastic caps, metal caps, cotton plugs, more even the screw-caps to secure the tube.

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- For the remaining dilutions of antibiotic agent, prepare it directly in the broth.
- The result is 1 mL of each dilution.
- For microdilution, the result is 0.1 mL in terms of volume.

Preparation of inoculum

- Select isolated colonies from an 18 to 24 hour agar plate for creating a direct broth suspension.
- Modify the turbidity of the created direct broth suspension equivalent to the turbidity of a 0.5 McFarland turbidity standard.
- Use a card with white background and black line to compare the inoculum tube and the 0.5 McFarland standard.
- Dilute the 0.5 McFarland suspension in 1:150 within 15 minutes after finishing up the preparation process. The eligible result in each tube is supposed to be around 5×10^5 CFU/mL.

Inoculation

- From the steps above, immediately add 1 mL of the adjusted inoculum to each diluted tube containing 1 mL of antibiotic agent. Also add it to the broth positive control tube.
- Mix it up until it results in a 1:2 dilution of each antibiotic concentration and a 1:2 dilution of the inoculums.

Incubation

- Incubate for 16 hours at 35 ± 2 ºC in an incubator. Caution, avoid stacking the trays more than 4 times high in order to prevent the difference in temperature.

Interpretation and Quality Control

- Measure the growth of bacteria in mm by comparing the growth amount in the wells against the amount of growth in the control well which is the one that has no antibiotic agent [27].
- The result of the test is “Valid” or “ Invalid”
    - Valid test, acceptable growth is more than or equivalent to 2 mm button. In terms of turbidity, the test is valid when definite turbidity can be identified.

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- Anything other than this is considered the test to be invalid.
- The procedure is shown in Figure 15 [29] below.

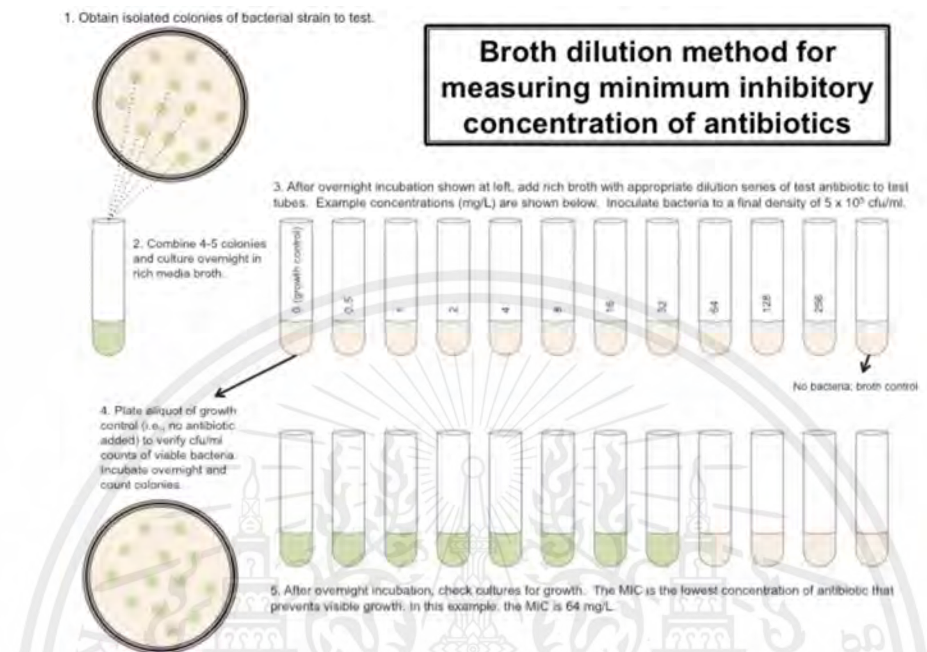

Figure 16: Broth dilution procedure [29].

- For quality control, use bacterial strains which have stable and acceptable MIC value as a control subject to test with 2 of our diluted tested subjects. One with MIC value above the MIC value of control subject. Another one with MIC value less than the MIC value of the control subject [29].
  - CLSI's Quality Control limitations for dilution susceptibility tests.
    i. *E. coli* ATCC 25922,
    ii. E. faecalisATCC 29212,
    iii. P. aeruginosa ATCC 27853
    iv. S. aureus ATCC 29213.

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2.3.2.1.3 Epsilometer (E-Test) Method

The Epsilometer (E-Test) method is the newest method for determining MIC value. E-Test is a method where standard reference MIC dilutions of an antibiotic have been repackaged onto a dry plastic strip. E-Test is based on the combination of both diffusion and dilution methods [32]. E-Test strip is a non-porous plastic rectangular strip with 5 mm in width, and 60 mm in length. It consists of predefined continuous and exponential gradients of antibiotic concentrations [32]. The strip is shown in Figure 16 [33] below.

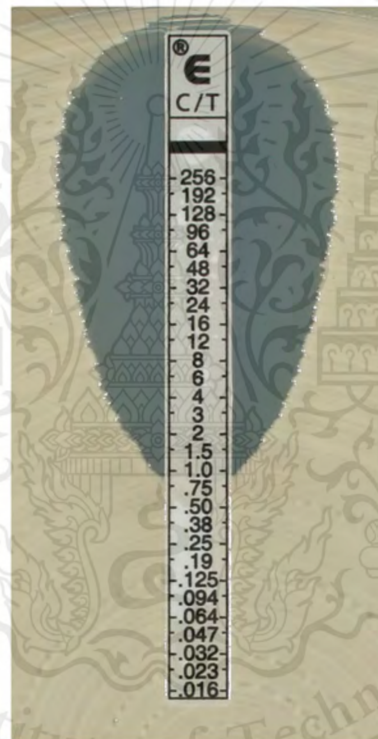

Figure 17: E-Test strip [33].

This method is useful in determining a direct quantification of antimicrobial susceptibility of bacterias [32]. Furthermore, it is also useful for determination of the MIC of fastidious, non-fastidious, nutritionally deficient bacterias, low level resistance, and lastly the normal or strange looking resistance patterns [32].

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2.3.2.2 Preparation of McFarland Turbidity Standards

McFarland turbidity standards is a standard for reference of turbidity for bacterial suspension [31]. The method of referring a suspension with the turbidity to the McFarland turbidity standard is to look at it visually. The result can be compared just like Figure 17 [34] below.

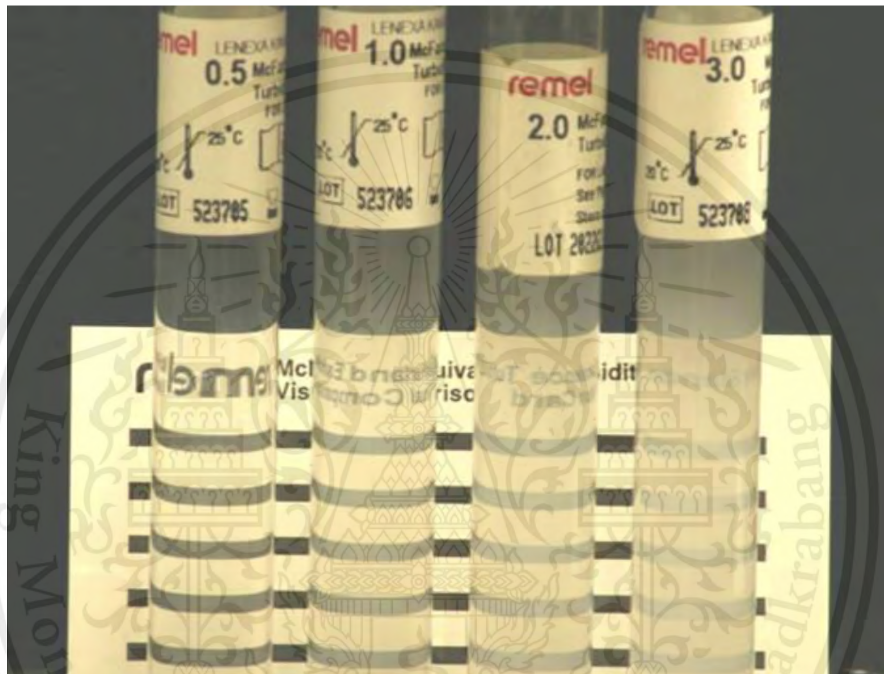

Figure 18: Turbidity test [34].

The McFarland turbidity standards are prepared by mixing 1% sulfuric acid and 1% barium chloride. For our case with DDM, the 0.5 McFarland turbidity standard is used as standard which is comparable to the density of a bacterial suspension 1.5x 10^8 CFU/ml [34].

<u>Procedure</u>

Firstly, prepare a 1% solution of anhydrous barium chloride (BaCl2). Then, prepare a 1% solution of sulfuric acid (H2SO4). Lastly, mix them up to form a turbid suspension and BaSO4 in a specific proportion for each McFarland turbidity standard as shown in Table 5 [34] below.

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Table 5: McFarland turbidity standard table [34].

McFarland turbidity standard no.	0.5	1	2	3	4
1% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1×1^8 CFU/ml)	1.5	3	6	9	12

After establishing the table above, put the mixture in a foil-covered screw–cap tube. Then, store at 25 °C and vortex it. Next, mark the tube to indicate the level of liquid. After that, identify that no evaporation occurs. Most importantly, compare the density of bacterial suspension with the McFarland turbidity standard by holding it in between a light source and a white background with black stripes. Within the period of 15 minutes, use saline or broth to dilute if the density is too much. On the other hand, add more bacteria if there is not enough density.

2.4 Automatic Disk Diffusion Assay

Automatic disk diffusion assay (ADDM) is a device that calculates the result of disk diffusion assay of an antibiotic and its corresponding bacteria where the output is one of the following, susceptible, resistant, or intermediate. To be more specific, these three types of results which are congruent to that of the traditional disk diffusion method (DDM).

Truthfully there are several ADDM devices available throughout the market for instance, the VITEK 2 by bioMérieux, the BD Phoenix System, OSIRIS Video Reader System, etc. Where in this case, 3 of them are in this chapter. These devices can effectively provide the susceptibility results of antimicrobial susceptibility tests, without the need for rooting the result. These needs are done by measuring the diameter of the zone of inhibition by hand, using that diameter value to compare it with antimicrobial susceptibility charts which are provided by CLSI or EUCAST for MIC values determination. Use this MIC value to determine the output as susceptible, intermediate, or resistant.

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In this chapter, we will be mainly discussing the idea of using automatic disk diffusion method over traditional disk diffusion method; its advantages over traditional disk diffusion method, its disadvantages to traditional disk diffusion method. While focusing on the convenience of using automatic disk diffusion method over traditional disk diffusion method. The 3 mentioned real life device examples will be demonstrated and explained in this chapter. To be more specific, aiming at describing their purposes, function, mechanism, efficiency and effectiveness, and finally their cost management.

Please note that our device is not congruent to the VITEK 2 and BD Phoenix System. It only shares similar theories and principles regarding the automated antimicrobial susceptibility test only. To be more specific, our device does not work exactly the same as both VITEK 2 and the BD Phoenix System, it only shares a similar mindset on how the automated disk diffusion method should be. Contrary to the VITEK 2 and BD Phoenix System, our device is moderately similar to the OSIRIS Video Reader System which will be deeply described in this chapter.

2.5 Modern Day ADDM Devices

There are exactly 3 modern day ADDM devices in this chapter which are as follows, the VITEK® 2: Healthcare System, the BD Phoenix™ automated identification and susceptibility testing system, and lastly the OSIRIS video reader system.

2.5.1 OSIRIS Video Reader System

Introduction

OSIRIS video reader system is an automatic disk diffusion method device that uses images or videos for analysis of the automatic determination of the zone of inhibition of disk diffusion [35]. This system is considered as an image processing technology which uses computer systems to operate and enhance its performance [36]. This device also has an ability to perform susceptibility tests on both conventional disk diffusion plates and microdilution panels. [36]. An algorithm is being used for finding the diameter while continuing on the radial analysis of the disk diffusion [35]. The input of this analysis is the pattern of a bacterial growth on agar

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plates around a paper disk containing a type of antibiotic [35]. The OSIRIS system transforms the image of the disk diffusion plates; taken by the camera, into computer digital signals using image processing which is triggered by the commands of the computer program [35]. This program then analyzes the images into 3 choices of output which are susceptible resistant, or intermediate of the bacterial growth against the antibiotics used to test [35].

Standards used as references for the susceptibility testing are the National Committee for Clinical Laboratory Standards (NCCLS), and the Comité de l'Antibiogramme de la Societe Franl'aise de Microbiologie (SFM) standards [35].

Method

Starting with using the camera to capture a picture of the cultured bacterial plates [37]. The picture then will be recorded and stored as an image in the PC [37]. Next, the analytic program will use that stored image by transforming it into digital computer signals which will then be calculated into the output choices of susceptible, resistant, or intermediate [37].

The OSIRIS system contains a processing unit, a camera, and the OSIRIS software which is based on the coding of Microsoft Visual Studio [37]. It will automatically measure and analyze the inhibition zones around the antimicrobial discs in the disk diffusion test plate [37]. The reading processes which include plate moving and CCD ‘s image capturing are controlled by a program implemented in CP-2051 V2.0 PLUS ETT controller [37]. The captured image is further transferred to the processing unit via Prolink Pixelview PV-BT878P [37]. The captured images of cultured plates are then operated with image processing techniques such as edge enhancing, edge detection, perimeter detection, and re-circulating of inhibition zones. It is also smoothed by a median filter and enhanced by a 5x5 Zobel element [37]. The enhanced zone will be detected and re-circulated as the white circles [37]. Lastly, the measured diameter of the zone of inhibition is interpreted and displays the mentioned output by the analytic program [37].

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Result

The OSIRIS system tended to read lower inhibition zones when compared with manual readings [37]. The time required for reading 16 antibiotics placed on 120-mm2 plates was less than 3 seconds, including categorisation of zone diameters as susceptible, intermediate and resistant [37]. In the OSIRIS system, time taken to provide results can be longer if the microbiologist decides to check the diameter inhibition zones in the monitor and to modify the automatic interpretation results [35].

In conclusion, OSIRIS system is a useful automatic antimicrobial susceptibility testing system [35]. However, readings need to be slightly modified to reduce inhibition zones deviations, which can enhance final perfor- mance of the OSIRIS system [35]. For the record, an example of the mentioned inhibition zone deviation values categorized by antibiotics are displayed in Table 6 below [35].

Table 6: An example of inhibition zone deviation table [35].

	Number of isolates displaying the following mm difference within indicated manual reference mm							Number and percentage of errors					
Antimicrobial agent	<−3	−3	−2	−1,0,1	2	3	>3	Minor		Major		Very major	
Ampicillin	1	2	5	50	2	2	1	4/63	6%	1/19	5%	0/41	0%
Amoxicillin–clavulanate	4	1	7	38	3	3	7	4/63	6%	1/46	2%	1/11	9%
Ticarcillin	1	0	4	47	3	5	3	1/63	2%	1/28	4%	0/33	0%
Cefazolin	1	1	5	53	0	0	3	1/63	2%	0/48	0%	1/14	7%
Cefuroxime	1	3	10	44	0	0	5	2/63	3%	0/54	0%	0/7	0%
Cefoxitin	2	1	9	41	1	1	8	4/63	6%	1/52	2%	0/10	0%
Cefotaxime	3	3	3	51	0	0	3	3/63	5%	0/58	0%	0/0	0%
Ceftazidime	1	2	5	46	5	1	3	1/63	2%	1/61	2%	0/2	0%
Imipenem	0	1	2	42	5	5	8	0/63	0%	0/63	0%	0/0	0%
TOTAL	14	14	50	412	19	17	41	20/567	3.5%	5/427	1.2%	2/130	1.5%

2.5.2 VITEK 2

VITEK 2 system is another device alternative for the OSIRIS video reader system. VITEK 2 detects bacterial growth and metabolic changes by using fluorescence based technology. The system has the advantage of reducing the handling time involved in manual methods for antimicrobial susceptibility testing.

It also has everything healthcare laboratories need for fast, accurate microbial identification, and antibiotic susceptibility testing [38]. VITEK 2 includes an

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expanded identification database for the comparison between the experimental results with the standard reference results [38].

Method

Figure 19: VITEK 2 mechanism and procedure for antimicrobial susceptibility testing [38].

The mechanism is based on phenotype matching. As visualized by the MIC pattern of a particular species and resistance mechanism, the VITEK 2 will design a distinctive color flag to separate specific groups of bacteria [38]. It creates a definite positive identification for all bacteria which is the bacteria's distinctive fingerprint or phenotype [38]. Basically, every bacteria has a form of identification which resembles a fingerprint which is a clear distinctive set of makers based on its individual phenotype and its susceptibility to different types of antibiotics [38]. This MIC pattern is a bacterial type's unique fingerprint and each known fingerprint of the type of bacteria is a part of a database of more than 3600 phenotypes with greater than 55000 MIC distributions [38]. When a match is found, the VITEK 2 then goes a step further clearly flagging each result (Green is for a match to known phenotype. Yellow is for a match except for one antimicrobial or test that is automatically stopped for review. Red is for no match.) [38]. The vast majority of results will get a green flag which is to be matched to a known phenotype [38].

Furthermore the availability of the VITEK 2 system brings a positive impact on both the workflow and the quality of the information. It only speeds the reporting

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of results, it also minimizes the chance for human error that is an inevitable part of manual review.

2.5.3 BD Phoenix Automated Identification And Susceptibility Testing System

The BD Phoenix Susceptibility System consists of an oxidation reduction indicator, turbidimetric growth detection, full on-panel antimicrobial concentrations and the BDXpert System. The results are further enhanced by specific delayed resistance features, providing additional assurance that Phoenix results are not only rapid but accurate. For the monitoring and the comparison of results is the duty of the BDXpert System where many reference standards can be used to calibrate the results which are the same types with the two previous devices. The results are then again displayed in either susceptible, resistant, or intermediate.

This system is the combination of the AP instrument, the M50 instrument, and the BD EpiCenter™ System for automated antimicrobial susceptibility testing. Without the combination, the automated antimicrobial susceptibility testing could not be completed.

2.5.3.1 AP Instrument

BD Phoenix AP, is a device that provides the laboratory workflow efficiency and standardized isolated inoculum [39]. The BD Phoenix AP is the first system to use automated nephelometry in antimicrobial susceptibility testing in which this step has traditionally been the most time consuming [39].

To inoculate a panel using BD Phoenix AP, starting with creating a heavy suspension of the bacteria preferred to be tested in Phoenix ID broth [39]. The system is capable of processing a starting McFarland of 0.20 to 4.0 to the appropriate testing McFarland [39]. The ID broth and corresponding AST broth are placed in a Phoenix AP rack and loaded onto the system for preparation [39]. Rack processing time is 5-7 minutes. Each Phoenix AP is capable of processing 200 ID/AST sets in less than 4.5 hours [39].

The system also contains the BD EpiCenter Barcode Printing Software which is the identification software for identifying the antibiotics when in operation [39]. By generating and scanning the barcodes, the different types of information that are going

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to be identified includes patient name, patient location, sample accession number, specimen type, Phoenix panel type, and isolate number [39]. Usage of the barcodes eliminates the risks of mixing up the subjects, which can lead to severe errors [39]. AP performance is displayed in Figure 19 [40] below.

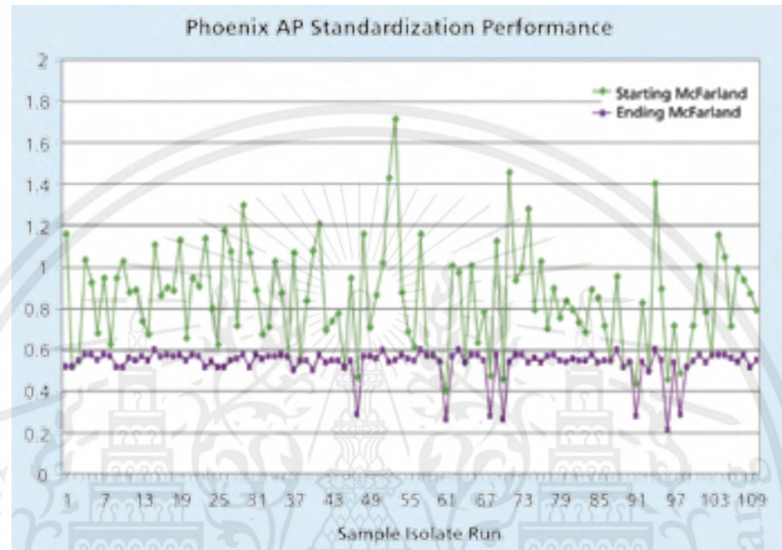

Figure 20: BD Phoenix AP standardization performance [40].

2.5.3.2 M50 instrument

The BD Phoenix M50 instrument provides the antimicrobial susceptibility results in those mentioned 3 types of final outputs [41]. The system uses unique dual growth detection technology and an innovative panel design with a wide range of antibiotics and dilutions to help provide the foundation for detection of emerging resistance [41].

The results are very accurate and very time efficient [41]. This aid the treatment procedure in the field of bacteria infections treatment. Leading to better judgement and decisions made by the patients for their suitable corresponding treatment programs [41].

Furthermore, the procedure contains 2 parts which are the panel preparation, and the identification of antimicrobial susceptibility testing [41].

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Part 1: Panel preparation

1. Suspend bacterial colonies in BD Phoenix ID broth and recep the tube.
2. Vortex
3. Place tubes in BD Phoenix AP sample rack
4. Uncap the ID and AST tubes
5. Place the rack in the input queue of the BD Phoenix AP instrument
6. Tap the start button: The instrument will automatically standardize the inoculum in ID broth tubes.
7. Adds BD Phoenix AST indicator and inoculates the BD Phoenix AST broth tubes.
8. Remove the rack and place it on the BD Phoenix inoculation station for data synching to the BD data management system.
9. Scan the rack and labels to connect each sample to the corresponding patient accession number.
10. Pour the contents of both tubes into the appropriate side of the panel and cap the panel.
11. Place the panels into the BD Phoenix M50 instrument. Output readings will be produced. A Summary of the procedure is shown in Figure 20 [42] below.

Figure 21: Summary of panel preparation procedure [42].

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Part 2: Identification of antimicrobial susceptibility testing

1. Use the tubes of part one and place it on the nephelometer
2. Add the BD Phoenix AST indicator to the BD Phoenix AST broth tube. Then, recep the tube. Mix by inverting it.
3. Add inoculum from the ID broth tube to the AST broth tube. Mix by inverting it.
4. Pour the contents of both tubes into the panel at the appropriate side of the panel. Then, cap the panel.
5. Place the panels into the BD Phoenix M50 instrument. The result is whether it is resistant, susceptible, or intermediate. A Summary of the procedure is shown in Figure 21 [43] below.

Figure 22: Summary of Identification of antimicrobial susceptibility testing procedure [43].

2.5.3.3 BD EpiCenter System

BD EpiCenter is basically the monitoring system for the antimicrobial susceptibility testing that provides real-time data access and analysis tools [39]. It compares the experimental results with the reference standards in order to come up with the best and the most suitable results [39].

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The system contains thousands of similar or related tests that have already been done. It uses these datas to compare with its own while in testing procedure [39]. It also compares it with the reference standards, whether it was the CLSI or the EUCAST or, etc. It compares it all, in a very short amount of time [39]. So, it is considered as one of the most time efficient monitoring data systems in the modern day [39].

The system also verifies that the existing test results in the data storage system really exist. Not just fake results [39]. To do this, it allows users to define rules based on any patient or facility demographic coupled with the result outcome and give authorisation to the managers to utilize the alerting capability integrated into the software [39]. This is to securely verify that the proper action is taken when each event is identified [39].

The system also makes sure that the authorized Infection Control Officer is able to ensure that the correct individuals are notified when a writing of new data occurs, for instance new types of resistance patterns or specific bacteria or detected [39].

The system also includes libraries which contain over one hundred predefined queries and reports [39]. These extra libraries provide the ability to write new or access raw data, use it in several types of formats, and most importantly share the new experimental data for other testers [39]. An example of the mentioned over one hundred predefined queries and reports is displayed in Table 7 [44] below.

Table 7: An example of the predefined queries and reports [44].

		Name Collection Date (Formatted)											
		Cefazolin		Cefoxitin		Clindamycin		Gentamicin		Levofloxacin		Oxacillin	
Name	Specimen Hospital Service	# Results	% S	# Results	% S	# Results	% S	# Results	% S	# Results	% S	# Results	% S
Staphylococcus aureus	3 BLUE	13	30.77%	13	30.77%	13	84.62%	13	100.00%	13	92.31%	13	30.77%
	3 ORANGE	32	37.50%	32	37.50%	32	84.38%	32	100.00%	32	78.13%	32	37.50%
	4 ADOLESCENT MED	30	30.00%	30	30.00%	30	50.00%	30	93.33%	30	60.00%	30	30.00%
	4 MEDICINE	28	32.14%	28	32.14%	28	67.86%	28	78.57%	28	71.43%	28	32.14%
	4 SOUTH	28	32.14%	28	32.14%	27	85.19%	28	100.00%	28	75.00%	28	32.14%
	5 NORTH	11	27.27%	11	27.27%	11	54.55%	11	81.82%	11	81.82%	11	27.27%
	ADOLESCENT MEDICINE CLINIC	15	20.00%	15	20.00%	15	93.33%	15	100.00%	15	73.33%	15	20.00%
	ALLERGY CLINIC	11	72.73%	11	72.73%	11	63.64%	11	100.00%	11	90.91%	11	72.73%
	BURN CENTER	33	57.58%	33	57.58%	33	60.61%	33	100.00%	33	69.70%	33	57.58%
	BURN TREATMENT	15	46.67%	15	46.67%	15	66.67%	15	100.00%	15	60.00%	15	46.67%
	EMERGENCY ROOM	182	19.23%	182	19.23%	179	93.85%	182	100.00%	182	86.26%	182	19.23%
	GEN PEDIATRIC EVENING CLINIC	68	25.00%	68	25.00%	62	93.55%	68	100.00%	68	80.88%	68	25.00%
	GENERAL PEDIATRIC CLINIC	25	16.00%	25	16.00%	25	96.00%	25	100.00%	25	72.00%	25	16.00%
	PULMONARY CLINIC	200	44.50%	201	44.28%	198	41.41%	201	88.56%	201	52.74%	201	44.28%
	Total	691	33.00%	692	32.95%	679	71.28%	692	95.23%	692	71.39%	692	32.95%
Total		691	33.00%	692	32.95%	679	71.28%	692	95.23%	692	71.39%	692	32.95%

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2.6 Comparison Between ADDM And DDM

In terms of convenience, the ADDM is much more convenient than DDM, because it requires less time, is easy to use, and does not require highly-skilled training beforehand.

In terms of performance, the ADDM outran the DDM in terms of minimizing errors. This is because the ADDM does not cause any human error, whereas the human errors for DDM is possible to occur. Leading to better performance for ADDM than DDM.

In terms of cost efficiency the ADDM is still victorious. This seems quite odd considering the traditional DDM is much cheaper than the ADDM when performed. This is because no machinery and software is involved in the procedure of DDM. Whereas for ADDM, software and machinery are its powerhouse. However the ADDM is much more cost efficient, because when performed several times (in the amount of worldwide or nationwide usage), ADDM is much cheaper to perform than the traditional DDM. This is because the salaries of the staff for traditional DDM can be replaced by the automatic machine which is the ADDM device. So, the ADDM provides better cost efficiency than traditional DDM.

2.7 Chapter Summary

In conclusion, there are 3 ADDM available in our modern day time. These 3 devices are OSIRIS video reader system, BD Phoenix System, and VITEK 2 system. OSIRIS video reader system is the one that shares the most similar principle and mechanism in terms of use with our device. This device uses a camera to record pictures or videos. Then, analyze it using a PC software. After that, the program will generate one of the 3 following choices of output which are susceptible, resistance, or intermediate. For the comparison between DDM and ADDM, there are 3 terms for our case. These 3 terms are in terms of convenience, performance, and cost efficiency respectively. Triple victory for ADDM in terms of convenience, performance, and cost efficiency.

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CHAPTER 3
METHODOLOGY

3.1 Introduction

In Chapter 2 we identified everything about ADDM. To be exact, it was about modern day ADDM devices including, OSIRIS video reader system, VITEK 2, and BD Phoenix Automated Identification And Susceptibility Testing System. For the BD Phoenix Automated Identification And Susceptibility System, we are mainly focusing on its three components of instruments which are the AP instrument, the M50 instrument, and the BD EpiCenter System. Moving on, we compare ADDM with traditional DDM. We use three categories in comparison which are in terms of convenience, in terms of performance, and in terms of cost efficiency; in which the ADDM takes the wins in all categories.

For the Microorganism plate and the antibiotic pair which will be used as the samples for the system to analyse, *E.coli* and Ampicillin was chosen to be the base sample type for the system. The reason why these two were chosen among the other microorganisms and antibiotics was that the E.coli itself is the common bacteria found all over the places so that it would cause less harm compared to other disease-causing germs if some mistakes happen during the project assembly and testing. And for Ampicillin, this antibiotic was chosen as the known antibiotic that works effectively against E.coli.

This chapter describes the design of our automatic disk diffusion assay device, a system that determines susceptibility results of a certain type of bacteria when engaging with a certain type of antibiotic in 3 choices of outputs which are susceptible, intermediate, or resistant. This chapter will be divided into two main parts which are the hardware part and the software part. Both parts then are divided into 3 psub-topics which are the design methodology, the system requirement, the interesting problems, and the proposed solution. Lastly, the chapter ends with the summary of the chapter.

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Our device mainly consists of 3 devices which are a so-called "box" which is the place where our experiment is performed, a web camera, and a computer program in which it calculates and displays output.

3.2 Hardware Part

3.2.1 Design Methodology

The design and development of the so-called "box" is the place where the experiment is performed. This box is a box with dimensions of 12 centimeters by 12 centimeter by 35 centimeter. Made with black acrylic sheet and merged together with acrylic welding solution, grout, super glue, and duct tape; carefully detailed by using grout; along with mounting some of its components with strong mounting tape; we create this box until it is the perfect match for our experiment. The reason why we choose black color is because it eliminates all interference from all the external lights that shine to the box, causing blur when taking pictures by the web camera. The steps for our construction of the box is as follows.

1. Cut the black acrylic plate into the mentioned dimensions, and merge it together with an acrylic welding solution. Compliment it with super glue and duct tape for extra protection.
2. Drill 6 holes at the same height measuring from the bottom of the box by using a power drill.
3. Insert 3 M6 knots and bolts into 3 drilled holes. These knots and bolts act as a foundation of transparent acrylic floor.
4. Place the transparent acrylic on both layers of M6 knots and bolts.
5. Merge the LED panel and the LED downlight with electrical switches by soldering. Cover it with electrical tape.

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Figure 23: Modified LED panel and LED downlight

6. Place the LED panel at the bottom of the box. Place the LED downlight at the top of the box.
7. Adjust the focus of the web camera by rotating the camera lens.

Figure 24: Blurred web camera (before len adjustment)

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8. Place the web camera at the top of the box. Adjust its angle and height for the best view.
9. Try covering the LED panel with multiple shades of transparent plastic paper. Do this to control the amount and color of light that shines from the LED panel. All the colors used are shown in the figure below.

Figure 25: Colors of papers and stickers used. From left to right: light blue paper, black paper, white paper, black sticker, gray sticker, and white sticker.

   a. Blue transparent plastic paper testing with LED panel. Result shown in the figure below.

Figure 26: Result by blue transparent plastic paper

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b. Black plastic paper testing with LED panel. Result shown in the figure below.

Figure 27: Result by black plastic paper

c. White transparent plastic paper testing with LED panel. Result shown in the figure below.

Figure 28: Result by white plastic paper

d. Black sticker testing with LED panel. Result shown in the figure below.

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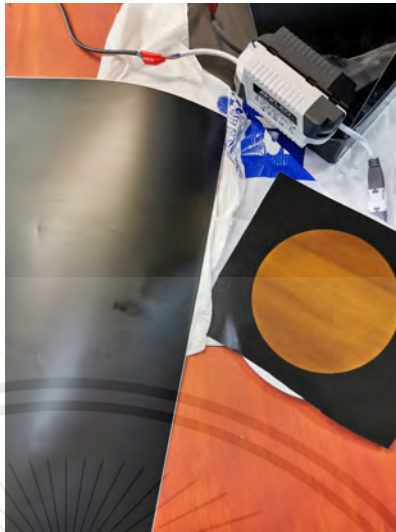

Figure 29: Result by black sticker

e. Gray sticker testing with LED panel. Result shown in the figure below.

Figure 30: Result by gray sticker

f. White sticker testing with LED panel. Result shown in the figure below.

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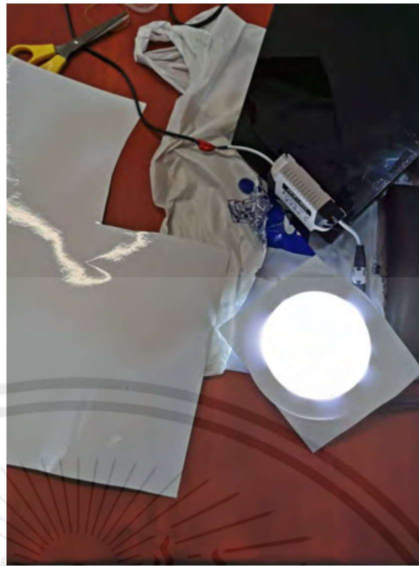

Figure 31: Result by white sticker

In conclusion, we decided to use the blue transparent plastic paper as it provides the best light filter for the plate when taking a photo by the webcam. As you can see from the figure below.

Figure 32: Result after filter.

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10. Identify the dimension of the fan to see whether it can fit inside the box or not.

Figure 33: Mini USB cooling fan

11. Try to break through the fan into its control system. Do this by using a power drill and a screwdriver.

Figure 34: After use a power drill

Figure 35: Using a screwdriver

12. Once through, remove the battery. The reason is to connect it with the system instead, so that we can directly turn on or turn off the mini fan by using the main switch. More convenient.

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Figure 36: Before battery removal.

Figure 37: After battery removal

13. We connect it with USB type A to micro USB B cord. Install it at the bottom of the box, underneath the plate and the LED panel light.

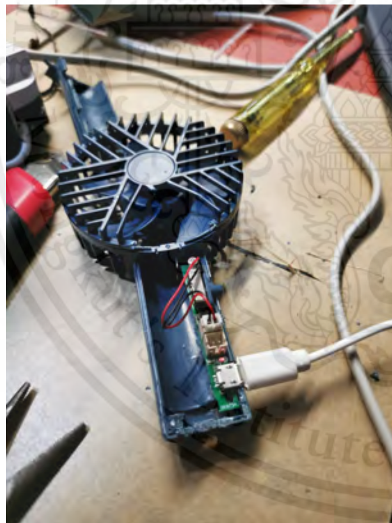

Figure 38: Connect the fan with the cord

Figure 39: Install it in the box.

14. Clean the box for excess debris. The output for the hardware part is shown in the figure below.

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Figure 40 : Final work of hardware part

3.2.2 System Requirement

Materials required for the device are listed as follows.

1. 4 Megapixel web camera
2. Black acrylic sheet
3. Clear acrylic sheet
4. Feature board
5. White transparent plastic paper
6. EVE Lighting’s LED Panel Circle 6 W
7. EVE Lighting’s LED Downlight COB Circle 5W Daylight
8. Sibyl’s mini usb cooling fan
9. M6 bolt and nut
10. Switch
11. Extended wire
12. Adapter
13. PC
14. USB type A to micro USB B cord

Tools required for the device are listed as follows.

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1. Acrylic welding solution
2. Grout
3. Super glue
4. Duct tape
5. Mounting tape
6. Clear tape
7. Electrical tape
8. Acrylic cutter
9. Acrylic axe
10. Soldering gun and soldering kit
11. Power drill
12. Standard toolset

3.2.3 Interesting Problems

There are 4 main problems with our hardware part. The first problem is that during the 16 hours of running our device, the temperature is too hot for the bacteria. Thus, affecting the growth of *E. coli* while also affecting the inhibition ability of ampicillin.

The second problem is that the heat from the LED panel inhibits the growth of the *E. coli* itself instead of the ampicillin in the disk.

The third problem is that the web camera cannot take full photos of the bacterial plate. The picture often displays the bacterial plate appearing to be either out of frame, or not located at the center of the frame. These situations cause the picture to be ineligible for the software later on.

The fourth problem is that in an air conditioned room or around 25°C, the plate appears to be surrounded by water droplets. These droplets interfere with the photo capturing of the plate by the web camera. So, the web camera cannot successfully take an eligible picture of the plate.

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3.2.4 Proposed Solution

Successful countermeasure for all of the four major problems of our device is deployed which are as follows.

For the first problem in which during the 16 hours of running our device, the temperature is too hot for the bacteria which affects the growth of *E. coli* while also affecting the inhibition ability of ampicillin, the proposed solution for successful countermeasure is to install a tiny fan below the disk. When the fan is running, the wind from the fan blows the heats within near the plate to some place else. Resulting in acceptable temperature for the *E. coli* to continue growing, and also allows the ampicillin to continue on inhibiting the growth of *E. coli*.

For the second problem in which the heat from the LED panel inhibits the growth of the *E. coli* itself instead of the ampicillin in the disk, the proposed solution for a successful countermeasure is to create some distance between where the LED panel is located, and where the plate is located. The best distance is 3 centimeters. So, we set the distance to be exactly 3 centimeters. Using black acrylic plate to separate between the LED panel and the plate is not acceptable, because black color of the acrylic plate blocks all the essential light from the LED panel. So, we used clear transparent acrylic instead.

For the third problem in which the web camera cannot take an acceptable picture of the plate, the proposed solution for a successful countermeasure is divided into two ways as follows.

1. Extend the height of the camera by using an extra black acrylic plate to form extra space for the extension. This solution is used when the captured image appears to be missing the outer part of the plate.
2. Install a plate holder which in our case is created by using a feature board and cut it to perfectly fit the shape of the plate.

For the fourth problem in which the water droplets at the plate block the sight of the web camera, the proposed solution for a successful countermeasure is to conduct the experiment in a normal ambient temperature at around 37 °C instead of conducting it in an air conditioned room at 25°C.

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3.3 Software Part

3.3.1 Design Methodology

The main concept behind the software design of this project is to make it more "YOLO" which stands for "You Only Looked Once". It means that you only need to look at the program and click on the button one time and the program will automatically do the rest of the work. We use PyCharm to create our program. According to the main concept, we design the software structure to be a large computer script that contains all the required processes starting from the following steps.

1. Users input the antibiotic amount that is used in the plate to the program's terminal. This step's purpose is to let the program estimate the appropriate dimensions of the zone of inhibition in the plate which will be used to compare with the actual results acquired from the camera. Our display where the user input antibiotic amount is shown in the figure below.

Figure 41: Antibiotic amount input display

2. The program window will be constructed, previewing the image that camera saw. After you make sure the plate is placed correctly within the slot on the acrylic plate, the only thing to do is pressing the shutter button to take the picture. In our case, we set the spacebar button to be the shutter button.
3. After acquiring image(s) from the camera, the image will undergo a set of image processing techniques and artificial neural network classification in order to determine the presence of a zone of inhibition in the plate and to measure the area of that zone. The final result of this step will be in the unit area. Our image processing and artificial neural network process is shown in the figure below.

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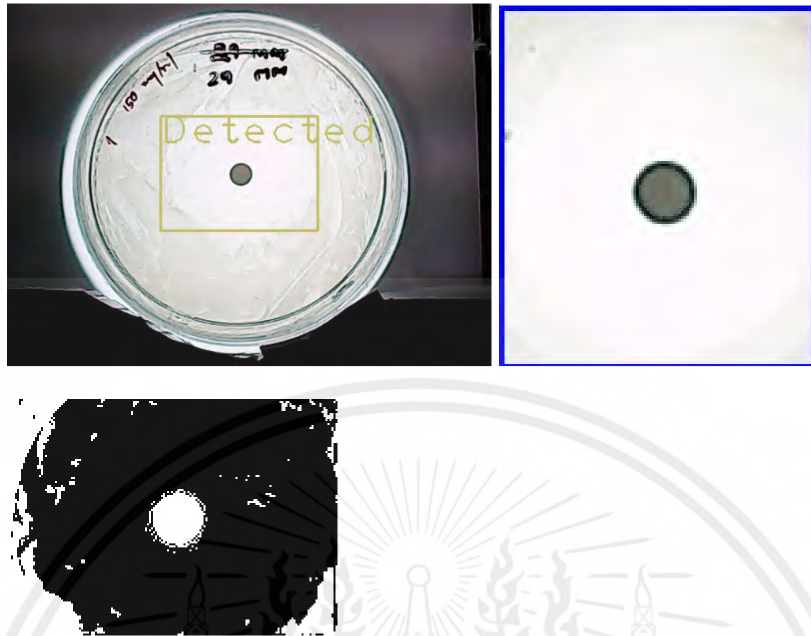

Figure 42: Our image processing and artificial neural network process.

4. Once the area of the zone of inhibition is acquired, the program will compare the measured result with the estimated value. If the measured value is not much different from the estimated one, the result will be categorised into the "Susceptible" group, meaning that the antibiotic in that plate can efficiently inhibit the bacteria at an acceptable rate. Otherwise, the result will be in the "Resistant" case as the zone of inhibition is not large enough or not inhibition at all.
5. The final result will be shown in the program terminal in the same location the input is typed. The display of our final output is shown in the figure below.

```
SingleShotMode
C:\Users\acer\PycharmProjects\ObjDetection\venv\Scripts\python.exe
Enter an Ampicillin Amount: 500
C:/Users/acer/PycharmProjects/ObjDetection/camera/SingleShotImg1.jpg
12735
Failed: Resistant

Process finished with exit code 0
```

Figure 43: Display of our final output

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This software is designed using python as the fundamental language with the aid of OpenCV libraries in order to process images, access to the camera and artificial neural network Packets. An overview of our program is shown in the figure below.

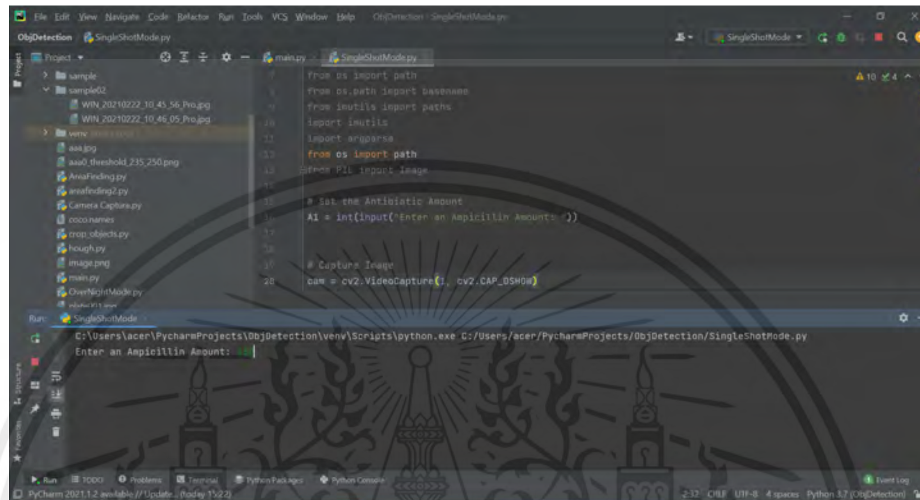

Figure 44: An overview of our program

3.3.2 Algorithm and Artificial Intelligence

In the part of the algorithm, in order to detect the presence of zones of inhibition efficiently, we introduce the deep-learning image classification using artificial intelligence to support this progress. The AI will be trained specifically to label the region in the image that is supposed to be the zone of inhibition.

After that, the program will crop the image, leaving only the labeled area. Then, the cropped image will be filtered via the image thresholding in order to turn the area in the zone of inhibition into black pixels and turn the rest into white pixels. The last process to measure the area is to count the number of black pixels in the image.

For the part of estimating the appropriate area, the Disc diffusion test is performed with a variety of antibiotics ranging from 50 mg/ml, 100 mg/ml, 150 mg/ml, until 500 mg/ml in total of 10 antibiotic levels. In each level, the range of pixel counts is recorded and constructs a data curve that represents the appropriate number of pixel areas. For later use, the program will use this line formula to

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calculate the value with the input data and compare with the measured area. The data curve is shown in the figure below.

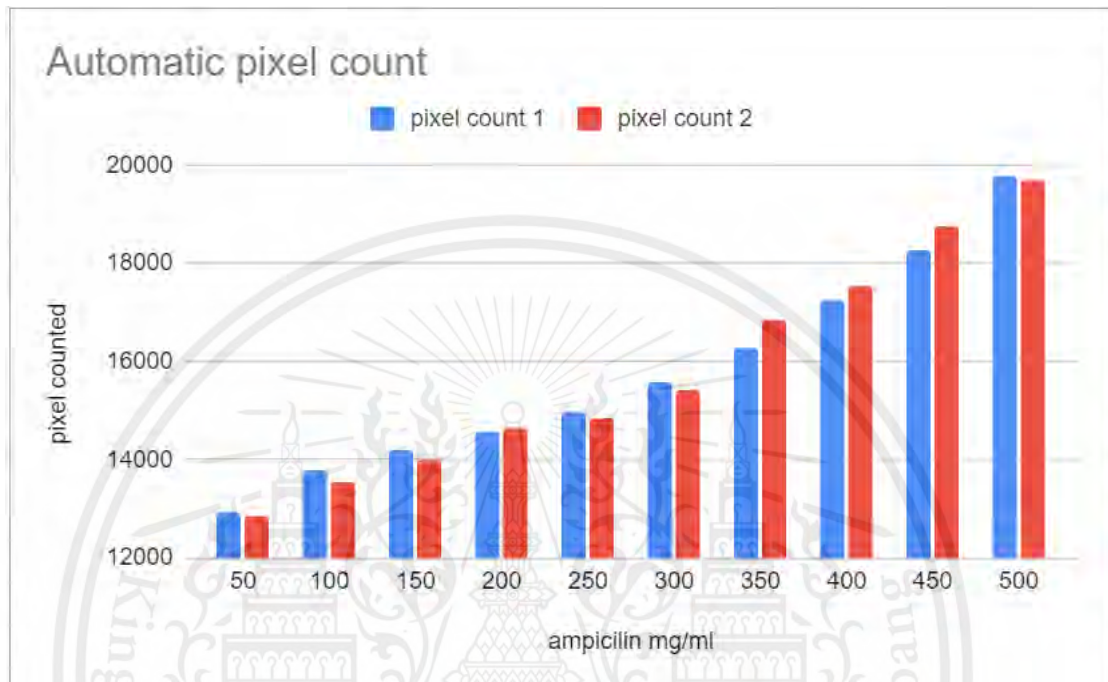

Figure 45: Our data curve

For the part of Artificial intelligence, we use the open source cloud computing platform Google Colab to develop. focusing on the deep learning algorithm called "YOLO". The set of images labeled the zone of inhibition are uploaded into the cloud storage via Google Drive and used to train the AI.

In order to save the time used in training the model, the pre-trained model is chosen to be the starter pack and the new dataset is embedded additionally. Therefore, this method of training the AI is operated with the training dataset containing around 40 images in total. After running it for an overnight period, the AI model is ready to be used.

3.3.3 Interesting Problems

During the development of the software, a problem is found at the method of detecting the circle in the image. At the first time the Hough transformation is selected to be used but the issue is that the function itself has too much sensitivity that

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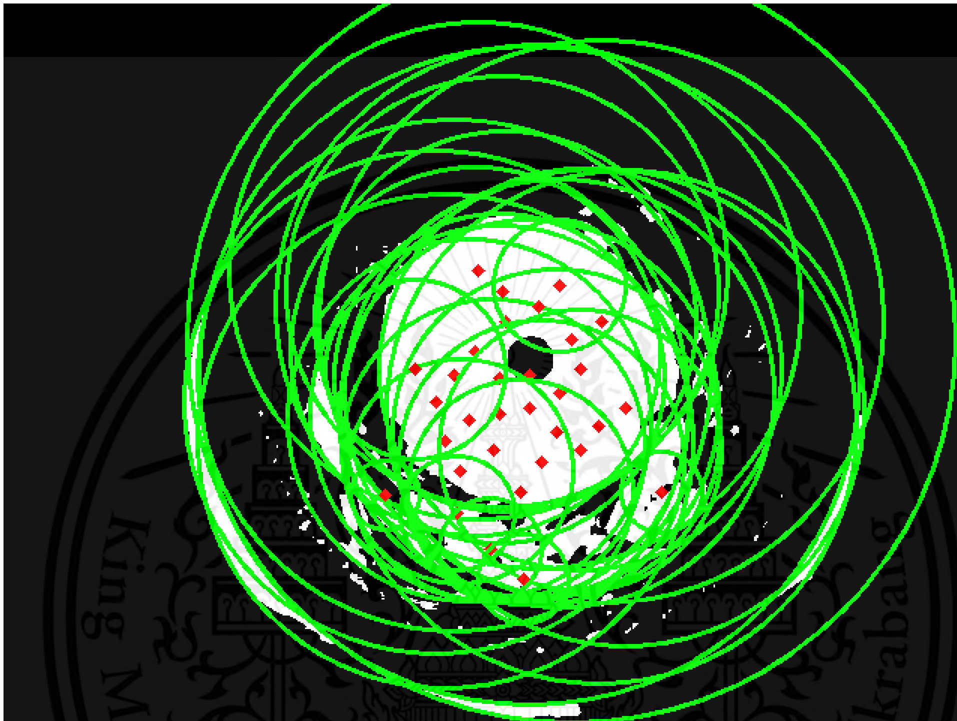
the adjustment applied cannot help much with the accuracy. The program detects the enormous number of small circles inside the images without tracking the desired region. The mentioned small circles are shown in the figure below.



.

Figure 46: Small circles

3.3.4 Proposed Solution

For the solution of the circle detection problem, we realized that the problem is the program cannot define the specific label for the zone of inhibition. We decided to come up with another method.

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Figure 47: Face detection deep learning result

Finally, we decided to use the deep learning algorithm, the same algorithm used in face detection, car tracking and object identification programs. The concept is to let the program identify the pixel patterns instead. The result is shown in the figure above.

3.4 Summary

In conclusion, our project has 2 parts which are the software part and the hardware part. For the hardware part, we design and construct a special project box where we perform. This project box mainly contains the experimental plate, a LED panel, a cooling mini fan, and a web camera. For the software part, we design and create a program for complete calculation of the susceptibility test of our experiment. Techniques used for building this program consist of image processing, deep learning algorithm, and AI training.

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CHAPTER 4
EXPERIMENTAL RESULT

4.1 Introduction

Chapters 3 described the design and implementation of the concept of Disc diffusion with the image processing techniques to be a system capable of identifying the result from the microorganism culturing whether the antibiotic used in that plate can suppress the bacteria or not, also it can be done automatically with the least human element as possible. In this chapter, we present a testing method and its results that show the accuracy and reliability of the image analysing algorithm to categorize the sample images taken from the project box into susceptible and resistant results. This chapter is organized as follows: section 4.2 is the testing result where section Section 4.2.1 introduces the concept of testing method of this algorithm and describes the microorganism and antibiotics used to set the sample plates, time used to cultivate the plate and environment conditions used during the cultivation. Next, section 4.2.2 presents the testing step in this experiment. The results of the tests are summarized in section 4.3, before the solution is evaluated in section 4.4.

4.2 Testing Result

In total over 120 tests were executed. Tests are categorized into two categories by the 2 tables which are located later on in this chapter. The tests illustrate the accuracy of our devices. These values of data then will be evaluated and also used as the extent to support the thesis.

4.2.1 Testing Concept

In this test, the concept to test the reliability and efficiency of the algorithm will be divided into 2 separate categories for 2 main functions of the software. The first one is the function to detect the presence of the zone of inhibition, the second one is the function to measure the area of the detected zone in the pixel form and compare it with the appropriate area from the antibiotic amount input into the program.

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For the microorganism culture plates, in order to standardize the conditions of the culture, we choose the same bacteria and antibiotic *Escherichia coli* and Ampicillin. Conditions and environments of the cultivation are 37 degree celsius and 60% humidity for 16 hours.

The method to check the zone of inhibition detections, the software itself can be used by checking the number of pixels measured because if there is no zone of inhibition, the number of pixels measured will be zero.

For the test of measuring function, we use the accuracy test in identifying the susceptible plate. all the plates will be susceptible so that if any result shows as resistant, it will be identified as false measurement. The testing results will be recorded and compre with the percentage version.

4.2.2 Testing Steps

Step 1: Prepare the cultured plate

- Prepare the E coli solution with 10^{-1} dilution factor from the *E. coli* stocks.
- Pipette 100 µL of the solution and spread onto the agar plate.
- Prepare the ampicillin solution with 10 different concentrations from 50 µg/mL to 500 µg/mL.
- Cut a piece of paper into a small circle, dip it into an ampicillin solution and place onto the plate.
- Incubate the plate with 37℃, 60% humidity for 16 hours.
- Prepare a total of 30 plates.
    - 10 plates without ampicillin
    - 20 plates with 10 different ampicillin levels, each level has 2 plates

Step 2: Check the result number

- For 30 plates, each plate will be used for a total of 4 tests, therefore 120 tests in total.
- For the result number
    - Non-zero: zone of inhibition detected
    - Zero: no zone of inhibition detected

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Step 3: Resistant or Susceptible

- All plates used in this experiment are the 20 plates with ampicillin, therefore every plate are susceptible case
- If result is resistant: record and use it to calculate the accuracy

Table 8: Test for the detection of inhibition zones

	Zone of inhibition detected	Nothing detected	True positive/true negative in percentage
Plate with zone of inhibition (80 test)	80	0	True positive 100%
Plate without zone of inhibition (40 test)	0	40	True negative 100%

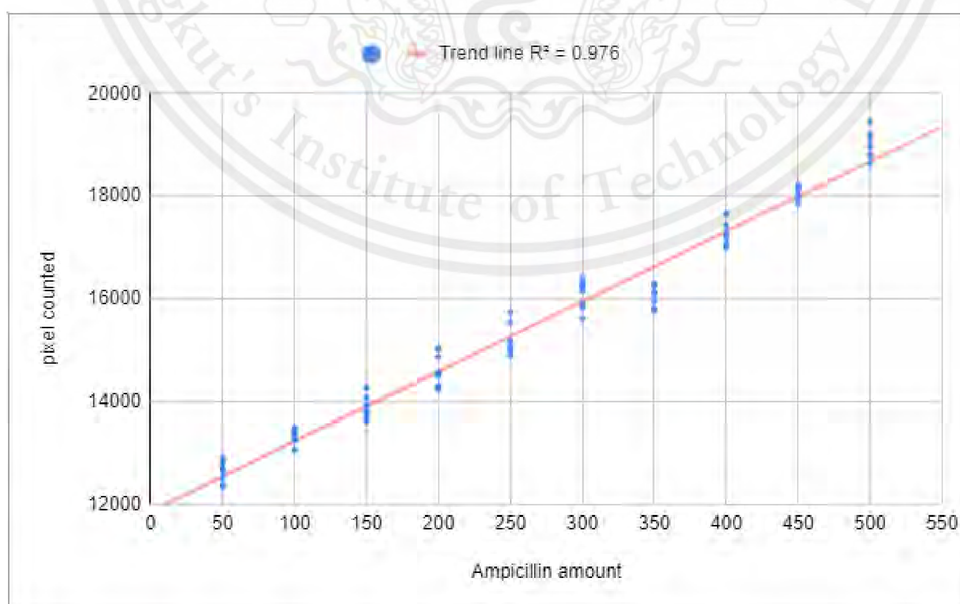

Figure 48: Data representing graph with R Square value and trendline calculated

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Table 9: Accuracy test for zone of inhibition size measurement

Ampicillin amount(ug/ml)	Susceptible result	Resistant Result	Accuracy in percentage
50 (8 test)	8	0	100%
100 (8 test)	8	0	100%
150 (8 test)	7	1	87.5%
200 (8 test)	6	2	75%
250 (8 test)	6	2	75%
300 (8 test)	7	1	87.5%
350 (8 test)	8	0	100%
400 (8 test)	7	1	87.5%
450 (8 test)	8	0	100%
500 (8 test)	8	0	100%
in total (80 test)	73	7	91.25%

4.3 Evaluation and Discussion

From section 4.2, a total number of 120 tests were performed in 2 different criterias; zone of inhibition presence detection (as shown in Table 7) and zone of inhibition size measurement accuracy (as shown in Table 8). After that the measured area data is being used to calculate the linearity of the dataset or R Square Test (as shown in Figure 47).

For the first criteria, we got the result of 120 out of 120 accurate results with 100 percent accuracy. showing that this software can accurately detect whether the zone of inhibition is present on the plate or not. The test of the plate which contains the zone of inhibition is conducted 80 times repeatedly using a pixel counter. The pixel counter is used to determine and identify the presence of pixels in the corresponding plates. Upon discovery, the plate is considered as detected. If not, then the plate is considered as nothing detected.

The second criteria, we got the result of 73 out of 80 accurate results, showing the accuracy of 91.25 percent. The cause behind the resistant results that we found is

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that the labeled area from the artificial neural network calculations may not cover the whole area of the zone of inhibition resulting in the measured pixel area being less than the actual area it should be. This lack of accuracy in the calculation of the Artificial Neural Network or ANN may come from a couple of factors. One of them is that the number of the sample images used to train the ANN is not enough to make it crop out the Zone of Inhibition completely.

The Data curves constructed from this test's result show the linear property RSQ of 0.976, similar to the RSQ value calculated from the data curve used to construct the Comparison Formula for the categorisation of the image to be Susceptible or Resistant. This means that the majority of the data in this test's results still shows the same relationship to the ampicillin amount.

From results in both criterias, it demonstrates that this algorithm has the potential to recognize the zone of inhibition with an accurate result but it may lack some accuracy in the dimension measurement.

4.4 Chapter Summary

This chapter introduced the image analysing algorithm for all of our experimental plates. Showing the process of concept and steps of our devices which leads to the foundation of our results. Breaking deep down into this is that our software has 2 main functions. The first function is to detect the presence of the zone of inhibition in our experimental plates. The second function is to measure that zone of inhibitions and calculate the output results. Our results then are shown in 3 categories as shown in the 2 tables and 1 figure respectively above within this chapter.

An evaluation and discussion of the results was then presented based on the mentioned 2 tables and 1 figure. For table 7, the result is 100 percent accuracy. The meaning of this is that our device can perfectly identify the presence of all the zones of inhibitions. For table 8, the result is 91.25 percent accuracy. Meaning, our device is able to perform the susceptibility test with acceptable results. Finally in Figure 47, the graph shows the raw data used for calculating the results which provide the aspects of the linearity of the data curve were discussed and came with a reliable result.

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CHAPTER 5
CONCLUSION

5.1 Conclusion

In this Chapter, we first summarize the work described in chapter 1, chapter 2, chapter 3, and chapter 4 all within the section of summary in this chapter. Then we draw a number of conclusions about key parts of the work undertaken within all every chapter into this section. Finally in section 5.2, we discuss future work and possible development that can apply on our project.

The aim of this project was to automate the process of Disc Diffusion Assay to simplify its usage and replicate the accuracy of the traditional process as much as possible. We chose to focus on the implementation of artificial intelligence and image processing techniques to create an algorithm for analysing images and to construct hardware environments that support its function.

We then designed and implemented a system that could automatically:

- capture an image of cultures microorganism plate
- recognize the presence of zone of inhibition within the plate
- measure the dimensions of the zone of inhibition
- determine whether the antibiotic effects on the microorganisms is considered as susceptible or resistant

These combined capabilities could simulate the process of examining the cultured plate within just 1 click inside the software. Therefore, less human elements involved, less chance of error to happen.

In Chapter 1 and 2 we state the general hypothesis that using image processing techniques with artificial neural networks, the software could analyse the image of a cultured plate with antibiotics and can determine the susceptibility efficiently. We have tested this thesis by culturing numbers of *E. coli* plates with Ampicillin as an antibiotic. Then we construct a camera box to capture images and analyse it using the algorithm, which were shown in Chapter 3 and 4. The results show that the software is capable of locating the zone of inhibition at a 100% rate and this software could

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also potentially measure its size with fairly high accuracy. These results are the proof of concept for automating the Disc Diffusion technique.

5.2. Suggestion

5.2.1 Zone of Inhibition Measuring Accuracy

From the results of the experiment, although the accuracy in the part of the zone of inhibition detections functions accurately, there are some errors in the section measuring the dimensions which opens the opportunity to further improve this ability. One example of the methods that could be possible to slightly improve the accuracy is to rearrange the order of the image processing procedures and reapply the ANN.

5.2.2 Base Microorganism and Antibiotic pairs

For this experiment we only use one type of both microorganisms and antibiotics which is e coli with 10^{-1} delusion factor and ampicillin concentrations of 50 - 500 µg/mL . Therefore this system can only be used efficiently with this pair combination within the range. Therefore, an opportunity to construct another test pair for other combinations of Microorganism and biotics is still open in order to generalize the usage ranges even more.

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APPENDIX A - Raw Data

A raw data obtained from recording the final answer of the software after running the test with the plates, consisting of the 3 data: Ampicillin amount inputted through the software, Pixel counted after analysing image, and the result category of the plate susceptible or resistant.

Test No.	Ampicillin Amount	Pixel Counted	Result
1	50	12903	susceptible
2	50	12904	susceptible
3	50	12905	susceptible
4	50	12906	susceptible
5	50	12907	susceptible
6	50	12908	susceptible
7	50	12903	susceptible
8	50	12904	susceptible
9	100	12905	susceptible
10	100	12906	susceptible
11	100	12907	susceptible
12	100	12908	susceptible
13	100	13281	susceptible
14	100	13277	susceptible
15	100	13400	susceptible
16	100	13484	susceptible
17	150	13822	susceptible
18	150	13937	susceptible
Test No.	Ampicillin Amount	Pixel Counted	Result
19	150	14088	susceptible
20	150	13598	resistant
21	150	13798	susceptible
22	150	13716	susceptible
23	150	13644	susceptible

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24	150	14275	susceptible
25	200	14247	resistant
26	200	14573	susceptible
27	200	15027	susceptible
28	200	15037	susceptible
29	200	14309	resistant
30	200	14501	susceptible
31	200	14876	susceptible
32	200	14556	susceptible
33	250	15740	susceptible
34	250	15539	susceptible
35	250	15186	susceptible
36	250	15062	susceptible
37	250	14897	resistant
38	250	15016	susceptible
39	250	14974	resistant
40	250	15097	susceptible
41	300	16405	susceptible
42	300	16322	susceptible
43	300	15912	susceptible
44	300	16269	susceptible
45	300	16242	susceptible
46	300	15834	susceptible
47	300	15625	resistant
48	300	16162	susceptible
49	350	16095	susceptible
50	350	16144	susceptible
51	350	16293	susceptible
52	350	16258	susceptible
53	350	16281	susceptible
54	350	15768	susceptible
55	350	15972	susceptible
56	350	15822	susceptible
57	400	17042	susceptible
58	400	17169	susceptible

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59	400	16998	resistant
60	400	17241	susceptible
61	400	17658	susceptible
62	400	17420	susceptible
63	400	17294	susceptible
64	400	17070	susceptible
65	450	18169	susceptible
66	450	17922	susceptible
67	450	18106	susceptible
68	450	18152	susceptible
69	450	18223	susceptible
70	450	18009	susceptible
71	450	18232	susceptible
72	450	17858	susceptible
73	500	18754	susceptible
74	500	18824	susceptible
75	500	19213	susceptible
76	500	19467	susceptible
77	500	18629	susceptible
78	500	19093	susceptible
79	500	18968	susceptible
80	500	18969	susceptible

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