INHIBITION OF MICROBIAL PATHOGENS BY RIBOFLAVIN, ULTRAVIOLET A AND SELECTED ANTIBIOTIC COMBINATIONS

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ABSTRACT

Blood infection remains as a major global public health problem. The World Health Organisation (WHO) estimates that 8 million new cases and 3 million deaths are directly attributed to the disease each year. The most common blood infections may lead to septicaemia if left untreated. In recent decades, pathogens have become resistant towards conventionally available antibiotics. This phenomenon persistently proliferates while increasing the number of individuals suffering from diseases and morbidity. In consequence, a pressing need arises for more effective antimicrobial agent to inhibit these infectious pathogens. One specific biomaterial of interest known as riboflavin or vitamin B2 has been shown to exhibit antimicrobial properties when activated with ultraviolet A (UVA) irradiation. Randomly selected blood borne pathogens *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Candida albicans* were tested by using disc diffusion method. The antimicrobial properties of riboflavin by UVA at 365 nm, with or without irradiation were tested. They were tested with (negative control), disc with stand-alone UVA irradiated riboflavin (50.0 µl), and disc with stand-alone riboflavin combined with pre-treated standard drugs for 5 minutes. The zones of inhibition were measured in millimetres. Collected data expressed as (mean ± standard deviation) and one way ANOVA and Post- Hoc Tukey's multiple comparison tests (p value was $p<0.05$) were done. The findings showed that stand-alone riboflavin without UVA exposure inhibited *S. aureus*, *E. faecalis*, *S. typhi*, and *P. aeruginosa* at a range of 20.0 ± 1.0, 17.7 ± 0.6, 17.3 ± 0.6, and 15.7 ± 0.6 respectively. Intermediate zones of inhibition were observed for *E. coli* and *C. albicans* in the range of 11.7 ± 0.6 and 11.7 ± 0.6 respectively. *K. pneumoniae* was the only pathogen that was resistant with a range of 7.7 ± 0.6 mm using the 50.0 µl of riboflavin solution. The mean difference of stand-alone riboflavin was also found to be significant against activated riboflavin with UVA

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at 365 nm at $p = 0.001$ level ($p<0.05$). Moreover, selected pathogens such as *S. aureus*, *E. faecalis*, *S. typhi*, and *P. aeruginosa* showed zones of inhibition significant at $p = 0.001$ after pre-treatment with standard drugs and then with riboflavin compared to standard drugs alone. The antimicrobial efficacy of riboflavin without the need for irradiation using UVA exposure for the microbial isolates tested was shown for the first time in this study. Furthermore, previous treatments with standard drugs and then with riboflavin application significantly inhibited the growth of the selected pathogens. This combinational or stand-alone riboflavin application could be further explored for enhanced management of blood borne pathogens.

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ABSTRAK

Pada zaman sekarang, jangkitan penyakit melalui darah menjadi suatu fenomena masalah kesihatan awam di dunia. Pertubuhan Kesihatan Dunia (WHO) menganggarkan bahawa 8 juta kes baru dan 3 juta kematian boleh dikaitkan secara langsung dengan jangkitan penyakit ini setiap tahun. Jangkitan darah yang biasa boleh menyebabkan septisemia jika tidak dirawat. Dalam beberapa dekad kebelakangan ini, patogen telah menjadi imun terhadap antibiotik yang sedia ada. Fenomena ini semakin berkembang disamping meningkatkan bilangan individu yang menghidap penyakit dan kematian. Akibatnya, satu keperluan mendesak timbul untuk mencipta antimikrob yang lebih berkesan untuk menghalang patogen daripada berjangkit. Satu komponen biologi yang berpotensi dikenali sebagai riboflavin atau vitamin B2 telah mempamerkan ciri-ciri antimikrob apabila digabungkan dengan sinaran ultraungu A (UVA). Beberapa patogen berjangkit melalui darah seperti *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* dan *Candida albicans* telah dipilih secara rawak dan telah diuji dengan riboflavin menggunakan kaedah cakera resapan. Ciri-ciri antimikrob riboflavin atau UVA pada 365 nm, dengan atau tanpa sinaran telah diuji. Ia telah diuji dengan kosong sebagai kawalan negative, cakera 50.0 µl riboflavin dalam keadaan larutan. Patogen yang dapat merencat pembiakan dengan larutan riboflavin telah dipilih untuk membuat kajian yang melibatkan penggabungan antibiotik. Semua patogen telah diuji dengan (kawalan negatif), cakera dengan berdiri sendiri UVA sinaran riboflavin (50.0 ul), dan cakera dengan berdiri sendiri riboflavin digabungkan dengan pra-dirawat standard antibiotik yang sedia ada selama 5 minit. Data yang dikumpulkan dinyatakan sebagai (min ± sisihan piawai) dan dianalisis menggunakan ujian ANOVA satu hala dan diikuti dengan ujian perbandingan berganda Post- Hoc Tukey (nilai p adalah $p < 0.05$) telah dilakukan. Dapatan kajian menunjukkan bahawa berdiri sendiri riboflavin tanpa pendedahan UVA

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merencat *S. aureus*, *E. faecalis*, *S. typhi*, dan *P. aeruginosa* pada julat 20.0 ± 1.0, 17.7 ± 0.6, 17.3 ± 0.6, dan 15.7 ± 0.6 masing-masing. Zon pertengahan perencatan diperhatikan untuk *E. coli* dan *C. albicans* dalam lingkungan masing-masing 11.7 ± 0.6 dan 11.7 ± 0.6. *K. pneumoniae* adalah satu-satunya patogen yang tahan pada julat 7.7 ± 0.6 mm dengan 50.0 ul daripada larutan riboflavin. Perbezaan min riboflavin berdiri sendiri juga didapati signifikan terhadap riboflavin diaktifkan dengan UVA pada 365 nm pada p = tahap 0.001 ($p <0.05$). Lebih-lebih lagi, patogen terpilih seperti *S. aureus*, *E. faecalis*, *S. typhi*, dan *P. aeruginosa* menunjukkan zon perencatan yang ketara pada $p = 0.001$ selepas pra-rawatan dengan ubat-ubatan standard dan kemudian dengan riboflavin berbanding ubat-ubatan standard sahaja. Keberkesanan antimikrob riboflavin tanpa memerlukan penyinaran menggunakan pendedahan UVA untuk mikrob telah ditunjukkan untuk kali pertama dalam kajian ini. Tambahan pula, rawatan sebelumnya dengan ubat-ubatan standard dan kemudian dengan riboflavin berkesan menghalang pertumbuhan patogen yang dipilih. Hasil kajian ini mengesyorkan bahawa kombinasi ubat-ubatan atau riboflavin bersendirian sahaja boleh digunakan sebagai rawatan yang lebih berkesan bagi merencat pembiakan patogen dalam darah seseorang tanpa sebarang kesan sampingan.

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LIST OF SYMBOLS AND ABBREVATIONS

S. aureus / SA	*Staphylococcus aureus*
E. faecalis / EF	*Enterococcus faecalis*
S. typhi / ST	*Salmonella typhi*
P. aeruginosa / PA	*Pseudmonas aeruginosa*
E. coli / EC	*Escherichia coli*
K. pneumonia / KP	*Klebsiella pneumoniae*
C. albicans / CA	*Candida albicans*
SPSS	Statistical Package for Social Sciences
UVA	Ultraviolet-A
MH	Mueller Hinton agar
°C	Degrees Celsius
µm	Micrometer
µl	Microlitre
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mg	Milligram
mm	Millimetre
g	Gram
ml	Millilitre
O_2	Oxygen
NO_3	Nitrate
NO_2	Nitrogen dioxide
%	Percentage
API	Analytical Profile Index
J	Joule

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CHAPTER 1

INTRODUCTION

1.1 Riboflavin

Riboflavin is a naturally available yellow-green fluorescent pigment also known as lactoflavin, vitamin G and vitamin B2, first studied in 1879 (Hertz, 1954). In 1936, in a conference held in Pittsburgh, the American Chemical Society (ACS) suggested that the term flavin should be used instead to describe the water soluble pigments, which have been demonstrated to be necessary for the normal nutrition of rat and for chicks' growth. In this conference, they also determined and terminated the terms such as lactoflavin, vitamin G and accepted riboflavin as vitamin B2 (Hertz, 1954).

Riboflavin (7, 8-dimethyl-10-((2R, 3R, 4S)-2, 3, 4, 5–tetrahydroxypentyl) benzo [g] pteridine-2, 4 (3H, 10H)-dione) is a naturally occurring compound considered essential as an important human nutrient (Figure1.1). Figure 1.1 a shows powder form of riboflavin while Figure 1.1b shows solution of riboflavin when diluted with phosphate buffered saline (PBS).

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Figure 1.1: Riboflavin as a powder (a) and in solution (b)

It is present in aerobic organisms and found in many types of foods such as cereals, variety of meats and fatty fish, certain fruits and dark-green vegetables in reasonably high concentrations. It is visible as orange-yellow crystal that is stable to heat, acid and oxidation. However, it is light sensitive especially to ultraviolet (UV) radiation, present in sunlight. Food subjected to washing and cooking for long period, will result in loss of some amount of riboflavin. Once consumed, vitamin B2 is easily absorbed from the small intestine into the blood, which transports it to the tissues. Excess intake leads to elimination through urine involving riboflavin in the form of other water soluble metabolites, such as 7-hydroxymethylriboflavin (7-hydroxyriboflavin) and lumiflavin (which can give it a yellow-green fluorescent glow). Riboflavin is non-toxic, as confirmed by a broad range of testing carried-out prior to its designation as “Generally Recognised as Safe” (GRAS) by the US Food and Drug Administration (FDA) (Select Committee on Gras Substances, 1979). Riboflavin is not stored in the body, except for a small quantity in the liver and kidneys (Elson and Haasan, 2003), so it is needed regularly in the diet. Current recommended nutrient intakes of riboflavin are 0.6 mg per 1000 kcal for persons of all ages (Select Committee on Gras Substances, 1979). Increment has been set to 0.3 mg during pregnancy and 0.5 mg during lactation to recover increased tissue synthesis for fatal and maternal development and riboflavin secretion in milk (Lindsay, 2012). A recent U.S. survey of over 20,000 persons, 1 to 74 years of age, revealed a mean average intake of 1.92 and a median of 1.69 mg per day (Select Committee on Gras Substances, 1979).

In water, riboflavin exhibits maximum absorption at 220, 265, 365 and 446 nm. The yellow-orange liquid solution containing riboflavin will be rehabilitated as lumichrome or lumiflavin once it is exposed to direct sunlight. Riboflavin also acts as one of the energy producing factor by activating the most important coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These coenzymes

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transport hydrogen to produce energy in the form of adenosine triphosphate (ATP) by breaking down carbohydrates and fats. Besides, riboflavin helps cells to consume oxygen well enough for respiration and healthier cell growth. It is also helpful for good sense of sight, skin, nails and healthy hair (Raymond *et al*., 2005).

Riboflavin is commonly used to treat and help prevent visual problems such as eye fatigue and cataracts thus ensuring good vision (Elson & Haasan, 2003). It seems to help with burning eyes, excess tearing and decreased vision resulting from eye strain. Riboflavin is also used for many kinds of stress conditions, fatigue and vitality or growth problems (Elson & Haasan, 2003). For people with allergies and chemical sensitivities, riboflavin-5-phosphate may be more readily assimilated than riboflavin. Riboflavin is given for various skin complications such as acne, dermatitis, eczema and skin ulcers. It also assists in maintaining healthy hair, skin, and nails. Riboflavin is also used in the treatment of liver disease, ulcers, digestive difficulties and leg cramps, and supplementing it may be advantageous for prevention or during treatment of cancer (Elson & Haasan, 2003; Powers, 2003). There is, however, not much published research to support these common uses (National Research Council, 1989).

Recent observations indicate that human beings lack riboflavin intake even though there are many varieties of riboflavin rich foods (Winters *et al*., 1992; Jakobsen & Jonsdottir, 2003) (Food and Nutrition Board, 1998). Riboflavin deficiency, termed ariboflavinosis, causes cheilosis is a condition characterized by dry scaling and fissuring of the lips, sore tongue, and scaly rash on scrotum or vulva. Deficiency has also been associated with night blindness, photosensitivity, lack of growth, inability to stand, cataracts, migraines, mild anaemia and fatigue/depression. Although rarely performed, tests like red blood cell glutathione reductive activity can be measured for evidence of deficiency, but usually clinical findings are sufficient to consider deficiency. Riboflavin deficiency presents a risk factor to cancer and also cardiovascular diseases (Yuvaraj *et*

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al., 2008; Hassan *et al*., 2012). Continuous supplementation of riboflavin may therefore prevent most of the diseases including pathogenic infections in human beings when a normal diet is consumed. In human, there is no evidence of riboflavin toxicity produced by excessive intakes. Even when 400 mg/d of riboflavin was given orally to subjects in one study for three months to investigate the efficacy of riboflavin in the prevention of migraine headache, no short-term side effects were reported (Schoenen *et al*., 1998; Yee, 1999; Sandor *et al*., 2000; Boehnke *et al*., 2004).

Riboflavin plays crucial roles in numerous medical and healing situations. For over 30 years, riboflavin supplements have been used as part of the phototherapy treatment of neonatal jaundice (Ennever *et al*., 1985; Ennever, 1988; Cavallo *et al*., 2012). The UV light used to irradiate the infants breaks down not only bilirubin, the toxin causing the jaundice, but also the naturally occurring riboflavin within the infant's blood, so extra supplementation is necessary. On the other hand, UV light can become a reason for the formation of pyrimidine dimers in DNA, which results in genetic damage to the cells (Kumar *et al.*, 2004; Goodrich *et al*., 2006). High or continuous exposures of UV light can cause photolysis, loss of colony-forming ability (death), enzyme inactivation and destruction of nucleic acid. A constant exposure of UV light on riboflavin can stimulate the latter and enables it to associate with nucleic acid (DNA/RNA) (Hardwick *et al*., 2004; Kumar *et al*., 2004; Goodrich *et al*., 2006). This connection causes a chemical adaptation, which presents an obstacle for pathogenic reproduction.

Several studies proposed on the bactericidal effects of photo-activated riboflavin using UVA. In 1965, Japanese scientists demonstrated that riboflavin, when exposed to visible or UV light could be used to inactivate RNA containing tobacco mosaic virus (Tsugita *et al*., 1965). Makdoumi, (2011) concluded that riboflavin photo-activation using UVA at 365 nm can achieve an extensive eradication of bacteria, and the

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combination is more potent in reducing bacterial number than UV alone. Indeed, the effectiveness of this new technique is reported in a number of articles in the literature. Recently, Schrier *et al*., (2009) reported a similar finding on success in using this technique for in-vitro killing of three important bacterias such as *S. aureus,* Methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa*. Martins *et al*., (2008) also noted that this technique were effective against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, MRSA and Drug resistant *Streptococcus pneumoniae* (DRSP), but was ineffective against *Candida albicans*. However, every study on treating blood infections so far has been carried-out with the combination of riboflavin and UVA irradiation.

Akompong *et al*., (2000) conducted a similar study but without the application of UVA, which involved the eradication of malarial infection in blood caused by *Plasmodium falciparum* in human blood (Akompong *et al*., 2000). His studies highlighted the successful inhibition of the growth of the parasites without the need for photo-activation of the riboflavin molecules and concluded that it can be used as an antimicrobial agent. However, to date no further riboflavin-only assisted antimicrobial studies on other types of pathogens were carried-out or repeated until the current investigation.

Through this research, we proposed a novel method of riboflavin assisted inhibition of the growth of a few selected Gram positive and negative bacterial and fungal isolates without UVA photo-activation. The bacterial strains studied were *Staphylococcus aureus* (SA), *Enterococcus faecalis* (EF*), Salmonella typhi* (ST), *Pseudomonas aeruginosa* (PA), *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP) while the fungal strain selected was *Candida albicans* (CA).

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1.2 Motivation

Increasing number of people affected by blood borne pathogens and pathogenic resistance to antibiotics are generally the motivation behind the need for alternative types of safer medication. These factors shape the motivation for the current research towards a naturally available organic source, which could provide a possible antimicrobial solution.

1.3 Objectives

The objectives of the study were to;

i. Identify minimum inhibitory concentration (MIC) of riboflavin without UVA irradiation to inhibit growth of pathogens.
ii. Study the antimicrobial effectiveness of selected riboflavin concentration in combination with UVA light source at 365 nm wavelength towards the inhibition of pathogens.
iii. Demonstrate the antimicrobial effects of the riboflavin co-treated with selected conventional antibiotics against pathogens.

1.4 Thesis Outline

Chapter 1 contains the introduction to the importance of riboflavin, UVA and the effects of current blood borne pathogens on human health. This chapter also lists the objectives of the study, and briefly explains the outline of the research approach.

Chapter 2 discusses the history of riboflavin and relevant previous works involving riboflavin. A thorough discussion on microbes and disease, background of UVA and history of blood borne pathogens and the importance to conduct the research was provided. In addition, the importances of antimicrobial agents are discussed and in-depth explanation provided on why pathogens become drug resistant.

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Chapter 3 meanwhile describes and explains the research methodology used in this study. The subtopic for this chapter includes research design and the research procedures adopted. It also explains the statistical methods employed.

In chapter 4, the results were analysed and discussed. The findings of the research were then compared with those of previous studies presented in the literature review.

Finally, chapter 5 summarizes the findings and their implications discussed. In this chapter, limitations and assumptions of the research work and suggestions for future works were also presented.

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CHAPTER 2

LITERATURE REVIEW

2.1 History of Riboflavin

Towards the end of the third quadrant of the 17th century, chemists have discovered a chemical compound or pigment that reflects yellow-green fluorescent. Initially it was referred as lactoflavin, vitamin G and vitamin B2 (Blyth, 1879). Continuous study and observation of this pigment by many researchers since 1925 revealed its more obvious chemical properties clearly.

Goldberger and Lily attempted to understand the nature of pellagra and its deficiency in rats (Goldberger and Lily, 1926). In this work, they expected the lesions to be heat-labile (Vitamin B1) but they noticed that the lesions were barred by a different factor, which was heat-stable. Goldberger named this factor as P.P (pellagra-preventing) factor and later on it was labelled as vitamin B2 in Great Britain and as vitamin G in United States of America (Sure, 1932). Further study on this vitamin revealed that there were other compounds such as B complex, which was considered as an additional factor for the prevention of rat pellagra.

Researchers have also carried-out experiments on a new oxidation enzyme collected from the aqueous extracts of yeast (Williams & Bruce, 2002). Through these studies, they characterised the enzyme as yellow-enzyme based on its appearance in yellow with a green fluorescence reflection. In addition, they also identified that this yellow-enzyme was present in almost all living cells. Comparative observation on this compound reported that it lack various characteristics of vitamin B2. However Goldberger and Lily (1926) reported that this attribute changes habitually (Gyorgy, 1934). In specific, there was deficiency of growth noticed by some researchers, while others were able to record the external features of dermatitis in some of the experimental animals.

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Another researcher, Gyorgy (1934) who was interested on the hypothesis of Goldberger and Lily furthered his investigation into trying to explain these phenomena (Gyorgy, 1934., Birch, 1935). His findings gave some important answers to what was observed by Goldberger and Lily. According to Gyorgy, the rats naturally live with no vitamin B in their food diet and as such, with intentionally added vitamin B1 and lactoflavin, it caused the increase of pellagra which later became worse when vitamin B2 was added. This drastic change produced lesions and became the reason behind the different characteristics observed by Goldberger and Lillie. This was later cured by using an unknown factor, which was recognised as vitamin B6 by Gyorgy. The author explained that some of the skin lesions can be due to lack of vitamin B2 or vitamin B6. Vitamin B2 was believed to have caused pellagra but later it was identified similar to nicotinic acids effect. Consequently, riboflavin was deemed necessary for growth of rat. Many researchers initiated further research to better understand the natural characteristics of riboflavin. Some isolated a strong yellowish green compound from egg white and named it as ovoflavin, which believed stimulated the growth of rats. In another study, Booher (1933) reported that riboflavin can be prepared from concentrated whey powder and it was also proved that the preparation boosted the growth of rat (Booher, 1933). These specific compounds were named as ovoflavin, lactoflavin and hepatoflavin according to their derivation from various substances (Bourquin & Sherman, 1931). They comprehended that these compounds can be identical to each other. As a confirmation test, they isolated these compounds in small quantities and attempted direct comparison but it was unsuccessful.

Finally in 1936, the term riboflavin was reserved for vitamin B2 while the stipulations lactoflavin and vitamin G were deemed redundant (Council on Pharmacy and Chemistry, 1937).

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2.2 Importance of Riboflavin

As discussed earlier, riboflavin, also known as vitamin B2 (Figure 2.1) is part of the vitamin B complex. It is an easily absorbed and water-soluble micronutrient with a key role in maintaining human health. Riboflavin is an orange powder and once dissolved in water displays intense greenish yellow fluorescence. Vitamin B2 cannot be stored by the body except in insignificant amounts and therefore it must be replenished daily.

Figure 2.1: The powder form of riboflavin-5- phosphate.

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Riboflavin has various functions in human and animal bodies. It supports energy production by aiding in metabolism of fats, carbohydrates, and proteins due to production of enzymes such as thyroid. It is basically used to prevent low levels of riboflavin or ariboflavinosis (riboflavin deficiency), cervical cancer, and migraine. It is also used for treating acne, muscle cramps, burning feet syndrome and blood disorders such as congenital methemoglobinemia (Akompong *et al*., 2000; Hirano *et al*., 2008) and red blood cell aplasia (Foy *et al*., 1961). Some people use riboflavin for eye conditions including eye fatigue, cataracts and glaucoma. It also plays an important role in treating nervous system related illness such as numbness and tingling sensations, Alzheimer's disease, epilepsy and multiple sclerosis (Powers, 2003., Ball, 2006; Yuvaraj *et al*., 2008). Riboflavin in combination with vitamin B6 was used to treat carpal tunnel

syndrome (Folkers *et al*., 1984). Besides this, riboflavin is also vital for normal reproduction, growth, repair and development of body tissues. It is proven to elevate energy level, boost immune system functions and facilitate and maintain healthy hair, skin and nails (Elson & Haasan, 2003, Powers, 2003). Riboflavin possesses ability to improve skin's secretion of mucus membranes which aids in clearing up skin pustules associated with rosacea (Richard *et al*., 2006).

Riboflavin has been used to delay aging, boost athletic performance, and treat ulcers like canker sores (aphthous ulcer), combat alcoholism, and cure liver diseases and sickle cell anaemia (Hilary, 2003). Riboflavin is also very useful in lactic acidosis, which is a serious blood-acid imbalance in people with acquired immunodeficiency syndrome (AIDS) (Saundra & Ali, 2004). There is preliminary clinical evidence that riboflavin may be useful for treating lactic acidosis in patients with AIDS caused by drugs called nucleoside analogue reverse transcriptase inhibitors (NRTI). Evidence suggests through increasing intake of riboflavin from dietary and supplements sources along with thiamine, folic acid, and vitamin B12 prevents cervical cancer (Hilary, 2003). It might decrease the risk of developing precancerous spots on the cervix.

Under some conditions, vitamin B2 can act as an antioxidant (Marziyeh & Ahmad, 2014). Vitamin B2 is an intermediary between the transfer of electrons in the cellular oxidation-reduction reactions, which generate energy from protein, carbohydrate and fat. The riboflavin coenzymes are also important for the transformation of vitamin B6 and folic acid into their respective active forms, and for the conversion of tryptophan into niacin. Riboflavin is involved in energy production as part of the electron transport chain that produces cellular energy.

Furthermore, riboflavin is an important nutrient for human found in green plants with higher percentage, especially in their leaves and also in aerobic organisms. For example, it is richest in organs such as liver, kidney, heart, fatty fish and certain plant.

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Riboflavin is heat stable but will leach into cooking water, reducing its amount in the food itself.

Riboflavin is easily destroyed by light and food stored in clear containers will lose their riboflavin content in a short period of time. Hence, food with riboflavin content should be stored in opaque containers. For example, breads and cereals which are often fortified with riboflavin.

The molecule of riboflavin is water soluble as it is a planar structure and has conjugated ring structure with sugar side chain (Figure 2.2). The planar structure is proficient on intercepting the bases of DNA/RNA in pathogens when exposed to the invisible light and near UV regions. It has been identified to contain antipathogenic and anticancer properties and has been passionately used in researches for decades in conjunction with UVA. Undeniably, various studies have been carried-out on the efficacy of riboflavin with the exposure of certain wavelengths on inactivating the pathogens (Mirshafiee *et at*., 2015).

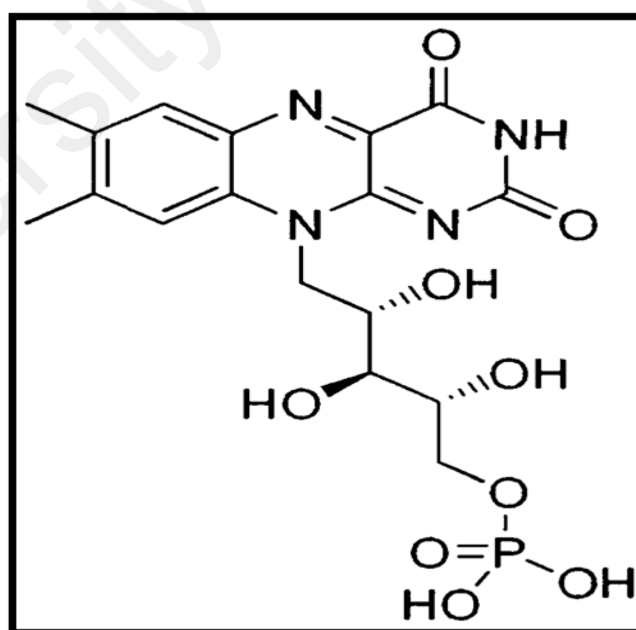

Figure 2.2: The molecular structure of Flavin mononucleotide (riboflavin -5-phosphate) produced from riboflavin by the action of the enzyme riboflavin kinase (Bruce, 2010).

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Riboflavin has been used in numerous medical and remedial circumstances (Siddiqui, 2012). For over 30 years, riboflavin supplements have been used as part of the phototherapy treatment of neonatal jaundice (Amin *et al*., 1992). The UV light used to irradiate the infants breaks down bilirubin and the toxin causing the jaundice besides naturally occurring riboflavin within the infant's blood. For that extra riboflavin supplementation is necessary (Amin *et al*., 1992).

Riboflavin is not toxic when taken orally, as its low solubility keeps it from being absorbed in dangerous amounts within the digestive tract (Select Committee on Gras Substances, 1979). Although toxic doses can be administered by injection, any excess above nutritionally relevant doses is excreted through urine, imparting a bright yellow colour when in large quantities. In humans, there is no evidence for riboflavin toxicity produced by excessive intakes. Even when 400 mg/day of riboflavin was given orally to subjects in one study for three months to investigate the efficacy of riboflavin in the prevention of migraine headache, no short-term side effects were reported (Boehnke *et al*., 2004).

According to the Research Development Association (RDA), riboflavin recommendation varies according to a person's weight, metabolic rate, growth, and caloric intake (National Research Council, 1989). Riboflavin requirement must equal the total energy needs and metabolism. The RDA for riboflavin is 1.7 mg/day for an adult man and 1.3 mg/day for an adult woman (National Research Council). The amounts found in many multivitamin supplements (such as 20 - 25 mg) are more than adequate for most people. Pregnant women require an additional 0.3 mg per day and those that are lactating require an additional 0.5 mg per day. In healthy adults, riboflavin concentration in blood is measured at (116 ± 46) nmol/L (Kirshenbaum *et al*., 1987).

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2.3 Deficiency of Riboflavin

Ariboflavinosis is a condition experienced by a person due to malnutrition of riboflavin. It has perceptive effects on the metabolism of carbohydrates, fats, and protein. All three of these basic food elements require riboflavin for the body to properly utilise the energy. A deficiency of riboflavin may result in bloodshot eyes, abnormal sensitivity to light, itching and burning sensation of eyes, inflammation in mouth, sore and burning tongue, and cracks on lips and in the corners of the mouth (Hilary, 2003). It may also result in dull or oily hair, oily skin, premature wrinkles on the face and arms, and split nails. Riboflavin deficiency also leads to the malfunctioning of hormone such as adrenal glands (Hilary, 2003). Riboflavin deficiency could also lead to chronic liver disease or lead to Total Parental Nutrition (TPN).

2.4 Background of Ultraviolet

The UV spectrum is divided into UVA (315 – 400 nm), UVB (280 – 315 nm), UVC (100 – 280 nm), Near UV (300 – 400 nm), Middle UV (200 – 300 nm), Far UV (122 – 200 nm), Hydrogen Lyman-alpha (121 – 122 nm), Vacuum UV (10 – 200 nm) and Extreme UV (10 – 121 nm) (Figure 2.3). The nuclear fusion in sun releases the UV radiation. As this radiation reaches earth, earth's magnetic field acts as a shield and protects us from its harmful effects. However, 2.3% of this radiation still passes through our atmosphere with positive and negative effects (Kishenbaum *et al*., 2006).

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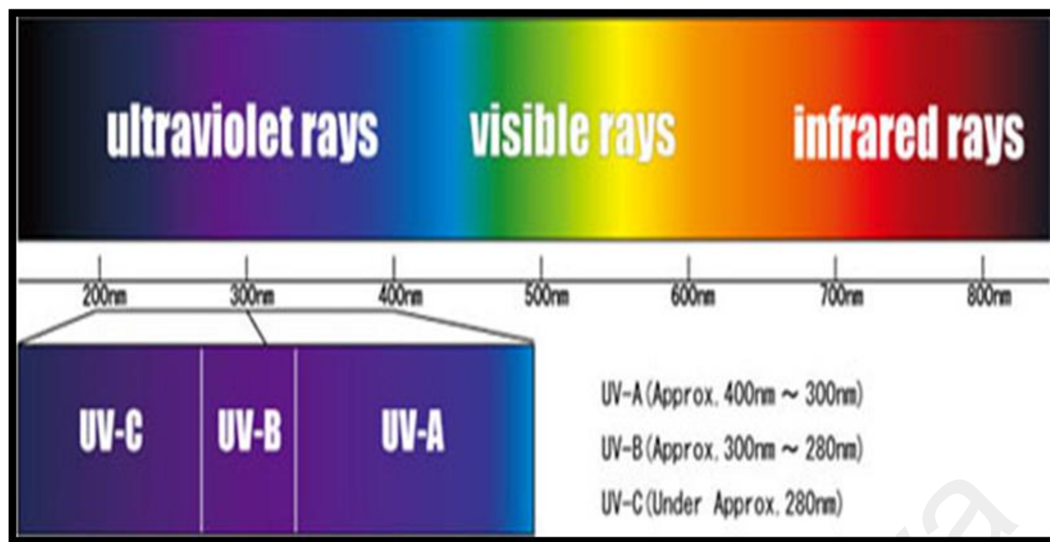

Figure 2.3: Types of ultraviolet (Kurt, 2001).

UVC radiation is completely absorbed in our atmosphere as with Far UV and Vacuum UV, making it to be almost invisible in our environment. Still, the ability of UVC radiation to kill bacteria has been utilised through germicidal lamps. As a negative impact, UVC is capable of causing severe damages to the outer layers of human epidermis such as sunburns, corneal burns and snow blindness if the particular spot of the skin is unintentionally over-exposed to UVC radiation. As a relief, these damages usually can be cured in a couple of days but still might be a very painful experience.

UVB radiation is the most dangerous radiation and has the strength to cause cellular DNA damages. Even so, it is essential to human as a pre-cursor for vitamin D production. Too much exposure to this UVB can cause erythema, cataracts and also contributes to the development of skin cancer. As such, people who work outdoors have the highest risk. Although UVB is blocked almost entirely by the ozone layer, it has been able to penetrate until the ground level due to the depletion of the ozone layer, increasing the probability of skin cancer occurrences.

The most frequently identified UV radiation in our environment is UVA radiation or also known as black light. This is due to the minimum absorption of its kind by the atmospheric ozone. Moreover, it is also partially needed by humans as a pre-

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cursor in the development of vitamin D. UVA lamps are used in phototherapy and tanning booths as an element of cosmetic treatment. This radiation has the capability to tan or darken the outer layers of dead skin. On continuous exposure, this can however lead to various problems such as erythema, cataract, toughening of the skin and weakening of the immune system.

The photochemical effects of UV radiation can be made worse by chemical agents including birth control pills, tetracycline, sulphathizole, cyclamates, antidepressants, coal tar distillates found in antidandruff shampoos, lime oil, and some cosmetics (The Fred Hutchinson Cancer Research Centre, 1972). UV Protection is provided by clothing, polycarbonate, glass, acrylics and plastic diffusers used in office lighting (The Fred Hutchinson Cancer Research Centre, 1972). Sun-block lotions can also be used as a short term solution against UV contact.

An individual might become an unexpected victim of UV radiation once the person is overly exposed to it. This occurs due to the characteristics of UV being invisible and exhibiting slow reaction rate. To avoid such incidents, every UV related materials and equipments are normally labelled with all necessary cautions and warnings on the product packaging cover, or at the entrance of the room containing the UV sources. As per record, accidents related to UV usually occur due to the carelessness of the individuals working with or next to the UV sources. This was due to improper protective coverings, or due to cracked or broken apparatus (Betsy, 1997). The consequence of such accidents depends on the type of UV radiation and the length of exposure.

2.5 Importance of Ultraviolet Radiation

Moderate amount of UV light are beneficial to human for producing vitamin D. Exposure to UV radiation are also helpful to treat most of diseases such as rickets,

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psoriasis, eczema and jaundice. In particular, UVA and UVB from the sun trigger vitamin D production. Vitamin D helps strengthen bones, muscles and the body's immune system (IARCH Handbooks for Cancer Prevention, 2001). It might also reduce the risk of acquiring cancers such as colon cancer (IARCH Handbooks for Cancer Prevention, 2001). UV light in fluorescent lamps is also used in the treatment of neonatal jaundice (Olsen *et al*., 1996). In this condition, the skin gets rid of its cells too quickly, develops itchy and scaly patches. Exposure to UV slows the growth of the skin cells and relieves the symptoms.

Some researchers also suggested that sunlight stimulates the pineal gland in the brain to produce certain chemicals called tryptamines, which improves our mood (Environmental Health Indicators, 2008). Furthermore, UV lamps have been employed to sterilize work spaces, laboratories and instruments. Its radiation has been applied in drinking water treatment plants and waste water recycling projects (Environmental Health Indicators, 2008). It is also used in food processing industry as non-thermal tool for sterilizing food (Environmental Health Indicators, 2008). Animals including birds, bees and reptiles are able to see into the near UV light to locate ripe fruits, flowers and seeds that stand out more strongly from the background (Environmental Health Indicators, 2008). The fruits, flowers and seeds often appear quite different from how humans see them. For example, when seen in UV light, some flowers have different line markings, which may help direct bees and birds to the nectar. Many insects use UV emissions from celestial objects as reference for navigating in flight. This is why light sometimes attract flying insects by disrupting their navigation process.

UV radiation has been used to study the chemical structure of various substances and widely employed in visible spectrophotometer to determine the presence of fluorescence in a given sample (Environmental Health Indicators, 2008). It is also employed in fine resolution photolithography and hence is extensively used in the field

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of electronics (Environmental Health Indicators, 2008). Detecting pollution or degradation of insulation in an electrical device can be carried-out with the help of UV radiation (Environmental Health Indicators, 2008). It has also been used as fire detectors (Environmental Health Indicators, 2008).

The Green Fluorescent Protein is used as a marker in experiments in the field of genetics (Environmental Health Indicators, 2008). Many substances, like proteins are capable of absorbing UV and emitting back at different wavelengths, which then can be finger-printed for protein detection. This property is now employed in genetics, biochemistry and other related fields. Artificial methods can also be devised to create a suntan effect using UV radiation such as in sun tan beds (Environmental Health Indicators, 2008).

2.6 Adverse Effects of Ultraviolet

The harmful effects from exposure to UV radiation can be classified as acute or chronic. The acute effects of UVA and UVB exposure are both short-lived and reversible. These effects include mainly sunburn or erythema and tanning or pigment darkening. The chronic effects of UV exposure can be much more serious, even life threatening, and include premature aging of the skin, suppression of the immune system, damage to the eyes, and skin cancer (Diffey, 1991).

UV is an environmental human carcinogen (US Department of Health and Human Services, 2014). It’s the most prominent and universal cancer-causing agent in our environment. There are three main types of skin cancer due to over exposure to sun such as basal cell carcinoma, squamous cell carcinoma and melanoma. Research shows that 90% of skin cancers are due to UV radiation (Koh *et al*., 1996). Sun burn occurs due to absorption of energy from UV rays, which causes damage to skin cells. Over exposure to UV radiation also has harmful suppressing effect on the immune system.

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Scientists believe that sunburn can change the distribution and function of disease-fighting white blood cells in humans for up to 24 hours after exposure to sun (United States Environmental Protection Agency, 2006).

Furthermore, prolonged exposure to UV or high intensities of UV can damage the tissues of the eyes and cause burning of the eye surface known as photokeratitis. The effects usually disappear within couple of days but may lead to further complications later in life. UV speeds up the aging process of skin since it destroys collagen and the connective tissue beneath the top layer of the skin. This causes wrinkles, brown liver spots and loss of skin elasticity (Environmental Health Indicators, 2008).

Industrial items such as many types of polymers used in consumer products, which include plastic, nylon and polystyrene are broken down or loses strength due to exposure to UV light. UV exposure also fade colours of many pigments such as food colouring, cosmetics, fabric, plastic, paint, ink and other materials (Environmental Health Indicators, 2008).

2.7 Previous Studies Using Riboflavin

Recently, riboflavin has been used in new treatment methods to slow down or stop the progression of the corneal disorder namely keratoconus (Martins *et al*., 2008). In corneal collagen crosslinking (CXL), riboflavin drops are applied to the patient's corneal surface. Once the riboflavin has penetrated through the cornea, UVA light therapy is applied. This induces collagen crosslinking, which increases the tensile strength of the cornea. This treatment has been shown in several studies to stabilize keratoconus. Several scientists also repeated the bactericidal effect of photo-activated riboflavin using UVA.

In 1965, Japanese scientists demonstrated that riboflavin, when exposed to visible or UV light could be used to inactivate RNA containing tobacco mosaic virus

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(Tsugita *et al*., 1965). Makdoumi (2011) concluded that riboflavin photo-activation using UVA at 365 nm could achieve an extensive eradication of bacteria, and the combination is more potent in reducing bacterial number than UV alone. Indeed, the effectiveness of this new technique is reported in a number of articles in the literature. Schrier *et al*., (2009) recently reported a similar finding on success in using this technique for in-vitro eradication of three important bacteria, *S. aureus*, MRSA, and *P. aeruginosa* (Schrier *et al*., 2009). Martins *et al., (*2008) noted that this technique was effective against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, MRSA, and DRSP, but was ineffective on *Candida albicans* (Martins *et al*., 2008). However, every study on treating blood infections so far has been carried-out with the combination of riboflavin and UVA irradiation.

The inactivation of pathogens is also dependent on the sources of UVA rays that diffuse into the bacteria cell. This circumstance became an obstacle for the development of existing researches for the treatment of blood borne infections deep within the biological tissues. In addition, the negative effects of UVA ionization on the biological tissues also hindered the advances in in-vivo investigations. Therefore, the need for optimising the strength and efficacy of riboflavin without the combination of UVA irradiation is very much necessary.

A study in year 2000 was conducted which involved eradication of malarial infection in blood using riboflavin without the application of UVA. Akompong *et al*., 2000 conducted his research without using UVA to treat malaria infection caused by *Plasmodium falciparum* in human blood. His study highlighted successful inhibition of the growth of the parasites. Through this work, riboflavin was demonstrated as safe to be used for treating patients infected with malaria and concluded that it can be used as an antimicrobial agent (Akompong *et al*., 2000). Furthermore Coimbra & Jungueira, 2003 in their studies discussed on the high dose of riboflavin and that the elimination of

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dietary red meat promoted the recovery of some motor functions in Parkinson's disease patients. These findings conclude the ability of riboflavin in treating patients suffering from the disease (Coimbra & Jungueira, 2003).

In contrast, Gariballa & Ullegaddi demonstrated that supplementation of riboflavin to 96 acute ischemic stroke patients with 5.0 mg of oral riboflavin reduces oxidative damage and cerebral oedema in their studies. 5 mg of riboflavin supplementation for 2 weeks significantly improved the health status of acute stroke patients (Gariballa & Ullegaddi, 2007).

Yuvaraj *et al*., in 2005 conducted a study to investigate if co-administration of Coenzyme Q10, (CoQ10), Niacin and riboflavin along with Tamoxifen which is a non-steroidal anti-estrogen drug most widely used as an adjuvant hormonal therapy in breast cancer, could augment the antioxidant status in post-menopausal women with breast cancer. They were evaluated in 78 post-menopausal breast cancer patients treated with Tamoxifen for 90 days. This supplementation effectively prevented the oxidative stress associated with Tamoxifen treatment. Through this study, it was concluded that riboflavin can also act as a photo-sensitizer, and this property may find importance in photodynamic therapy of cancer.

Later a mouse model was used to assess the effect of riboflavin in combination with cisplatin, one of the most effective anti-cancer agents. Under infrared spectroscopy light exposure, riboflavin administration reduced cisplatin-induced DNA damage in the liver and kidneys. This study gave a promising result, but further clinical studies involving higher primates and humans are necessary to examine whether riboflavin might be an effective adjuvant to chemotherapy in humans (Hassan *et al*., 2012).

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2.8 Microbes and Diseases

One of the foremost public health crises in the world is blood infection. It is devastating and life threatening response to infection. It is a state of infectivity which can lead to tissue damage, organ failure, and death through release of toxins into blood cells and plasma by pathogens. Patients who developed sepsis have an increased risk of complications and death and face higher healthcare costs and needs longer treatment. According to the World Health Organisation (WHO), 8 million new cases and 3 million deaths on average were directly related to such diseases every year (Morse, 2009; Smyth *et al*., 2008) (Figure 2.4). The struggle for control, far from being over, has reached a critical stage. Based on a WHO survey in 1996, infectious diseases remain the world's leading cause of death, accounting for at least 17 million (about 33%) of the 52 million people who die each year (WHO, 1996). Apart from those 17 million, about 9 million of them are young children and up to half the world's population of 5.72 billion people are at risk of many endemic diseases (WHO, 1996). CDC's National Centre for Health Statistics (NCHS) estimates that the number of people in the hospital with septicaemia increased from 621000 in the year 2000 to 1141000 in 2008. In 2008, the percentage of hospitalized patients who were discharged to other short stay in hospitals or long term care institutions was higher for those with septicaemia or sepsis (36%) than for those with other conditions (14%). Meanwhile 17% of septicaemia hospitalizations ended in death, whereas only 2% of other hospitalization ended in death (WHO, 2014).

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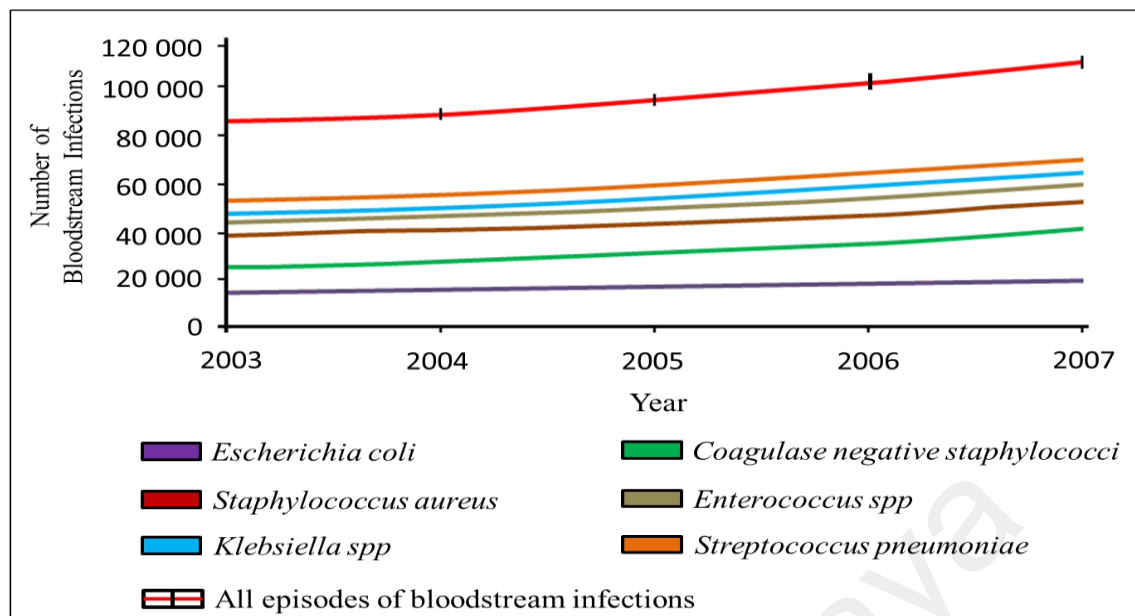

Figure 2.4: Healthcare Associated Infections Report Year 2003 – 2007 (WHO, 2003-2007).

The lack of treatment for blood infections may lead to septicaemia. As of now, there are positive treatments towards blood infections using currently available antimicrobials. The evolution of microbes has made them to become more resistant towards antibiotics and caused chromosomal mutation, inductive expression of a latent chromosomal genes or exchange of genetic material via transformation, bacteriophage transduction and plasmid conjugation (Randall & Michael, 1996). This condition is hampering treatment with present antimicrobial agents.

In simple terms, antimicrobial resistance means that microbes have come up with a way of stopping a drug from killing or damaging them. The principle behind the development of antimicrobial resistance is survival of the pathogens through resistance to an antimicrobial so that most microbes multiply very rapidly. Naturally most of the microbes' are susceptible to different antimicrobial drugs; therefore different microbes develop resistance to different drugs. Only some bacteria have always been resistant to certain antibiotics and that each antibiotic only has an effect on specific microorganism. But sometimes bacteria can become resistant to antibiotics that previously killed or damaged them. Pathogens can develop resistance in few ways such as a random change

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in the genetic material of the bacteria known as a mutation. This can cause the genetic material to make the bacteria resistant to harm by the drug.

Pathogens can also become resistant by picking up genetic material that contains instructions that code for antibiotic resistance. This genetic material can come from viruses and bacteria cells or plasmids, which are loops of DNA in a bacterial cell that are separate from its chromosome. This is due to bacteria having only one single chromosome, unlike us humans who have 23 pairs in each cell. These plasmids can move from bacteria to bacteria, picking up and depositing bits of genetic material as they leave (Gyles & Boerlin, 2014). If the plasmid contains a bit of genetic material those codes for antibiotic resistance, this can be spread to many other bacteria. So this explains how antibiotic resistance can develop but it doesn't explain what effect these genetic instructions have on stopping the antibiotic from harming the bacteria.

Furthermore pathogens are able to become resistant to antibiotics through inactivation of the antibiotics before it reaches the bacteria cell. Resistant occur as the pathogens plummet the uptake of the antibiotic into the bacterial cell and increase the amount of the antibiotic that is pumped out of the cell. Through this process the pathogen multiplies the antibiotic's target on the bacterial cell. At this time pathogen implies different ways of multiplying, feeding and maintaining its structure enabling it to function despite the effect of the antibiotic (Albert *et al.*, 2002).

Antimicrobial resistance is a dilemma due to less choice of effective drugs with which to treat infections. The more an antimicrobial drug is used, the more resistance develops in the microorganisms it is used to treat (Eileen *et al*., 2010). For example, only a few years after penicillin was developed, resistance to it was discovered in *S. aureus.* This is a bacteria commonly found on our skin as part of our microflora. Some *S. aureus* is now resistant to almost all antibiotics and can be very difficult to treat if they cause illness (Eileen *et al*., 2010). Following years of heavy use of penicillin,

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several species of bacteria are now resistant to this drug. Minor infections that were or are easily treated may become more serious if this trend continues as the range of effective antimicrobials is reduced. The biggest problem facing us is the development of multi-resistant microorganisms (Eileen *et al*., 2010). This is when a pathogen becomes resistant to more than one antimicrobial drug. The more pathogens become resistant, the harder it will be to be treated. This is the problem with infections such as MRSA (Eileen *et al*., 2010).

An effective antimicrobial drug should possess the following properties; potent and selectivity at target site, good absorption from the site of administration, appropriate distribution in the body, adequate persistence in tissues and non-toxic to patients (Franklin & Snow, 2005a). These drugs may either inhibit the cell wall, protein or nucleic acid synthesis, or alternate cell membrane permeability (Jawetz, 1980).

2.9 Infections cause by Microorganisms

2.9.1 *Staphylococcus aureus*

Staphylococci are facultative anaerobes. They are Gram positive, occur in grape like-clusters and are catalase positive. They are major components of the normal flora of skin and nose in each and every individual. *S. aureus* is a one of the common cause of opportunistic infections in hospital and community, including pneumonia, osteomyelitis, septic arthritis, bacteraemia, endocarditis, abscesses/boils and other skin infections. It is also present in golden colour. *S. aureus* is an extremophile that can survive in extreme temperatures or other inhospitable circumstances. About 70-90% of population carry this strain of *Staphylococcus* in their nostrils at some time (Williams, 1963). Although present on the skin of only 5-20% of healthy people, as many as 40% carry it elsewhere, such as in the throat, vagina, or rectum, for varying periods of time, from hours to years, without developing symptoms or becoming ill. It is still one of the

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five most common causes of nosocomial infections and is often the cause of post-surgical wound infections. Each year, some 500,000 patients in American hospitals contract staphylococcal infection (Bowersox, 1999). The bacteria may disseminate throughout the body and causes fatality if remain untreated.

S. aureus flourishes in hospitals, where it infects healthcare personnel and patients who have surgery, acute dermatitis, insulin-dependent diabetes, dialysis-dependent kidney disease or one who receives frequent allergy-desensitization injections. *Staphylococcus* bacteria can also contaminate bedclothes, catheters, and other objects. *S. aureus* causes a variety of infections. Boils and inflammation of the skin surrounding a hair shaft (folliculitis) are the most common. Toxic shock (TSS) and scalded skin syndrome (SSS) are among the most serious implications. TSS is a life-threatening infection characterized by severe headache, sore throat, fever as high as 105ºC, and a sunburn-like rash that spreads from face to the rest of the body. The symptoms appear suddenly and also cause dehydration and watery diarrhoea. Inadequate blood flow to peripheral parts of the body (shock) and loss of consciousness might occur within the first 48 hours. Between the third and seventh day of illness, skin peels from the palms of the hands, soles of the feet, and other parts of the body. Kidney, liver, and muscle damage can occur consecutively.

Rare in adults and most common in new-borns and children under the age of five, SSS originates with a localized skin infection. A mild fever or an increase in the number of infection-fighting white blood cells may occur. Bright red rashes spread from face to other parts of the body and eventually form scales. Large, soft blisters develop at the site of infection and elsewhere. When they burst, they expose burnt-like inflamed skin.

S. aureus can also cause respiratory disease, pockets of infection and pus under the skin (carbuncles), tissue inflammation that spreads below the skin, causing pain and

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swelling, inflammation of tissue that enclosed and protects the spinal cord and brain (meningitis), inflammation of bone and bone marrow (osteomyelitis), pneumonia and inflammation of the valves and walls of the heart (endocarditis)

2.9.2 *Enterococcus faecalis*

E. faecalis is family of *Enterococcaceae* and class of bacilli. It is Gram positive cocci, facultative anaerobic, occurs singly, in pairs or short chains, no haemolysis on blood agar after 24 hours but it may show alpha haemolysis after 48 hours. *E. faecalis* is commonly found in root canal-treated teeth in prevalence values ranging from 30-90% of the cases (Molander *et al*., 1998). The root canal treated teeth are about nine times more likely to be infected with *E. faecalis* than cases of primary infections (Rocas *et al*., 2004). Normal inhabitant of intestinal tract (10^5-10^8 CFU's per gram of stool) and female genital tract, it is occasionally associated with urinary tract infection, bacteraemia and bacterial endocarditis (Murray, 1990; Hidron *et al*., 2008).

E. faecalis is a hub element of the intestinal flora of human and a leading cause of nosocomial infections worldwide (Richards *et al*., 2000). Enterococci are allied with a variety of pathologies, including pelvic infections, intra-abdominal abscesses, postsurgical infection, bacteraemia, endocarditis, and urinary tract infections (Evans & Chinn, 1947; Jett *et al*., 1994; McBride *et al*., 2007). The capability of *E. faecalis* to cause serious infection is connected to the intrinsic resoluteness of the bacterium, which enables it to tolerate aridness, persist in hospital environment, and then tolerate host defences (Jett *et al*., 1994; Kramer *et al*., 2006). In addition, enterococci are particularly capable at acquiring resistance to antibiotics and disseminating these elements within and beyond the genus (Weigel, 2003; McBride *et al*., 2007).

Moreover cytolysin toxin produced by *E. faecalis* is responsible for bursting a variety of target membranes and contributes to the toxicity or lethality of the infection.

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By inactivating the cytolysin, the number of the bacterium in circulation might be reduced, while not harming many of the natural microorganisms in the body. This can be accomplished through drug combination treatment because of its highly specific targeting.

Other characteristics of *E. faecalis* includes it is non-motile, micrometer sized diameter, facultative anaerobes (prefer anaerobic), complex and variable nutritional requirements, perform simple fermentation, mechanism of pathogenicity is unknown, members of genus streptococcus, belongs to Lancefield's serologic group D *Streptococcus*, catalase negative, optimum growth at 37ºC and sensitive to chlorination.

The enterococcus group is a subgroup of the faecal streptococci that includes at least five species such as *S. faecalis*, *S. faecium*, *S. durans*, *S. gallinarum*, and *S. avium*. They can be distinguished from other streptococci by their ability to grow at high pH (9.6-10), high temperature (45ºC) and high salt concentration (6.5% sodium chloride). The enterococci are generally resistant to many Gram positive antibiotics such as tetracyclines, aminoglycosides, sulphonamides, some penicillin, and lincosamides. *E. faecalis* and *E. faecium* are the most frequently found species in human.

E. faecalis is the only enterococcus species that has been genetically characterised so far (Gilmore *et al*., 2014). Its genome is 3 million bases in length. The two genetic mechanisms first discovered in the enterococci were conjugative transposes and sex pheromone plasmids. Some strains require vitamin B and amino acids for growth.

The enterococci are used as bacterial indicator for determining the extent of faecal contamination in foods and in recreational surface waters. Water quality guidelines based on enterococcal density has been proposed for recreational water. The guideline is 33 enterococci/100 ml for recreational fresh waters. For marine waters, the guideline is 35 enterococci/100 ml (Benoit & Denis, 2007). The guidelines are based on

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the genomic mean of at least five samples per 30 days period during the swimming season. There are two types of selection methods namely membrane filter technique and multiple-tube technique (Benoit & Denis, 2007). The membrane filter technique is used for samples of fresh and saline waters. However, it is unsuitable for highly turbid waters. The multiple-tube technique on the other hand is applicable to fresh and marine waters, but is primarily used for raw and chlorinated waste water.

2.9.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a member of Gamma Proteobacteria class of bacteria. It is a Gram negative, aerobic rod belonging to the bacterial family *Pseudomonadaceae*. Almost all strains are motile by means of a single polar flagellum. The bacterium is ubiquitous in soil and water, and on surfaces in contact with soil or water. Its metabolism is respiratory and never fermentative, but will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor (Todar, 2007).

The bacterium is adept at infecting many different organs and tissues. Since it causes disease primarily in person whose health is compromised in some manner, it is considered as an opportunistic pathogen. Mechanical ventilation, for instance, predispose patients to pneumonia caused by *P. aeruginosa*. Likewise, the presence of a urinary catheter is associated with an increased risk of urinary tract infections. Patients with cancer who have neutropenia resulting from chemotherapy or hematologic malignancies are prone to bacteraemia and burn affected patients often experience wound infections. Although each of these infections is most often categorised as hospital-acquired, *P. aeruginosa* frequently causes community-acquired disease in patients with cystic fibrosis.

The reason *P. aeruginosa* so often infects hospitalised patients is likely to be multifactorial. This bacterium is capable of metabolising an impressive list of

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compounds for the generation of energy and thus often contaminates intravenous solutions, hospital equipments and even disinfectants. Such contamination has led to epidemics in which multiple patients have been infected with a single strain that originated primarily from a single source. Even in the absence of such epidemics, *P. aeruginosa* is a frequent cause of nosocomial disease. In hospitalized patients exposed to numerous antimicrobial agents, the intrinsic and acquired resistance of this organism undoubtedly confers on it a selective advantage and allows for colonization and subsequent infection (Fleiszig *et al*., 1997). Once established, *P. aeruginosa* infections often pose a therapeutic dilemma because of these same properties of resistance.

Given the number of compounds active against *P. aeruginosa*, it might be expected that treatment of infections caused by this bacterium would be straightforward. This situation, however, is complicated by the predilection of *P. aeruginosa* to develop resistance to nearly any antimicrobial agent. Resistance is problematic at three levels, which are intrinsic resistance, acquired resistance and emergence of resistance during therapy. Each of these must be considered when choosing an antibiotic regimen for patients infected with *P. aeruginosa.*

In general, *P. aeruginosa* is naturally less susceptible than other Gram-negative bacilli to many antibiotics, such as ampicillin (Principen), most cephalosporins and the macrolides. This is because of its relatively impermeable outer membrane and its ability to actively transport some antibiotics out of the cell, preventing accumulation. *P. aeruginosa* also harbours an inducible chromosomally encoded beta-lactamase, referred to as AmpC beta-lactamase, which is capable of degrading many beta-lactams even though it is found naturally at very low levels.

Biofilm formation by *P. aeruginosa* may also contribute to antibiotic resistance in infections (Costerton *et al*., 1999), although the importance of this mode of growth in hospital-acquired infections that do not involve foreign bodies is less well-understood.

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When grown as biofilms, which are organized communities of bacteria that grow on surfaces, individual bacteria are much more resistant to antibiotics than when grown planktonically (dispersed in fluid) (Costerton *et al*., 1999).

In addition to its intrinsic resistance, *P. aeruginosa* has the ability, through mutational changes or acquisition of exogenous genetic material, to develop resistance to each of the anti-pseudomonal antibiotics (Todar, 2007). For example, the chromosomally encoded AmpC beta-lactamase is capable of degrading beta-lactams, such as piperacillin and ceftazidime, when mutations result in production of large amounts of this enzyme. Of note, the beta-lactamase inhibitors tazobactam, sulbactam sodium and clavulanate are not active against AmpC beta-lactamase. Resistance to aminoglycosides may occur by mechanisms that differentially affect members of this class. Thus, resistance to gentamicin and tobramycin is often not accompanied by resistance to amikacin. *P. aeruginosa* can also express several efflux pump systems. Over-production of these pumps can prevent accumulation of antibiotics within the bacterium and result in simultaneous loss of susceptibility to multiple antibiotics.

Not surprisingly, the many and varied ways by which *P. aeruginosa* can resist the actions of antibiotics have resulted in documentation of relatively low susceptibility rates. Although in different patient populations and different geographic locations, two troubling trends are evident. First, susceptibility rates appear to be decreasing with time, especially with regard to Piperacillin, Ceftazidime, Imipenem and Ciprofloxacin susceptibility rates decreased from 89% in 1990 through 1993 to 68% in 2000. Secondly, 10% or more of the recovered isolates are not adequately treated with most empirically chosen single agents.

Even when antimicrobial agents to which an isolate is susceptible are chosen, a successful therapeutic outcome is not ensured. One reason for this uncertainty is that *P. aeruginosa* has shown a regrettable propensity to develop resistance to antibiotics

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during therapy. Resistance develops because of the natural occurrence of mutations essential for antibiotic penetration or activity. Within infected tissue, selection for individual bacteria that harbour these mutations occurs because the antibiotic is present, and eventually these organisms compose the majority of the bacteria population.

2.9.4 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram negative, non motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin and intestines. It can be found in singly, pairs or short chains. Diplobacillary forms are commonly found in-vivo (Ristuccia & Cunha, 1984). It is clinically the most important member of *Klebsiella* genus of *Enterobacteriaceae*. It is *Enterobacteriaceae* family of the γ-Proteobacteria class in the phylum Proteobacteria (Ryan & Ray, 2004).

In recent years, *Klebsiella* have become important pathogen in nosocomial infections. *K. pneumoniae* can cause the disease *Klebsiella pneumonia*. They cause destructive changes to human lungs by inflammation and haemorrhage with cell death or necrosis that sometimes produces thick, bloody and mucoid sputum. Typically these bacteria gain access after a person aspirates. The most common infection caused by *Klebsiella* bacteria outside the hospital is pneumonia, typically in the form of bronchopneumonia and also bronchitis. These patients have an increased tendency to develop lung abscess, cavitation, empyema, and pleural adhesions. It has a high death rate of about 50% even with antimicrobial therapy. The mortality rate can be nearly 100% for persons with alcoholism and bacteraemia (Ryan & Ray, 2004).

Furthermore, it also can cause infections such as lower biliary tract, and surgical wound sites. The range of clinical diseases includes pneumonia, thrombophlebitis, urinary tract infection, cholecystitis, diarrhoea, upper respiratory tract infection, wound

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infection, osteomyelitis, meningitis, and bacteraemia (Podschun & Ullmann, 1998; Ryan & Ray, 2004). A patient with invasive device in their body such as respiratory support equipment and urinary catheters faces increased risk factor. In addition, the use of antibiotics can be a factor that increases the risk of nosocomial infection with *Klebsiella* bacteria (Ryan & Ray, 2004). Entry of the bacteria into the bloodstream can cause sepsis and septic shock.

2.9.5 *Escherichia coli*

Escherichia coli are one of several types of bacteria that normally inhabit the intestine of humans and animals (commensal organism). It is a Gram negative bacterium, facultative anaerobic, rod shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm blooded organisms are capable of causing disease under certain conditions when the immune system is compromised or disease may result from an environmental exposure (Singleton, 1999).

E. coli strain does not cause disease but its virulent strains can cause gastroenteritis, urinary tract infections and neonatal meningitis. This organism may cause septicaemia, infantile gastroenteritis, tourist diarrhoea and hemorrhagic diarrhoea (Todar, 2007). An *E. coli* infection may also arise due to environmental exposure. Infections with this type of bacteria pose a serious threat to public health with outbreaks arising from food and water that has been contaminated with human or animal faeces or sewage. This type of bacteria has been used as a biological indicator for safety of drinking water since the 1890s (Edberg *et al*., 2000). Exposure may also occur during hospitalization, resulting in pneumonia in immune compromised patients or those on ventilator.

The symptoms of infection and resulting complications are dependent upon the strain of *E. coli* and the site of infection. These bacteria produce toxins that have wide

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range of effects. Symptoms caused by some *E. coli* infections range from mild to severe, bloody diarrhoea, acute abdominal pain, vomiting and fever. Gastrointestinal complications that can cause *E. coli* infections include irritable bowel syndrome (IBS) ischemic colitis, appendicitis, perforation of the large bowel, and in some instances gangrene in the colon (Kamada *et al*., 2005). Other known *E. coli* causing infections may include chronic renal failure, pancreatitis and diabetes mellitus. Some neurological symptoms such as drowsiness, seizure and coma may occur. In infants, *E. coli* infections are present in cases of infantile gastroenteritis and neonatal meningitis.

Strains of *E. coli* that produce diarrhoea were initially distinguished by their O (somatic) antigens found on the bacterial surface. Although there is an overlap in characteristics between strains, they may be classified into four main groups; enterohemorrahagic (0157), enteropathogenic (055, 0111), enterotoxigenic (06, 078), and enteroinvasive (0124, 0164). The O157:H7 strain is the member of the group most often associated with a particularly severe form of diarrhoea. The O indicates the somatic antigen, while the H denotes the flagella antigen, both of which are found on the cell surface of the bacteria. The bacterium was discovered in 1977 (Azadi *et al*., 2010), and first reports of infections followed in 1985 (Todar, 2007). *E. coli* O157:H7, as it is frequently referred to by researches, causes bloody diarrhoea in many infected patients. It accounts for about 2% of all cases of diarrhoea in the western world, and at least one-third of cases of hemorrhagic colitis, or about 20,000 cases per year (Hamer & Sherwood, 1997).

E. coli O157:H7 is also the most common cause of unique syndromes, known as the Haemolytic-Uremic Syndrome (HUS) and Thrombocytopenic Purpura (TTP), which causes kidney failure, haemolytic anaemia and thrombocytopenia (Phillip *et al*., 2005). Usually, infection with this strain of bacteria will subside without further complications. However, about 5% of people who are infected will develop HUS/TTP. This infection

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also accounts for the majority of episodes of HUS, especially in children (Phillip *et al*., 2005).

This strain of bacteria produces a potent toxin called verotoxin, named for the toxin's ability to kill green monkey kidney or "vero" cells. Bacteria that produce verotoxin are referred to as Vero toxin-producing *E. coli* (VTEC). The numbers of bacteria that are necessary to reproduce infectious levels of bacteria are quite small, estimated at 10-100 viable bacteria. These toxins are lethal for intestinal cells and those that line vessels (endothelial cells), inhibiting protein synthesis causing cell death. It is believed that the damage to blood vessels result in the formation of clots, which eventually leads to the HUS. HUS/TTP is a serious, often fatal, syndrome that has other causes in addition to *E. coli* O157:H7; it is characterized by the breaking up of red blood cells (haemolysis) and kidney failure (uraemia). The syndrome occurs most often in the very young and very old.

After incubation period of three to four days on average, watery diarrhoea begins, which rapidly progresses to bloody diarrhoea in many victims, in which case the bowel movement may be mostly blood. Nausea, vomiting and low-grade fever are also frequently present. Gastrointestinal symptoms last for about one week, and recovery is often spontaneous. Symptomatic infection may occur in about 10% of infected individuals. About 5-10% of individuals, usually at the extremes of age or elevated leukocyte count, develop HUS/TTP, and ultimately, kidney failure (Hall & Glickman, 1988; Ruggenenti *et al*., 2001). Patients taking antibiotics or medications for gastric acidity may also be at risk. Neurological symptoms can also occur as part of HUS/TTP and consist of seizures, paralysis and coma. Rectal prolapsed may also be a complication, and in some cases colitis, appendicitis, perforation of the large bowel and gangrene in the bowel. Systemically, the most prevalent complications of *E. coli* 157 infections are HUS and TTP.

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Many strains of *E. coli* produce verotoxin, but not strains of O157. There have been as many as one hundred different types implicated in the development of diseases. Strain OH111 was found to be involved in outbreaks in Australia, Japan and Italy. The O128, O103 and O55 groups have also been implicated in diarrhoea outbreaks. In Britain, cases of infantile gastroenteritis in maternity hospitals and neonatal units have been attributed to the *E. coli* (non-O157 group). Many of these organisms have been identified in cattle. Two toxins may be produced by this group, the heat-labile enterotoxin (LT) that can produce enteritis in infants, and a heat-stable enterotoxin (ST), the action of which has yet to be determined.

Some strains of the enteroinvasive *E. coli* have been involved in the development of gastroenteritis in infants. These organisms do not produce an enterotoxin. The cells of the intestine are affected with the development of symptoms that are typical of a shigellae infection.

2.9.6 *Salmonella typhi*

Salmonella is a Gram negative enteric bacillus. It is a facultative anaerobe and rod shaped bacterium in the same proteobacteria family as *Escherichia coli*, the family *Enterobacteriaceae*, trivially known as “enteric” bacteria. Salmonella is well studied as *E. coli* from a structural, biochemical and molecular point of view, and as poorly understood as *E. coli* from an ecological point of view. Salmonella live in the intestinal tracts of warm and cold blooded animals. Some species are ubiquitous and other species are adapted to a particular host. In humans, *Salmonella* are the cause of two diseases called salmonellosis which is enteric fever (typhoid), resulting from bacterial invasion into the bloodstream and acute gastroenteritis, resulting from a food borne infection/intoxication (Hirose *et al*., 2002).

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As with all *Enterobacteriaceae*, the genus *Salmonell*a has three kinds of major antigens such as somatic, surface, and flagellar; Somatic (O) or cell wall antigens (Todar, 2005). Somatic antigens are heat stable and alcohol resistant. Cross-absorption studies individualize a large number of antigenic factors, 67 of which are used for serological identification. O factors labelled with the same number are closely related, although not always antigenically identical. Surface (Envelope) antigens, are commonly observed in other genera of enteric bacteria such as *Escherichia coli* and *Klebsiella*. It may be found in some *Salmonella* serovars. Surface antigens in *Salmonella* may mask O antigens, and the bacteria will not be agglutinated with O antisera. One specific surface antigen is well known; the Vi antigen. The Vi antigen occurs in only three *Salmonella* serovars from 2 200 as Typhi, Paratyphi C and Dublin. Strains of these three serovars may or may not have the Vi antigen.

Flagellar antigens are heat-labile proteins. Mixing salmonella cells with flagella-specific antisera gives a characteristic pattern of agglutination (Todar, 2007). The antiflagellar antibodies can immobilize bacteria with corresponding H antigens. A few types of Salmonella enteric serovars such as *Enteritidis typhi* produce flagella and have the same antigenic specificity. Such H antigen is then called monophasic. The H antigen is then called diphasic. For example, Typhimurium cells can produce flagella with either antigen i or antigen 1, 2. If a clone is derived from a bacterial cell with H antigen i, it will consist of bacteria with i flagellar antigen. However, at a frequency of 10^{-3}- 10^{-5}, bacterial cells with 1, 2 flagellar antigen pattern will appear in this clone.

The principal habitat of the salmonellae is the intestinal tract of humans and animals (Todar, 2005). Typhi and Paratyphi A are strictly human serovars that may cause grave diseases often associated with invasion of the bloodstream. Salmonellosis in these cases is transmitted through faecal contamination of water or food. In the pathogenesis of typhoid, the bacteria enter the human digestive tract, penetrate into

intestinal mucosa without causing lesion and are stopped in the mesenteric lymph nodes in which bacterial multiplication and part of the lyses occurs, in mesenteric lymph nodes, viable bacteria and LPS release its endotoxin into bloodstream resulting in septicaemia. Release of endotoxin is responsible for cardiovascular collapse in typhoid due to action on the ventriculus neurovegetative centres (Todar, 2007).

Salmonella excretion by human patients may continue long after clinical cure and they are considered as asymptomatic carriers (Todar, 2007). Asymptomatic carriers are potentially dangerous when unnoticed. About 5% of patients clinically cured from typhoid remain carriers for months or even years. However, infants and young children are much more susceptible to infection, easily achieved by ingesting a small number of bacteria (Todar, 2005). In infants, the contamination could be through inhalation of bacteria-laden dust. The germ multiplies in the intestinal lumen causing an intestinal inflammation with diarrhoea that is often mucous-purulent and bloody. In infants, dehydration can cause a state of severe toxicity. The symptoms are usually mild. Extra intestinal localizations are possible, especially salmonella meningitis occurs in children.

In Germany, between 1990 and 2005, the number of salmonella infections officially recorded cases approximates 50000 (Ivanoff & Levine, 1997; Todar, 2007). It is estimated that every fifth person in Germany is a carrier of salmonella. In USA, there are approximately 40000 cases of salmonella infection reported each year (Ivanoff & Levine, 1997; Todar, 2007). According to WHO, over 17 million people worldwide are infected with typhoid fever each year, with 500000 to 600000 fatal cases (Ivanoff & Levine, 1997; Todar, 2007).

2.9.7 *Candida albicans*

Candida albicans also referred as monilia is a fungus that is normally present on the skin and in mucous membranes such as the vagina, mouth, or rectum. The fungus

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can also travel through the blood stream and affect the throat, intestines and heart valves. *Candida albicans* becomes an infectious agent when there is change in the body environment that allows it to grow out of control. Nevertheless, fungi can cause trivial symptoms to death, especially in immune-suppressed patients. Fungi are eukaryotes that have more biochemistry similarities with mammalian cells compared to bacteria (Franklin & Snow, 2005a). Thus, they cause the difficulty of specific action and subsequently limited therapeutic options (Franklin & Snow, 2005b).

Candida albicans growth from a single cell to two type of forms such as yeast or mold which become a causal agent of opportunistic oral and genital infections in humans (Ryan & Ray, 2004). Systemic fungal infections including those by *C. albicans* have emerged as important cause of morbidity and mortality in immune compromised patients. *C. albicans* is commensal and biofilm may form on the surface of implantable medical devices. In addition, hospital-acquired infections of *C. albicans* have become a cause of major health concerns.

C. albicans lives in 80% of the human population without causing harmful effects, although overgrowth of the fungus results in candidiasis. Candidiasis causes infection of the skin, eye, oral cavity, oesophagus, gastrointestinal tract, vagina and vascular system of humans. In addition, candida species plays an important role in the pathogenesis of nosocomial infections in the newborn population. They colonise the neonatal skin and gastrointestinal tract. *Candida albicans* is the most preventable fungal pathogen, which causes the neonatal diseases. However the incidence of infection caused by *Candida parapsilosis* also increased dramatically (Bendel, 2003). *Candida albicans* virulence factors include host recognition biomolecules (adhesions), morphogenesis (the reversible transition between unicellular yeast cells and filamentous growth forms), and secretion of aspartyl proteases and phospholipases that help in the

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morphogenesis into invasive filaments and the adherence to the host cells (Calderone & Fonzi, 2001; Jackson *et al*., 2007).

2.10 Drug-resistance of Microbes

In recent times, living microorganisms have demonstrated their resistance to the toxic hazards of antimicrobial drugs through evolution. Many antibiotics have only cytostatic action but do not cause any detectable morphological changes on the microbes (Franklin & Snow, 2005b). These were proven by highly resistant organisms that emerge after prolonged or repeated exposure of the microbes to the drug. It was confirmed that bacteria can transfer drug resistance not only to the same species but also to different species and genera. Resistance to antibacterial drugs is now widespread, and the increasing resistance trend of antifungal drugs is also a major concern (Franklin & Snow, 2005d).

General mechanisms for most types of resistance include (a) conversion of the active drug to an inactive derivative by enzymes synthesized by the resistant cells, (b) loss or down-regulation of an enzymatic mechanism required to convert an inactive drug precursor to an active antimicrobial agent, (c) loss of sensitivity of the drug target site as a result of; (i) modification of the target size by enzymatic activity in the resistant cells, (ii) mutations in the microbial chromosome affecting the target, (iii) horizontal acquisition of genetic information encoding a drug-resistant form of the target enzyme, over production of the drug-sensitive enzyme, or proteins that protect the target site from inhibition, (d) removal of the drug from the cell by drug efflux pumps, and (e) reduction in cellular permeability to drugs caused by changes in the cell envelope (Franklin & Snow, 2005e).

Inappropriate consumption of antibiotics contributes to bacterial resistance. Therefore proper intake of antibiotics might boost up our immune system and protect

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organs to function properly against pathogens infections. In this study some bacteria were able to be inhibited through the application of riboflavin solution, which acts as an antibiotic. They are tested through disc diffusion method such as *S. aureus*, *E. faecalis*, *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *S. typhii*, and *C. albicans*.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Selection Range of Riboflavin Concentration

To find the range of riboflavin concentrations that could be effective to inhibit growth of pathogens, 5.0 to 100.0 g of riboflavin powder with1:1 ratio were prepared with Phosphate buffered saline (PBS). Based on FDA requirements (Food & Nutrition Board, 1998), the stock solution riboflavin was prepared at 1.0 g/ml. According to the protocol used in our work, each disc contained 50.0 µl per 6 mm disc. The main objective was to find the optimum concentration of riboflavin to inhibit growth of blood borne pathogens. An appropriate concentration, primarily on the basis of its demonstrated in-vitro spectrum of activity was chosen. Based on our studies, the optimum riboflavin's range of concentration in phosphate buffered saline (PBS) with a ratio 1:1 was from 20.0 to 100.0 g/ml. Through minimum inhibitory concentration (MIC) studies, riboflavin concentration of 25.0 g/ml was confirmed as appropriate to demonstrate riboflavin efficacy towards blood borne pathogens. Spectral analysis of riboflavin was performed using UV-Visible spectrophometer (Perkin Elmer UV Win Lab 6.0.4.0738/1.61.00 Lambda 900) to generate absorbance spectrum of riboflavin at 25.0 g/ml concentration.

3.2 Preparation of Riboflavin Stock Solutions

The images from a to h in Figure 3.1a to 3.1h shows the preparation of riboflavin stock solutions and the method used to pipette the 50.0 µl of solution into sterile 6 mm discs paper before placing on the cultured agar plates. Riboflavin-5-phosphate (Sigma-Aldrich, Malaysia) was used to prepare the stock solution (Figure 3.1a). Using a weighing balance, 5.0 to 100.0 g of riboflavin powder in increments of 5.0 g was measured and placed in light sensitive bottles (Figure 3.1a), Figure 3.1b

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shows PBS (Invitrogen), in ratio of 1:1 was used to the dissolve riboflavin powder, and while Figure 3.1c shows that the same method was used for other concentration too. Figure 3.1d shows the prepared solution, pipette 50.0 µl of riboflavin solution from the light sensitive bottle (Figure 3.1e) and placed into the multiwall culture plates (Figure 3.1f) which was placed earlier with 6 mm sterile discs (Figure 3.1g).

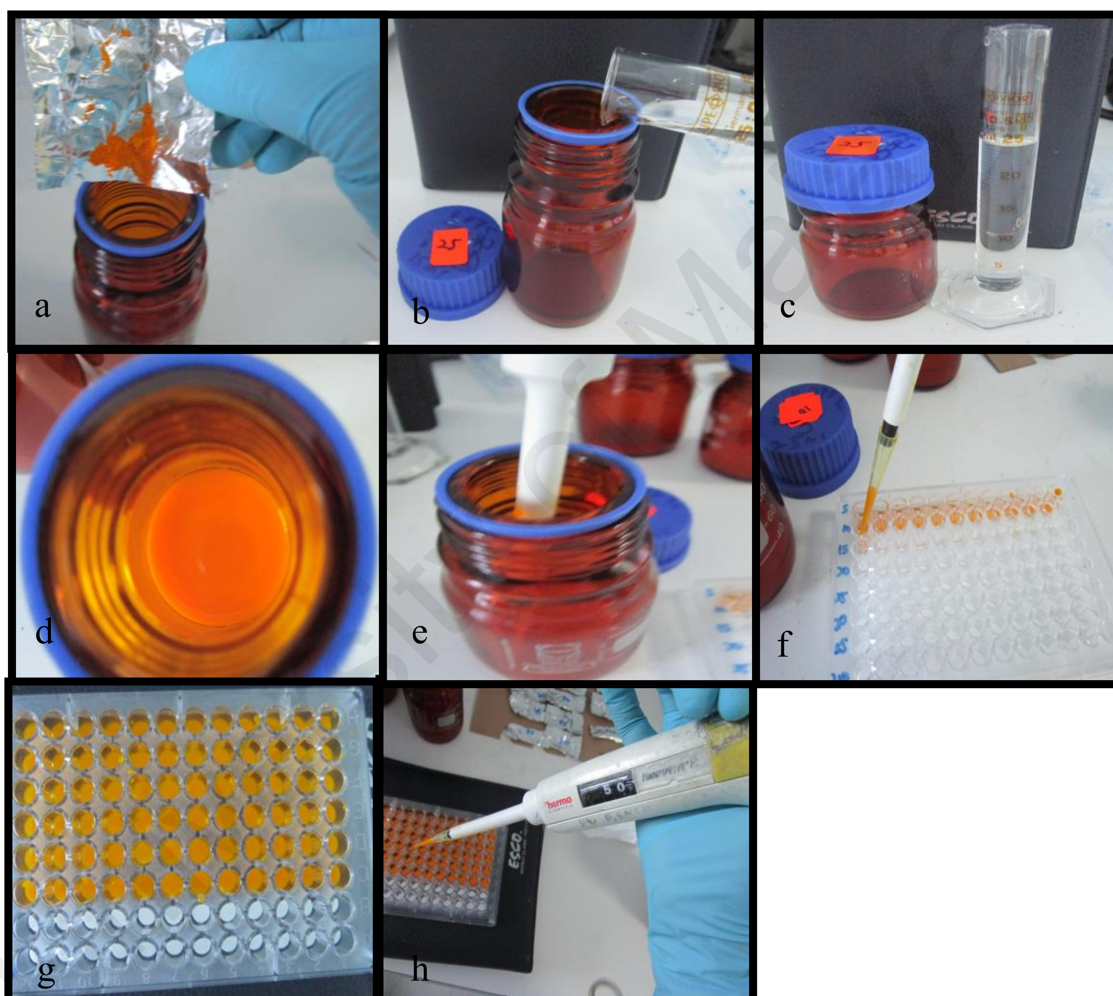

Figure 3.1: Images show the steps (a to h) involved in preparing riboflavin stock solutions used in this work. The labels above refer as following (a) light sensitive bottle, measured riboflavin powder with aluminium foil, (b) PBS with light sensitive bottle, (c) light sensitive bottle with the prepared PBS, (d) prepared riboflavin solution, (e) pipette riboflavin solution from light sensitive bottle, (f) release the riboflavin solution into the multiwall culture plates, (g) riboflavin solution with 6 mm sterile disc in the multiwall culture plate, (h) smoothly press the 6 mm sterile disc to absorb riboflavin solution.

The solutions were further protected from light source by storage in light sensitive dark bottles. The prepared solutions were used within 30 minutes of

preparation to avoid loss of its efficacy and reduce possible light contamination and also other measurement errors. From the prepared stock solutions, 50.0 µl of riboflavin solution were applied onto 6 mm sterile discs in multiwall culture plates until it absorbs all the solution (Figure 3.1h). Finally, added the prepared 6 mm sterile disc with riboflavin on the cultured agar plates by using sterile forceps (Sigma-Aldrich, Malaysia).

3.3 Exposure of Riboflavin to Ultraviolet A

The selected optimum concentration (25.0 g/ml) of riboflavin solution was exposed to UVA light (365 nm) of power density 10.4 J. A comparative study of riboflavin efficacy with and without UVA activation was then undertaken. 50.0 µl of solution was pipette into multiwall culture plates, followed by exposure to UVA light for 60 minutes before use. Then the solution was placed onto 6 mm disc papers before being transferred onto agar plates.

3.4 Selection of Pathogens

The blood borne pathogens were selected and obtained from the Microbiology Laboratory of Gribbles Pathology (M) Sdn. Bhd. Petaling Jaya. Bacterial strain studied were *Staphylococcus aureus* (SA), *Enterococcus faecalis* (EF), *Pseudomonas aeruginosa* (PA), *Klebsiella pneumoniae* (KP), *Escherichia coli* (EC), and *Salmonella typhii* (ST) and the fungus (yeast) was *Candida albicans* (CA). All pathogens were kept in cryogenic vials at -20ºC freezer to maintain its viability (Baker & Jeffries, 2006).

3.5 Biochemical Tests to Identify the Selected Pathogens

The following tests were done to confirm all the pathogens selected for this study. BioMerieux's API®Analytical Profile Index (API) tests were carried-out as a

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confirmation test for the selected pathogens. The tests kits were used for identification of Gram positive and negative bacteria, and fungus. The system offers a large and credible database, which can be accessed through the internet based APIweb™ service. APIweb™ is a software product containing all of the API strip databases for a reliable automated interpretation of API strip results when used on any compatible PC workstation. It is rapid and easy to use; works by keying in the biochemical or numerical profile of the strip to obtain microorganism identification. The software provides a detailed report that is displayed on the screen and can be printed out. It offers manual identification of microorganisms.

The following tests were carried-out to identify the species of each microorganism;

3.5.1 API Gram Negative Identification

API 20E is an 18 to 24 hour identification of *Enterobacteriaceae* and other non-fastidious Gram negative bacteria.

API Rapid 20E was used to identify *Enterobacteriaceae* in duration of 4 hours.

API 20NE is a 2 hour identification of Gram negative non *Enterobacteriaceae*.

3.5.2 API Gram Positive Identification

API Staph was used to identify clinical *Staphylococci* and *Micrococcus* within overnight.

3.5.3 API Yeast Identification

API 20 AUX is a 48 to 72 hour identification of fungus.

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3.5.4 Gram Stain of the Pathogens

Before and after the experiment, each microorganism was Gram stained to observe the morphology. The smears were fixed through heat fixation. The procedure was as follows;

A loop of fresh colonies from culture media was obtained and smeared on a clean glass slide. Then the slide was dried by passing over a Bunsen burner three times. The heat causes the microorganisms to adhere to the glass slides. After 10 minutes, the slides were stained with crystal violet, Gram iodine, acetone and safranin. The staining processes were as follows;

Labelled slides were arranged on staining rack. The slides were covered with crystal violet and allowed to stand for one minute. This was the primary stain. Then, the slides were gently washed with distilled water. Secondly, Lugol's iodine were poured on the slides and left for one minute. This was a mordant. The iodine was discarded by holding the slides on slanted position, washed with acetone until all stains were removed. The slides were washed with distilled water. Finally, the slides were stained with safranin and allowed to stand for one minute. This was the counter stain. The slides were washed gently, dried in air and observed under oil immersion objective. Gram positive organisms stained with crystal violet appeared violet coloured, while Gram negative organisms stained with safranin appeared pink coloured. Each of the images was observed, photographed using a 16 megapixel digital camera (Canon A3300IS) and analysed.

3.5.5 Preparation of the Wet Film

A loop of fresh colonies from culture media of *C. albicans* was obtained and smeared on a clean glass slide. Then a drop of saline was added on the smear. After that the slide was covered with clean cover slip. Finally the wet film was observed under

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microscope. The image of *C. albicans* was photo photographed using a 16 megapixel digital camera (Canon A3300IS) and analysed.

3.6 Preparation for In-vitro Experiments

3.6.1 Preparation of Mueller-Hinton Agar Plate

Mueller-Hinton (MH) agar is considered the best medium to use for routine susceptibility testing of non-fastidious bacteria. MH agar preparation included the following steps;

MH agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions (Clinical and Laboratory Standards Institute, 2006). Immediately after autoclaving, it was allowed to cool in a 45 to 50°C water bath. The freshly prepared and cooled medium was poured into glass or plastic, flat-bottomed petri dishes on a level and horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm. The agar medium was allowed to cool to room temperature and, unless the plate is used the same day, it was stored in a refrigerator at 2 to 8°C. Plates were used within seven days after preparation unless adequate precautions, such as wrapping in plastic, are taken to minimize drying of the agar. A representative sample of each batch of plates was then examined for sterility by incubating at 30 to 35°C for 24 hours or longer (Clinical and Laboratory Standards Institute, 2006).

pH of the MH agar was kept between 7.2 and 7.4 at room temperature after solidification. If the pH is 7.2, certain drugs such as aminoglycosides, quinolones and macrolides, will appear to lose potency while other agents like tetracycline may appear to have excess activity. If the pH is 7.4, the opposite results may occur (Clinical and Laboratory Standards Institute, 2006).

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To avoid false positive or false negative, MH agar should be tested weekly with known strains of microorganisms in order to verify that the media and discs are working as expected.

3.6.2 Preparation of Inoculum

Selected strains were streaked on horse blood agar at 35-37ºC and left overnight. Using a sterile inoculating loop or needle, four or five isolated colonies of the microorganisms to be tested were selected. All the microorganisms were suspended in 2 ml of sterile saline. The saline tube was then vortexed to create a smooth suspension. The turbidity of these suspensions was adjusted to a 0.5 McFarland standard by adding more microorganisms if the suspension was too light or diluted with sterile saline if the suspension were too concentrated. It is advisable to use these suspensions within 15 minutes of preparation.

Furthermore, the microorganisms to be tested must be alive in order to get valid results. For the susceptibility test, it is best to sub-cultures the microorganisms before the day of testing. Extremes in inoculum density, undiluted overnight broth cultures or other un-standardized inoculum for inoculating plates should not be used. The growth method of preparing the inoculum should be used instead, if the microorganisms are difficult to suspend directly into a smooth suspension (Clinical and Laboratory Standards Institute (CLSI), 2007).

3.6.3 Inoculation of the Mueller-Hinton Plates

Each microorganism was tested by MH agar plates. It was preferable to allow the plates to remain in the plastic sleeve while they warm to minimize condensation. A jar was placed on top of the agar plate and the plate inverted. The excessive liquid slowly flowed out from the agar into the jar and later evaporated. Plates were placed in

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a laminar flow hood at room temperature to dry for at least 10 to 30 minutes. Each plate was labelled according to each microorganism to be tested.

A sterile cotton swab was dipped into the inoculums tube. The swab was rotated against the side of the tube, above the fluid level using firm pressure to remove excess fluid. Inoculation of the dried surface of a MH agar plate by streaking the swab three times over the entire agar surface; the plate rotated approximately 60°C each time to ensure an even distribution of the inoculums. The plate was rimmed with the swab to pick up any excess liquid. They were discarded into an appropriate container to avoid spread of pathogens (Franklin *et al*., 2012; Villanova, 2003). The lid slightly leaved ajar, the plate was allowed to sit at room temperature at least 3 to 5 minutes but not more than 15 minutes to avoid drying of the agar culture plate.

3.6.4 Experimental Design

Riboflavin treatments were tested against three settings of organisms which were Gram positive and negative bacterial and fungal isolates. In this study, riboflavin was tested with and without exposure to UVA to justify their antimicrobial efficacy towards blood borne pathogens. In another set of experiments, 5.0-100.0 g of riboflavin powder was measured and dissolved in PBS (ratio 1:1) to determine the MIC of riboflavin solution towards blood borne pathogens. The selected pathogens were *Staphylococcus aureus* (SA), *Enterococcus faecalis* (EF), *Escherichia coli* (EC), *Salmonella typhii* (ST), *Pseudomonas aeruginosa* (PA), *Klebsiella pneumoniae* (KP) and *Candida albicans* (CA). The Kirby-Bauer test, also known as the disc diffusion method was used for antibiotic susceptibility test. For this study, a culture medium, specifically the MH and Sabaroud's agar was used. The plates were uniformly and aseptically inoculated with the tested microorganisms. Then the sterile 6 mm discs were impregnated with a 50.0 µl of stand-alone and UVA exposed riboflavin solution concentrations were placed on the

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cultured agar plates (Franklin *et al.*, 2012). Prepared riboflavin solutions were exposed to UVA (365 nm) for 60 minutes at 10.4 J. If the microorganism is susceptible to riboflavin solution, there will be no growth around the disc. Thus this area was considered as the zone of inhibition. The effectiveness of the solution determined was based on the area of inhibition. Each plate consists of a control (C), which was impregnated with distilled water. Positive control involved standard antibiotic (Vancomycin used on SA and EF, Gentamicin used on PA, EC, and KP, Imipenem used on ST, while Nystatin used on CA) to each tested microorganism (Clinical and Laboratory Standards Institute, 2006). Experiments were performed in triplicates for each of the microorganism.

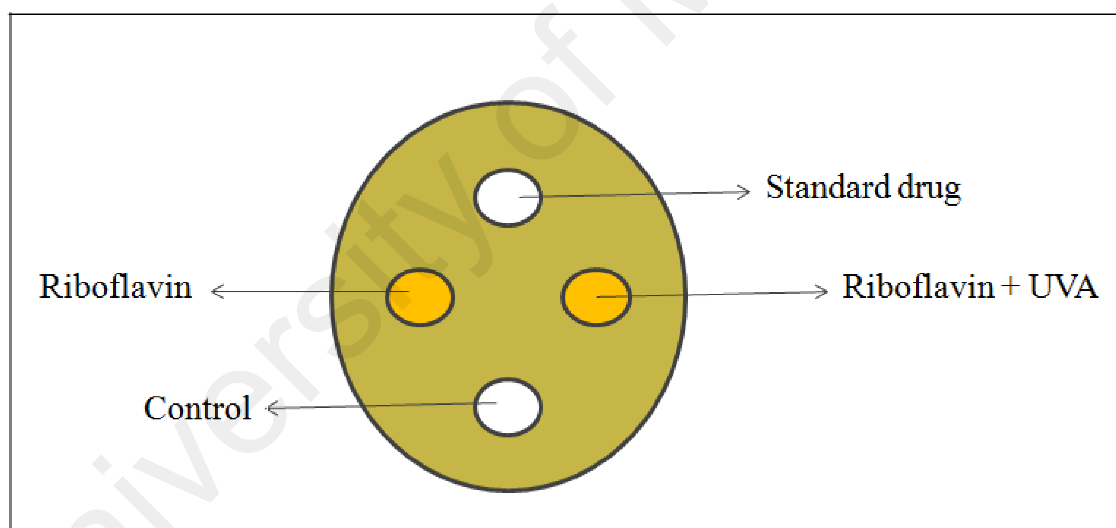

Figure 3.2: Experimental design and data analysis of the plates. Kirby-Bauer discs were used on the cultured plates. Standard drug used were conventional antibiotics and the control were 6 mm blank disc paper impregnated in distilled water. Riboflavin: consist of 50.0 µl, Riboflavin + UVA: consist 50.0 µl + 365 nm at 10.4 J for 60 minutes.

3.6.5 Placement of the Antibiotic Discs

The impregnated discs with riboflavin solution were either exposed to UVA or not exposed and then placed on the surface of the inoculated agar using sterile forceps. Cultured plates were placed on templates during addition of the drugs. The forceps were sterilised by cleaning with a sterile alcohol pad and allowed to air dry or by immersing

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the forceps in alcohol followed by igniting with fire. The lid of the Petri dish was then partially removed. The discs on the plate placed over one of the desired spots and the discs were gently pressed with the forceps to ensure complete contact with the agar surface. Once all the discs were placed on the inoculated plates, the plates were inverted and placed in a 35 ± 2℃ incubator for 18 to 24 hours (Isenberg, 2004).

3.6.6 Incubation of the Cultured Plates

To incubate the cultured plates, a temperature range from 35 to 37℃ is required. Plates should also not be placed in a CO_2 incubator as this will decrease the pH of the agar and might result in errors due to incorrect pH of the media. These microorganisms were incubated for 24 hours and then it was observed.

3.6.7 Measuring Zone of Inhibition

Diameter of the zone of inhibition was measured to the nearest millimetre using a calliper and ruler. To do this, the plate was placed on a black non-reflecting surface illuminated with reflected light. The plate was viewed by using a direct, vertical line of sight to avoid any parallax that might result in misreading. The zone sizes of each plate were recorded and the image was captured. Sometimes the zone of inhibition of microorganisms overlaps. When this occurs, measurement was taken from the centre of the disc to a point on the circumference of the zone where a distinct edge is present. This would be the radius and measurement multiplied by two to determine the diameter.

3.6.8 Interpretation and Reporting of the Results

After the plates have been incubated, there should be a noticeable “clearing” zone around each of the testing riboflavin discs. The diameter of each zone was measured and recorded in millimetres (mm). Each measurement can be compared to a

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zone size interpretative chart. Using the chart, the microorganism can be characterized as being resistant (R), intermediate (I), or susceptible (S) to the riboflavin drug. Intermediate susceptibility means that some inhibition from the prepared solution occurred but not sufficiently enough to inhibit the growth of the microorganism in the body. In these studies, commercially available antibiotics and its interpretative criteria for standard drugs according to Clinical and Laboratory Standards Institute (CLSI) guidelines were such as Vancomycin (S ≥ 15 mm; I and R no zone) used on SA and EF (S ≥ 17 mm; I 15-16 mm and R ≤ 14 mm), Imipenem (S ≥ 16 mm; I 14-15 mm, and R ≤ 13 mm) on ST, Gentamicin (S ≥ 15 mm; I 13-14 mm, and R ≤ 12 mm) on PA, EC and KP, and Nystatin (S ≥ 15 mm; I 10-14 mm, and R no zone) used on CA (Franklin et al., 2012). Each disc and its surrounding agar area were photographed using a 16 megapixel digital camera (Canon A3300IS).

3.7 Statistical Analysis

Obtained results were analysed using SPSS version 18.0 and Microsoft Excel 2007. Data were expressed as mean with their standard error. Furthermore, data from the tested drug were analysed by using One Way ANOVA and Student-t test. One Way ANOVA followed by Post-Hoc Tukey's multiple comparison tests to analyse the concentration of stand-alone and UVA exposed riboflavin. The significance was tested by using the multiple range tests at 95% least significance difference. Outcome of the result was stated as p-value. Values of $p < 0.05$ were considered statistically significant.

3.8 Pre-treatment with Selected Antibiotics

Selected pathogens such as from Gram positive bacteria SA and EF and Gram negative bacteria ST and PA pre-treated with standard drugs for 5 minutes and then riboflavin (50.0 µl) was added on the 6 mm sterile disc before apply on cultured plates.

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All the plates were incubated overnight. Vancomycin was used for SA and EF, Imipenem on ST and Gentamicin on PA. They were tested by using Kirby-Bauer discs with empty disc (control), (R) - riboflavin 50.0 µl alone, SD - Standard drugs alone and then the combination of SD (5 minutes) + R (50.0 µl). The mean zones of inhibition (mm) were measured around the discs (Clinical and Laboratory Standards Institute, 2006).

3.9 Method for Preparing a Sample for Confocal Microscopy

Laser scanning confocal microscopy represents one of the most significant advances in optical microscopy. It primarily enables visualization deep within both living and fixed cells and also tissue. It has ability to sharply collect the defined optical sections from which three-dimensional image can be created (Lattante et al., 2014)

A drop of prepared riboflavin solution was placed on a cleaned glass slide and then it was covered with cover slip. After that it was subjected to laser confocal scanning microscopy for imaging.

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CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Optical Microscope Characterization of the Riboflavin Solution

Using optical microscope (Olympus BX40) riboflavin was observed as fine and long yellow cylinder-like crystals as shown in Figure 4.1. The length of the crystal-like cylinders was varied from the highest at 1.14×10^{-2} mm to the shortest at 7.5×10^{-3} mm.

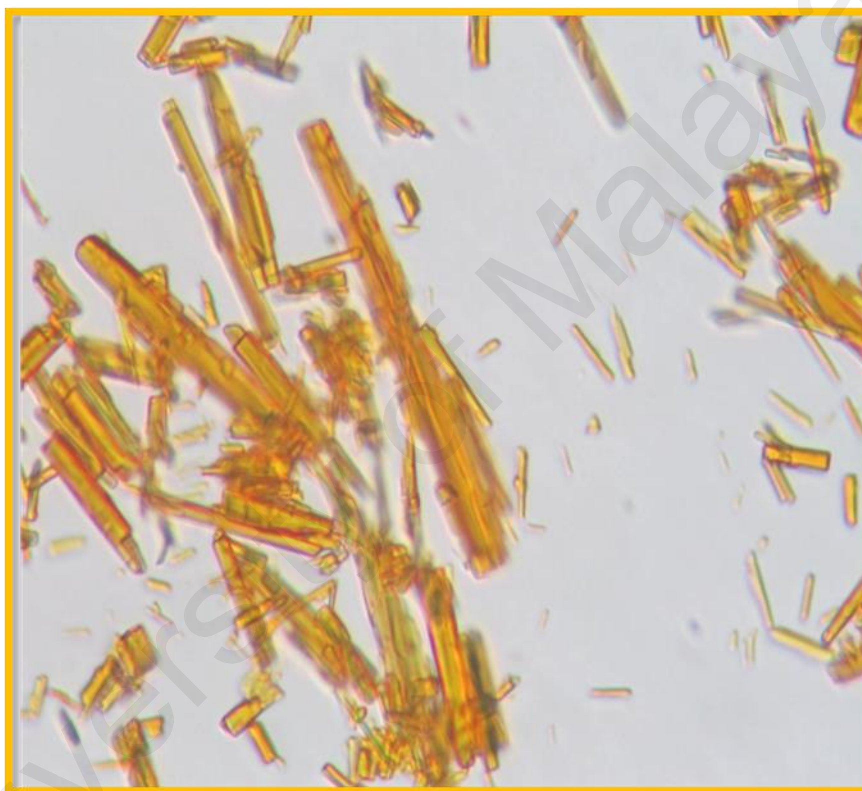

Figure 4.1: Riboflavin crystals observed under light microscope (1000 X magnification).

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4.2 Photo-activation of Riboflavin

4.2.1 Spectral Analysis of Riboflavin Using UV-Visible Spectrophotometer

Riboflavin in its oxidized state has light absorption peaks in UV range of wavelengths of 100-280, 280-315, and 315-400 nm and in visible light spectrum in the

region of 445 nm. The irradiation using UV-visible spectrophotometer leads to excitation of riboflavin and later followed by its degradation (Figure 4.2).

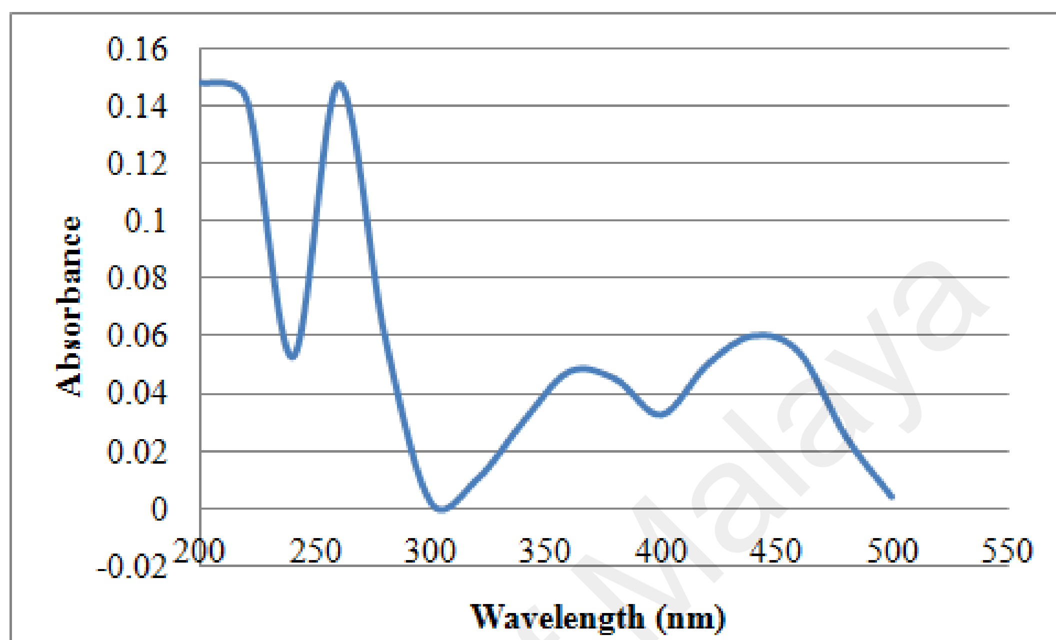

Figure 4.2 Absorption spectrum of riboflavin at 25.0 mg/ml concentration. The spectrum peaks at 220, 260, 370 and 440 nm, with absorbance of 0.143, 0.148, 0.05 and 0.06, respectively.

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4.2.2 Characterization of Confocal Laser Microscope

Confocal laser scanning microscope involves a laser beam passing through a light source aperture and focused by an objective lens into a small focal volume within/or on the surface of the prepared solution of riboflavin. During this process, fluorescent light from the illuminated spot passes back through the objective lens (Lattante *et al*., 2014). After undergoing some filtering and blocking of the original excitation wavelength, the fluorescence was detected by a photo-detection device before transduced into electrical signals acquired by a computer (Lattante *et al*., 2014).

In our work, the excitation leads to fluorescence in the green light spectrum at a wavelength of 520-560 nm (Figure 4.3). Image a-d shows the fine crystals shape of riboflavin. After illumination of the riboflavin, it was observed to be bleached (Wokken, 1969). They are some crucial limits on confocal laser scanning microscope of

fluorophore photo-dynamics. The fluorescence photo bleaching limits the total number of features of available fluorescence photons. Also, fluorosphore excited-state saturation limits the fluorescence emission rate and thus the available imaging intensity. Irreversible photo bleaching limits the number of available fluorescence photons per molecule. Therefore after illumination, riboflavin was observed to be bleached (Lattante *et al*., 2014).

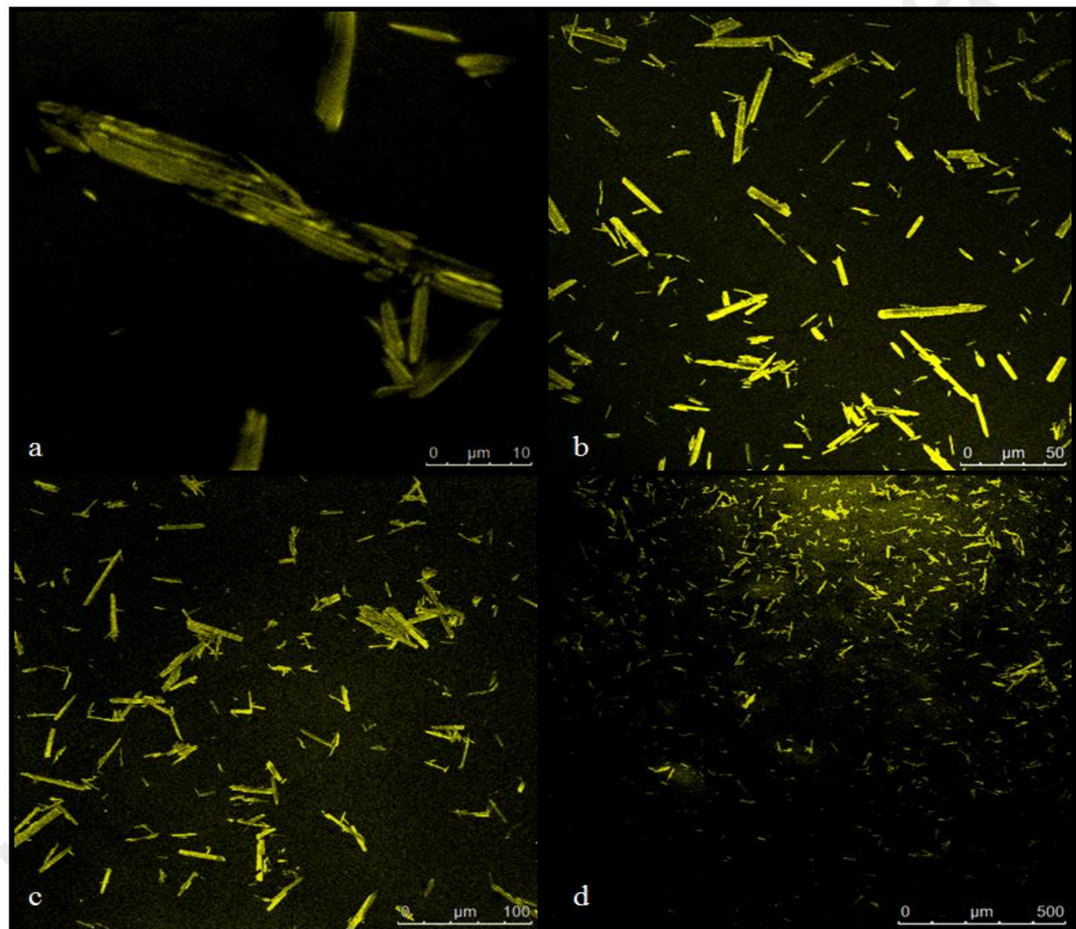

Figure 4.3: The image of multiple wavelength of riboflavin crystal is shown under confocal laser microscope; a-0-10 µm, b-0-50 µm, c-0-100 µm and d-0-500 µm.

In the confocal laser microscope the excitation leads to fluorescence in the green light spectrum at a wavelength of 520-560 nm (Figure 4.4). Image e shows the fine crystals of riboflavin (×1000 magnification). After illumination, it was observed that riboflavin was bleached (Wokken, 1969).

A confocal microscope creates sharp images of a specimen rather than conventional microscope which appear blurred when viewed. This is achieved by excluding most of the light from the specimen that is not from the microscope's focal plane. The image has less haze and better contrast than that of a conventional microscope and represents a thin cross-section of the specimen. Thus, apart from allowing better observation of fine details it is possible to build three-dimensional (3D) reconstructions of a volume of the specimen by assembling a series of thin slices taken along the vertical axis.

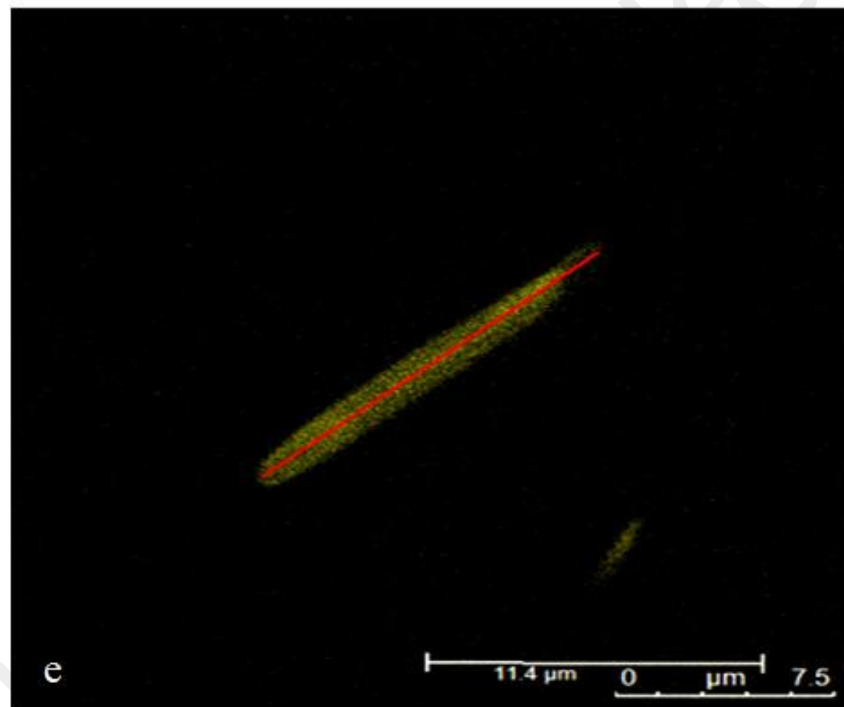

Figure 4.4: The image shows the size of the single strand of riboflavin under confocal laser microscope; 11.4 µm.

4.3 Macroscopic and Microscopic Observation of Selected Blood Borne Pathogens

4.3.1 *Staphylococcus aureus*

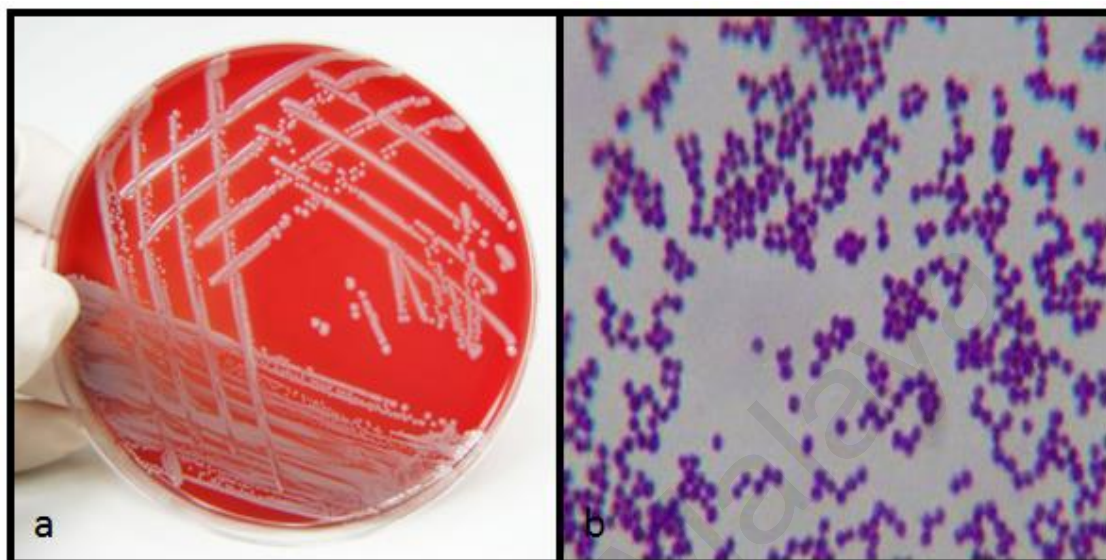

Figure 4.5: *S. aureus* on (a) Columbia blood agar plate and (b) Gram stain of *S. aureus* under optical microscope (1000X magnification).

S. aureus was cultivated on Columbia agar with 5% defibrinated sheep blood agar. Plates were then incubated for 24 hours at 37°C. A zone of clear beta - haemolytic, surrounds the white colonies that turn a buff-golden colour with time, which is the basis of the epithelial *aureus (golden)* (Figure 4.5(a)). It was observed as yellow-pigmented colonies (Figure 4.5 (a)) (Franklin *et al*., 2012).

Characteristics of *S. aureus*

S. aureus is a facultative anaerobic, Gram positive coccus, which appeared as grape-like clusters when viewed through a microscope and has large, round, convex, golden-yellow colonies on Columbia blood agar plate. Some forms single cell, pairs, and short chains are also seen. It has typical Gram positive peptidoglycan cell wall structure. Staphylococci grow greatest aerobically but are facultative anaerobic. They are non-flagellate, non-motile, and non spore forming. It is 1- 4 mm in diameter with sharp borders (Figure 4.5 (b)).

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4.3.2 *Enterococcus faecalis*

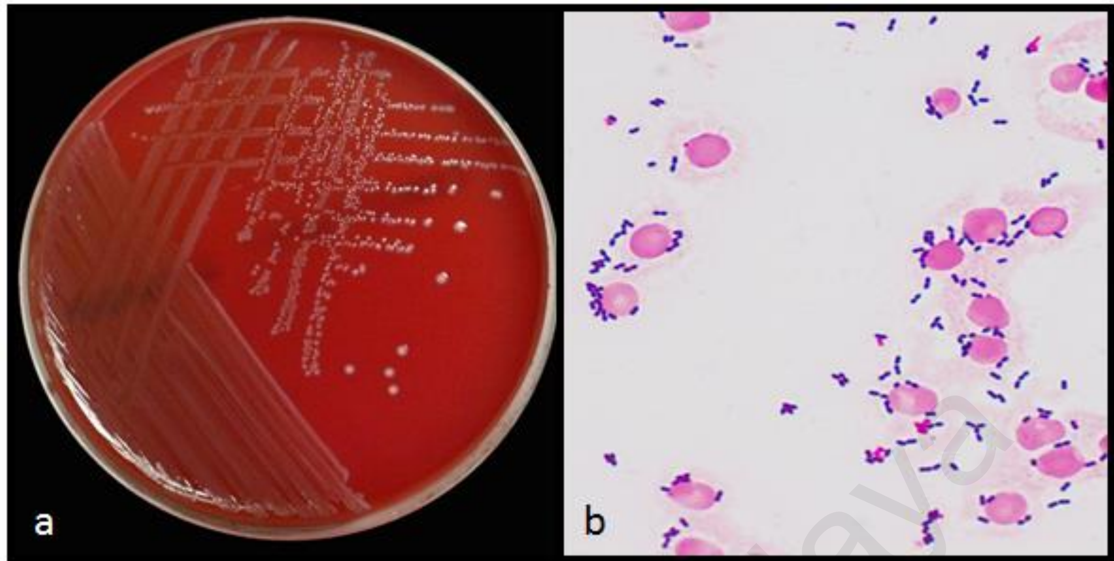

Figure 4.6: *E. faecalis* on (a) Columbia a blood agar plate and (b) Gram stain image of *E. faecalis* under optical microscope (1000X magnification).

E. faecalis was cultured on Columbia agar with 5% defibrinated sheep blood. The plates were incubated at 37°C overnight and gamma-haemolytic colonies of *E. faecalis* were observed. It typically appears as small gray colonies that lack of hemolyzed cells surrounding the colony but was able to observe alpha haemolysis after 48 hours (Figure 4.6 (a) (Franklin & *et al*., 2012).

Characteristics of *E. faecalis*

The microscopic observation of *E. faecalis* was cocci in pairs, clusters or short chains. It also divides by binary fission to form chains of bacteria. The organism appeared purple using this staining technique and was Gram positive. *E. faecalis* is a facultative anaerobic bacterium. It is non-spore forming and non-motile (Figure 4.6 (b)).

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4.3.3 *Salmonella thyphi*

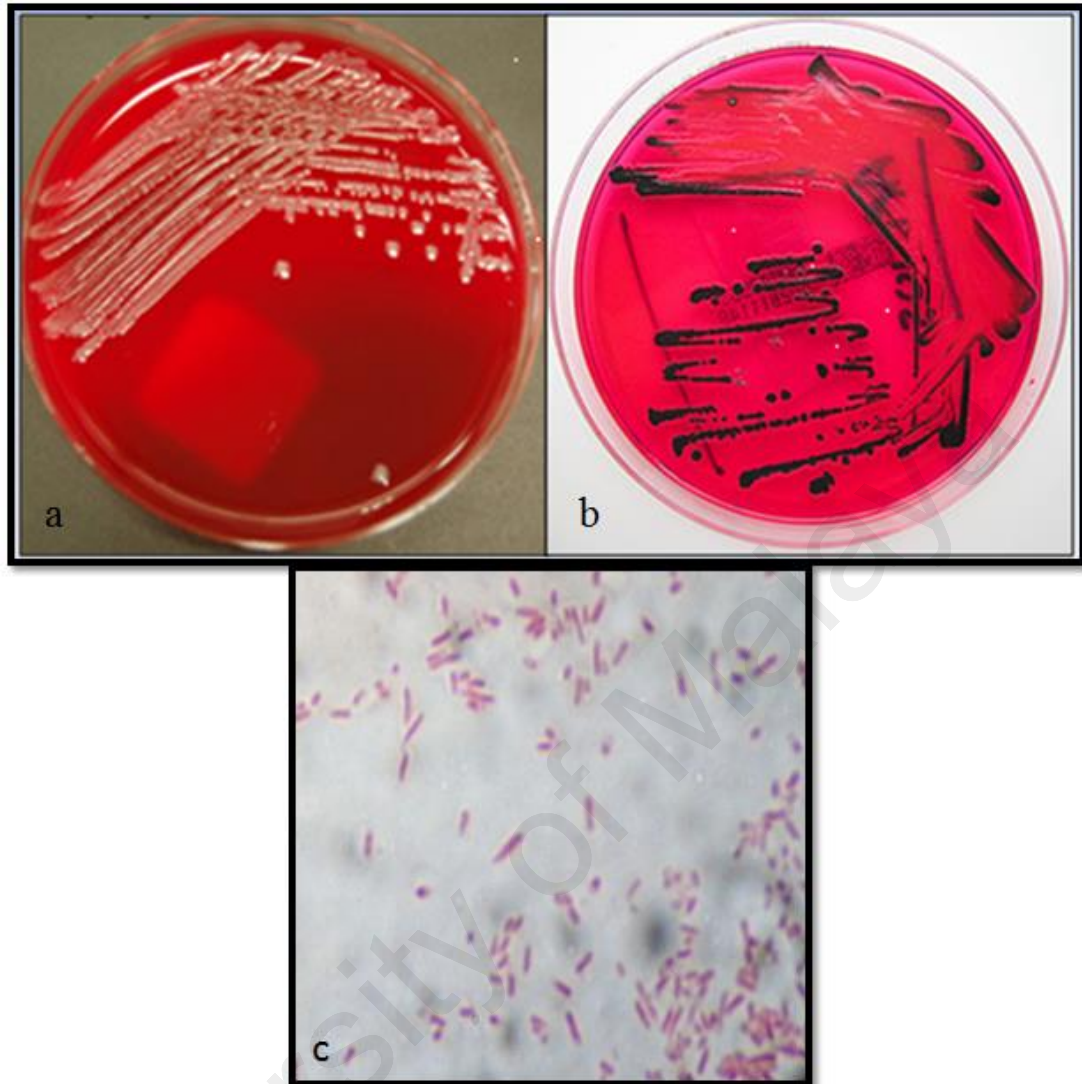

Figure 4.7: *S. thyphi* on (a) Columbia blood agar plate, (b) XLD agar plate and (c) Gram stain of *S. thyphi* under optical microscope (1000X magnification).

S. thyphi colonies were cultured on Columbia agar with 5% defibrinated sheep blood and XLD agar plate. Cultured plates were placed in an aerobic condition at 37°C for 24 hours. The bacteria are firmly non-lactose fermenting. It also does not produce gas when grown in TSI a medium, which is used to differentiate it from other *Enterobacteriaceae* [Figure 4.7 (a)(b)] (Franklin *et al*., 2012).

Characteristics of *S. thyphi*

It is an obligate parasite. *S. thyphi* is Gram negative facultative rod shaped enteric bacillus with diameter of 0.7-1.5 µm and length 2-5 µm. It is a motile by means

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of peritrichous flagella. Most species produce large colonies. Many colonies appear opaque and translucent Most of the species produce large colonies. Many colonies appear opaque and translucent. It is facultative anaerobic susceptible to various antibiotics [Figure 4.7 (b)]. *Salmonella* live in the intestinal tracts of warm and cold blooded animals but some are ubiquitous. Some species are adapted to a particular host. In humans, *Salmonella* are the cause of two diseases such as salmonellosis which is typhois fever resulting of invasion of bacterial into bloodstream, and acute gastroenteritis which is caused through food-borne infection.

4.3.4 *Pseudomonas aeruginosa*

P. aeruginosa colonies were cultured on Columbia agar 5% defibrinated sheep blood. Cultured plates were placed in aerobic condition at 37°C for 24 hours and able to grow at temperatures as high as 42°C. Some strains produce a diffusible green pigment and a distinctive fruity odour [Figure 4.8 (a)] (Franklin *et al*., 2012).

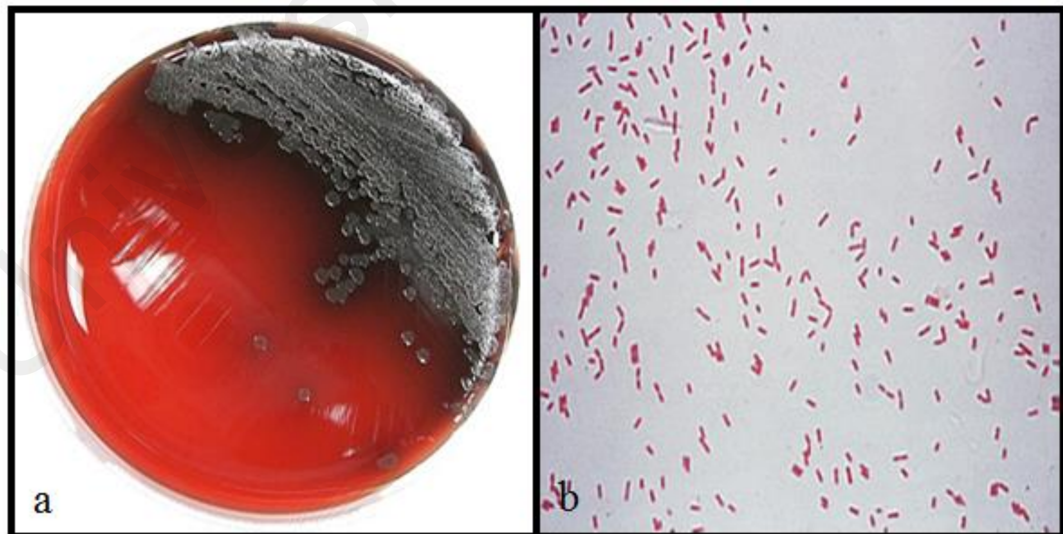

Figure 4.8: Image of *P. aeruginosa* on (a) Columbia blood agar and (b) Gram stain image of *P. aeruginosa* under optical microscope (1000X magnification).

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Characteristics of *P. aeruginosa*

It is a Gram positive rod-like bacterium measuring 1-5 μm long and 1.0 μm wide. It obligates aerobic respiration. Most of the strains are motile with single polar flagellum and has a mucous appearance [Figure 4.8 (b)].

4.3.5 *Escherichia coli*

Figure 4.3.5 shows the image of *E. coli* on (a) Columbia blood agar. The cultured plate was then incubated for 24 hours at 37°C in an aerobic atmosphere. Colonies of *E. coli* were observed to grow without haemolysis but many strains isolated from infections were surrounded by a zone of beta–haemolytic [Figure 4.9 (a)] (Franklin *et al*., 2012).

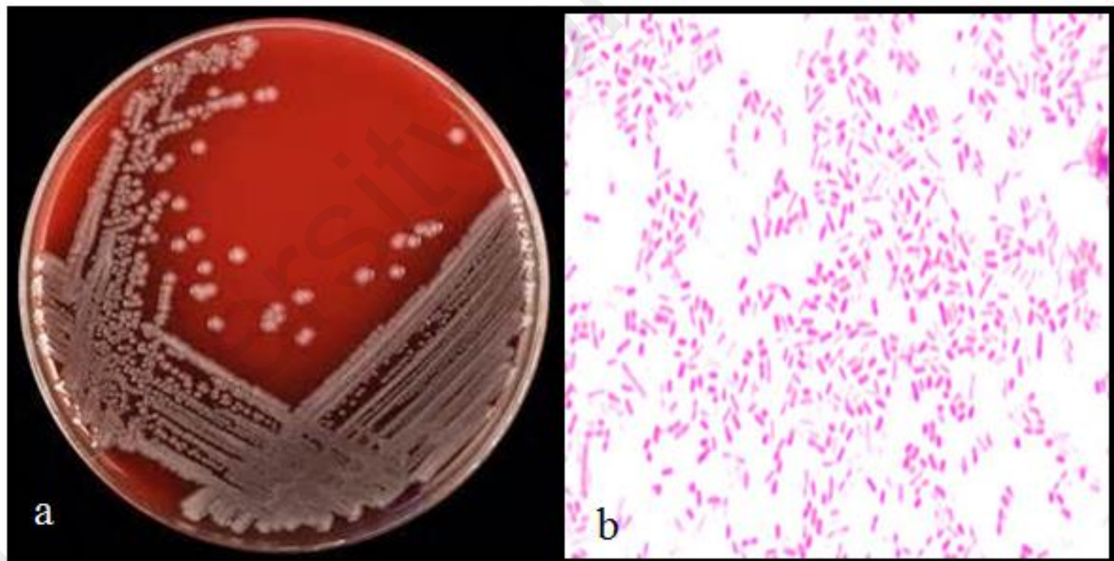

Figure 4.9: Image of E. coli on (a) Columbia blood agar and (b) Gram stain image of *E. coli* under optical microscope (1000X magnification).

Characteristics of *E.coli*

E. coli is a Gram negative rod and motile bacterium. The bacteria exist in cocco-bacillus forms. They are shiny, mucous colonies with entire margins and are slightly raised. Older colonies often have a darker centre. Individual bacterial cell's size was

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approximately 2 µm of length and a diameter of 0.5 to 0.7 µm. *E. coli* cell stains Gram negative because they have a thin cell wall with only 1 to 2 layers of peptidoglycan. It is a facultative anaerobic. *E. coli* has lack of fimbriae. It is known to express type IV pili, which are associated with the twitching motility of this pathogenic strain [Figure 4.9 (b)] (Franklin *et al*., 2012).

4.3.6 *Klebsiella pneumoniae*

K. pneumoniae was inoculated on Columbia sheep blood agar. Cultured plates were then incubated for 24 hours in aerobic condition at 37°C. The colonies produce a distinctive yeast odour and bacterial colonies have a viscous or mucous appearance. It looked raised, translucent, and white to creamy in colour and showed a very slimy mucous (Figure 4.10 (a)) (Franklin *et al*., 2012).

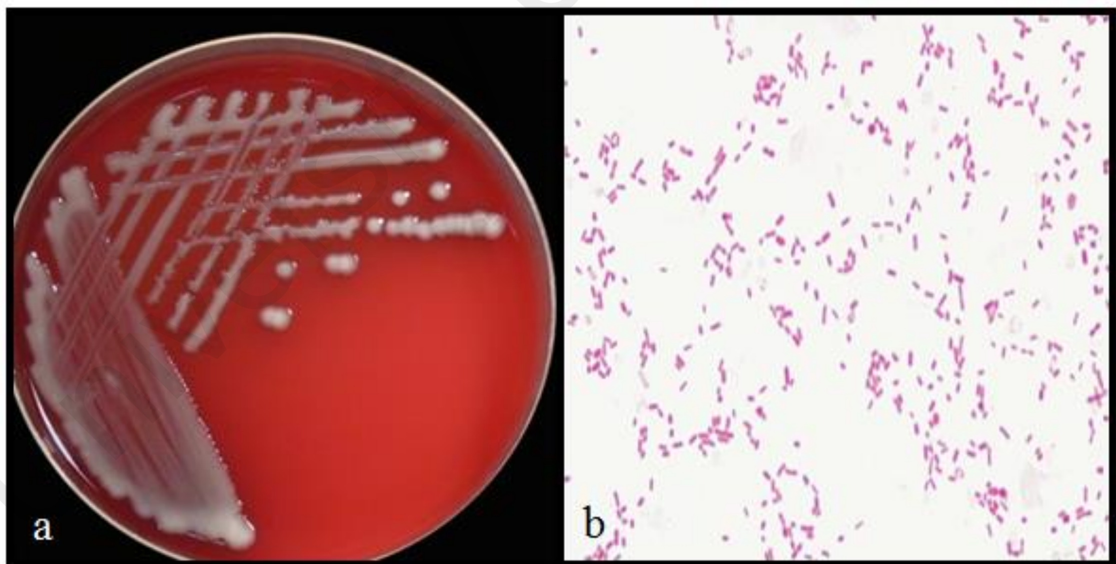

Figure 4.10: Image of *K. pneumoniae* on (a) Columbia a blood agar and (b) Gram stain image of *K. pneumoniae* under optical microscope (1000X magnification).

Characteristics of *K. pneumoniae*

It is known as Friedlander's bacillus. It is small in size 0.5 - 0.8 by 1 - 2 mm and Gram negative coccobacilli. It is non-motile, encapsulated, lactose fermenting,

facultative anaerobic and rod shaped bacterium. It also does not form spores. *K. pneumoniae* are arranged in singles, in pairs or cluster forms (Figure 4.10 (b) (Franklin *et al*., 2012).

4.3.7 *Candida albicans*

C. albicans are grown on blood agar. It forms white, creamy and opaque coloured colonies. The colonies have a distinctive yeast smell and budding cells can be seen by direct microscopy. It can grow in normal room temperatures or even anaerobic conditions. It reproduces through budding and usually its size is 10 to 12 µm in diameters (Figure 4.11 (a)) (Franklin *et al*., 2012).

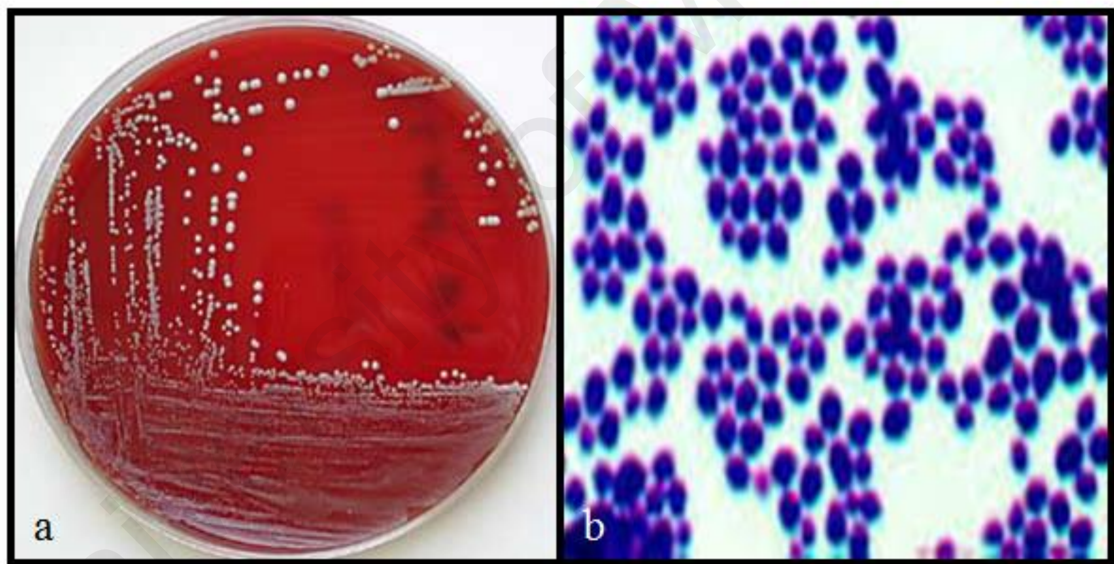

Figure 4.11: Image of *C. albicans* on (a) blood agar and (b) Gram stain image of *C. albicans* under optical microscope (1000X magnification).

Characteristics of *C. albicans*

C. albicans is a unicellular and oval shaped diploid fungus. In direct stained smear of the pathogenic sample, the yeast is seen attached to pseudohyohae (Figure 4.12). Both yeast and pseudohyphae is Gram positive. It is a form of yeast that lives on various mucosal surfaces of the body. It's dimorphic, which means it can change from one morphological form to the next in different environment conditions. It also

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produces mycelia. It is opportunistic, occurring when a patient has some lower immunity. Infections caused by endogenous yeast and also many nosocomial. Candidiasis is the most common pathogen among HIV patient. The pathogen is more invasive when the host debilitate (Figure 4.11 (b)) (Franklin *et al*., 2012).

In physiological conditions such as body temperature, pH and the presence of serum, it may grow into a hyphal form called pseudohyphae. The formation of pseudohyphae which was observed through wet film occurs through polarized cell division when yeast cells growing by budding have elongated without detaching from adjacent cells thus the cells remain attached to each other (Figure 4.12). Chlamydospores also formed on the hypha. Its structures are round, refractive spores with a thick cell wall (Figure 4.12). An overgrowth of the invasive, multicellular filamentous pseudohyphae form results in the fungal infections called candidiasis or thrush.

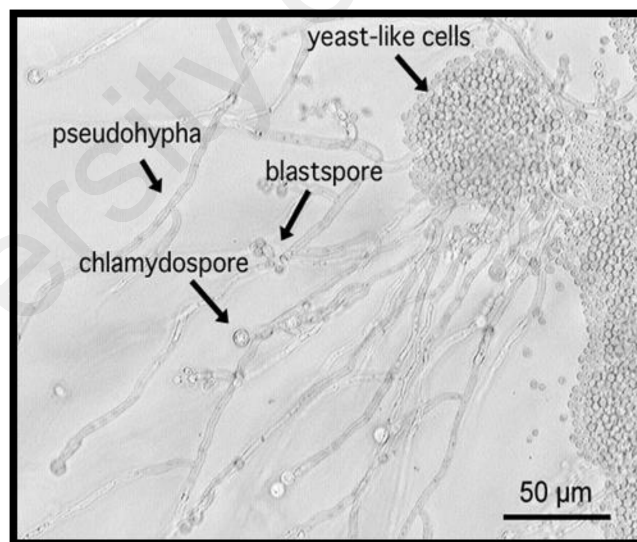

Figure 4.12: Various morphological forms of *C. albicans* (Calderone & Fonzi, 2001).

4.4 Biochemical Tests to Identify Selected Blood Borne Pathogens

The following tests were done to confirm all the pathogens selected for this study. They were analyzed using API tests as shown below;

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Table 4.1: Confirmation tests and procedures for the selected blood borne pathogens

Pathogens	Confirmation Test	Procedure
SA	Coagulase test: Plasma clot observed.	In a test tube, 1 ml citrated plasma was taken. This was inoculated with 0.1 ml culture to be identified and incubated at 37ºC. Plasma clot which was observed within 4 hours was confirmed as S. *aureus*, as if it is not, the plasma clot would not form even after 24 hours.
EF	API Rapid 20E Bile: Colour changed to dark brown.	One colony from cultured plate was swabbed on bile plate and left standing for 30 minutes. Colour change to dark brown indicates presence of the microorganism.
ST	API 20E Serological test (Polyvalent): Observed agglutination in all reagents.	One black colony from horse blood agar plate was mixed with reagents and observed to confirm the species. It is confirmed through the presence of antigens H, O and Vi
PA	API 20 NE Oxidase test: Colour changed from pink to maroon and finally black.	Organisms were grown on horse blood agar. 2-3 drops of tetramethyl-p-phenylenediamine dihydrochloride were added. Observed for colour changes from pink to maroon and finally black. If no colour changes are observed, the test is negative.
EC	API 20 E Methyl Red (MR) test: Colour remains red.	In a glucose phosphate broth culture, if 2 drops of 0.04% methyl red is added, it will remain as red colour in acidic condition. Therefore, the presence of red colour indicates positive MR test, while the absence indicates negative MR test.
KP	API 20 E Voges – Proskauer (VP) test: Colour changed to pink red.	The organisms were suspended in glucose broth. After 5 minutes of incubation, 0.6 ml of α-naphtol and 0.2 ml of 40% KOH were added and shaken for 5 seconds. A positive test is indicated by the appearance of pink red colour between 2 to 4 hours after addition of reagents.
CA	API 20 AUX Wet film test: Observed budding yeast and pseudohyphae	One drop of saline was placed on sterile slide. Then, 2 colonies were mixed with the saline. The slide was covered with glass cover slip and observed under microscope. Budding yeast and pseudohyphae should be observed for positive results.

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All the tested API kits results are as shown below in Figure 4.13.

(a) Image "A" and API Staph strip shows the identification test towards *S. aureus*.

(b) API Rapid 20E inoculated with of *E. faecalis* before and after incubation at 37ºC for 24 hours.

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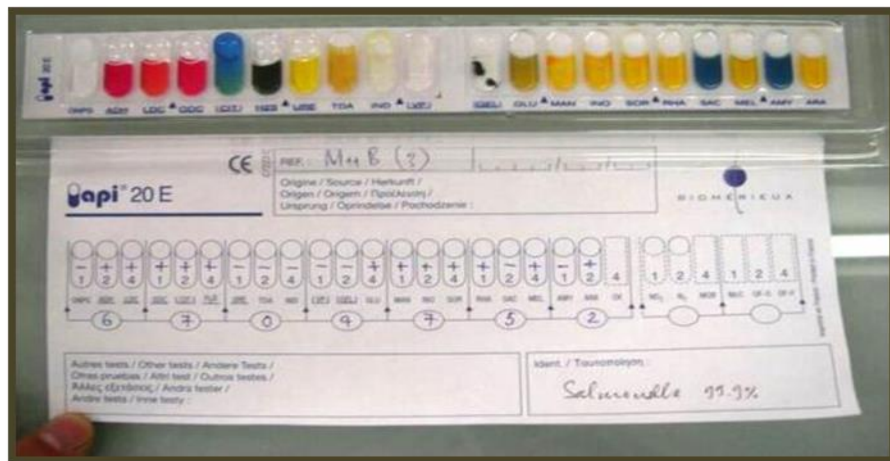
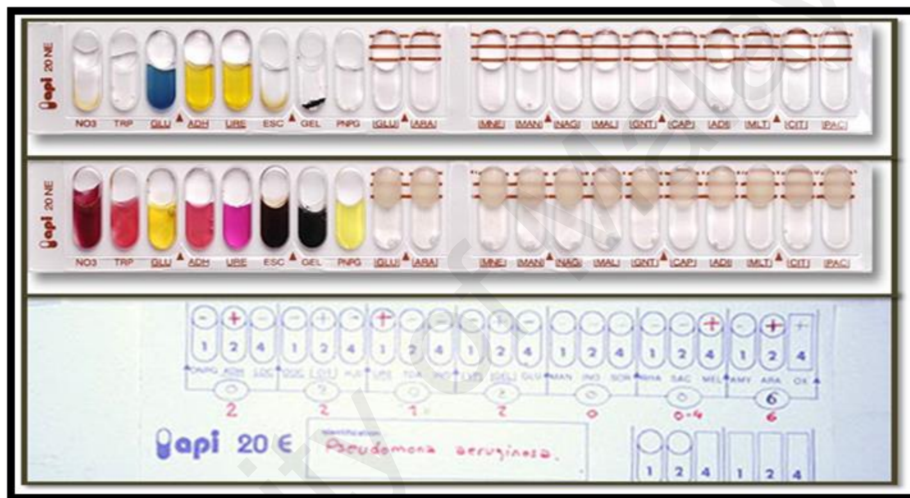
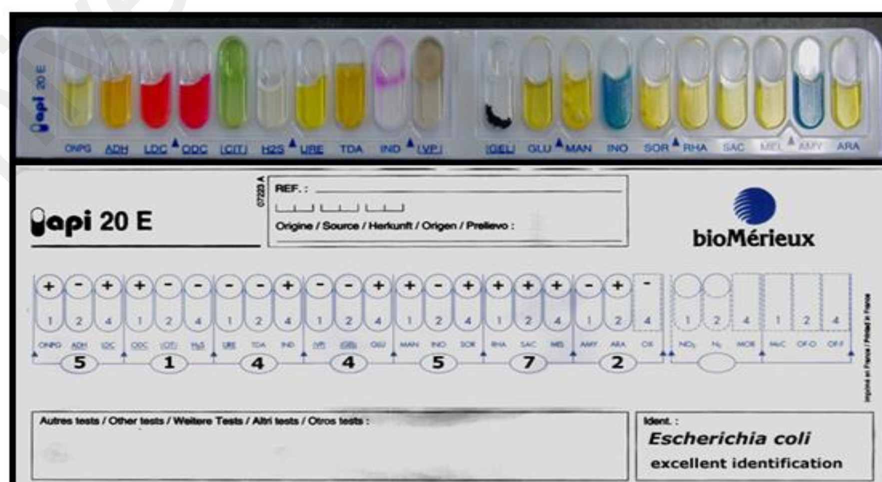


(c) API 20E used to identify Salmonella spp. and through Serological test with the present of black colonies confirmed the *Salmonella typhi*.



(d) *P. aeruginosa* was confirmed through API 20NE and oxidase tests. The above API strip show before and after the incubation period and the presence of turbid confirmed the presence of *P. aeruginosa*.



(e) *E. coli* also identified through API 20E as the confirmation test.

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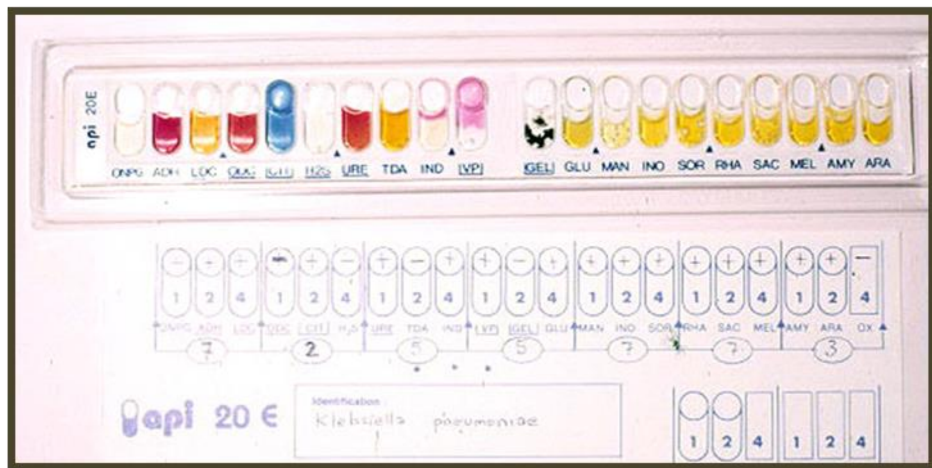
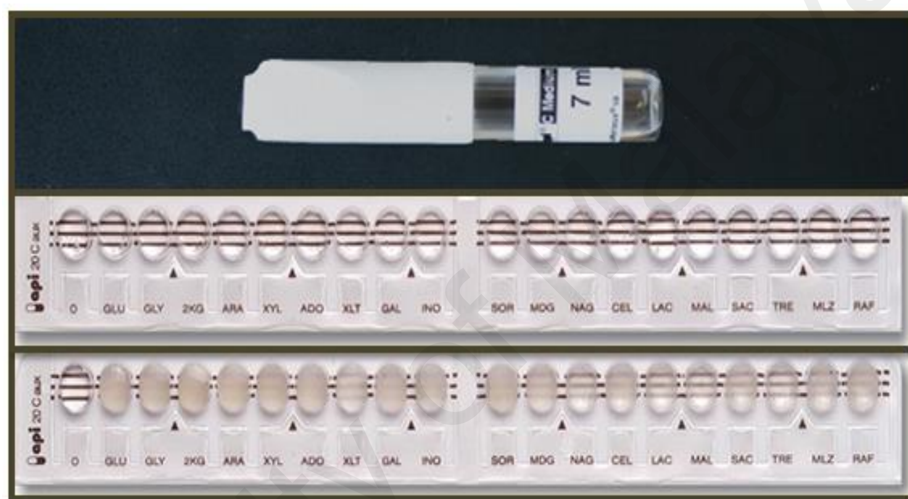


(f) Image show the API 20E as a confirmation test for *K. pneumoniae*.



(g) The API 20AUX kit with the solutions, which was used to identify *C. albicans*. The presence of turbidity on top of the solution confirms the presences of *C. albicans*.

Figure 4.13: Identification of pathogens using API test strips (a) *S. aureus,* (b) *E. faecalis,* (c) *Salmonella typhi,* (d) *P. aeruginosa,* (e) *E. coli,* (f) *K. pneumoniae,* and (g) *C. albicans.*

4.5 Minimum Inhibitory Concentration (MIC) of Riboflavin

Table 4.2 shows the minimum and maximum zone of inhibition on selected riboflavin concentration for SA, EF, ST, PA, EC, KP, and CA.

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Table 4.2: Minimum inhibitory concentration (MIC) of riboflavin to inhibit growth of the selected pathogens. All the measurements were recorded in triplicate.

Stock solution of riboflavin at ratio 1:1 (g/ml)	Pathogens [Zone of inhibition (mm) (mean ± SD)]						
	SA	EF	ST	PA	EC	KP	CA
0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5.0	16.3 ± 1.5	7.7 ± 0.6	6.0 ± 0.0	6.0 ± 0.0	6.3 ± 0.6	6.0 ± 0.0	6.0 ± 0.0
10.0	16.4 ± 1.4	7.7 ± 0.6	6.0 ± 0.0	6.0 ± 0.0	6.3 ± 0.6	6.0 ± 0.0	6.0 ± 0.0
15.0	17.8 ± 0.5	7.7 ± 0.6	6.0 ± 0.0	6.0 ± 0.0	6.3 ± 0.6	6.0 ± 0.0	6.0 ± 0.0
20.0	18.7 ± 0.3	11.2 ± 0.1	11.0 ± 0.1	11.0 ± 0.1	9.8 ± 0.1	6.0 ± 0.0	8.2 ± 0.1
25.0	19.3 ± 0.3	16.5 ± 0.1	14.7 ± 0.2	11.9 ± 0.1	11.8 ± 0.1	8.1 ± 0.1	11.3 ± 0.1
30.0	18.7 ± 0.1	16.5 ± 0.1	14.6 ± 0.3	11.9 ± 0.2	11.7 ± 0.1	8.1 ± 0.1	11.3 ± 0.2
35.0	18.6 ± 0.2	16.6 ± 0.1	14.6 ± 0.3	11.8 ± 0.1	11.5 ± 0.1	8.2 ± 0.1	11.7 ± 0.1
40.0	18.6 ± 0.1	16.4 ± 0.1	14.6 ± 0.1	11.8 ± 0.1	11.5 ± 0.2	8.2 ± 0.1	11.3 ± 0.1
45.0	18.5 ± 0.1	16.4 ± 0.0	14.6 ± 0.2	11.8 ± 0.1	11.4 ± 0.1	8.1 ± 0.1	12.4 ± 0.1
50.0	18.5 ± 0.1	16.4 ± 0.1	14.5 ± 0.1	11.8 ± 0.2	11.5 ± 0.1	8.1 ± 0.1	12.3 ± 0.1
55.0	18.5 ± 0.1	16.3 ± 0.1	14.5 ± 0.1	11.8 ± 0.1	11.4 ± 0.1	8.1 ± 0.1	12.3 ± 0.2
60.0	18.4 ± 0.1	16.3 ± 0.2	14.3 ± 0.1	11.8 ± 0.3	11.4 ± 0.1	8.1 ± 0.1	12.2 ± 0.1
65.0	18.4 ± 0.1	16.3 ± 0.1	14.2 ± 0.0	11.7 ± 0.1	11.3 ± 0.1	8.1 ± 0.1	11.3 ± 0.1
70.0	18.4 ± 0.1	16.3 ± 0.2	13.9 ± 0.2	11.7 ± 0.2	11.3 ± 0.1	8.1 ± 0.1	10.3 ± 0.2
75.0	17.9 ± 0.2	16.3 ± 0.1	13.8 ± 0.5	11.7 ± 0.1	11.3 ± 0.1	8.1 ± 0.1	10.3 ± 0.1
80.0	17.8 ± 0.2	16.3 ± 0.1	13.8 ± 0.4	11.7 ± 0.2	11.3 ± 0.1	8.1 ± 0.1	8.5 ± 0.1
85.0	17.8 ± 0.3	16.3 ± 0.1	13.8 ± 0.2	11.7 ± 0.2	11.3 ± 0.1	8.1 ± 0.1	8.6 ± 0.1
90.0	17.8 ± 0.1	16.2 ± 0.1	13.8 ± 0.3	11.7 ± 0.2	11.3 ± 0.1	6.0 ± 0.0	8.5 ± 0.1
95.0	17.8 ± 0.1	16.2 ± 0.1	13.8 ± 0.2	11.7 ± 0.1	11.3 ± 0.1	6.0 ± 0.0	8.7 ± 0.2
100.0	17.8 ± 0.1	16.2 ± 0.2	13.7 ± 0.3	11.7 ± 0.2	11.3 ± 0.1	6.0 ± 0.0	8.7 ± 0.1

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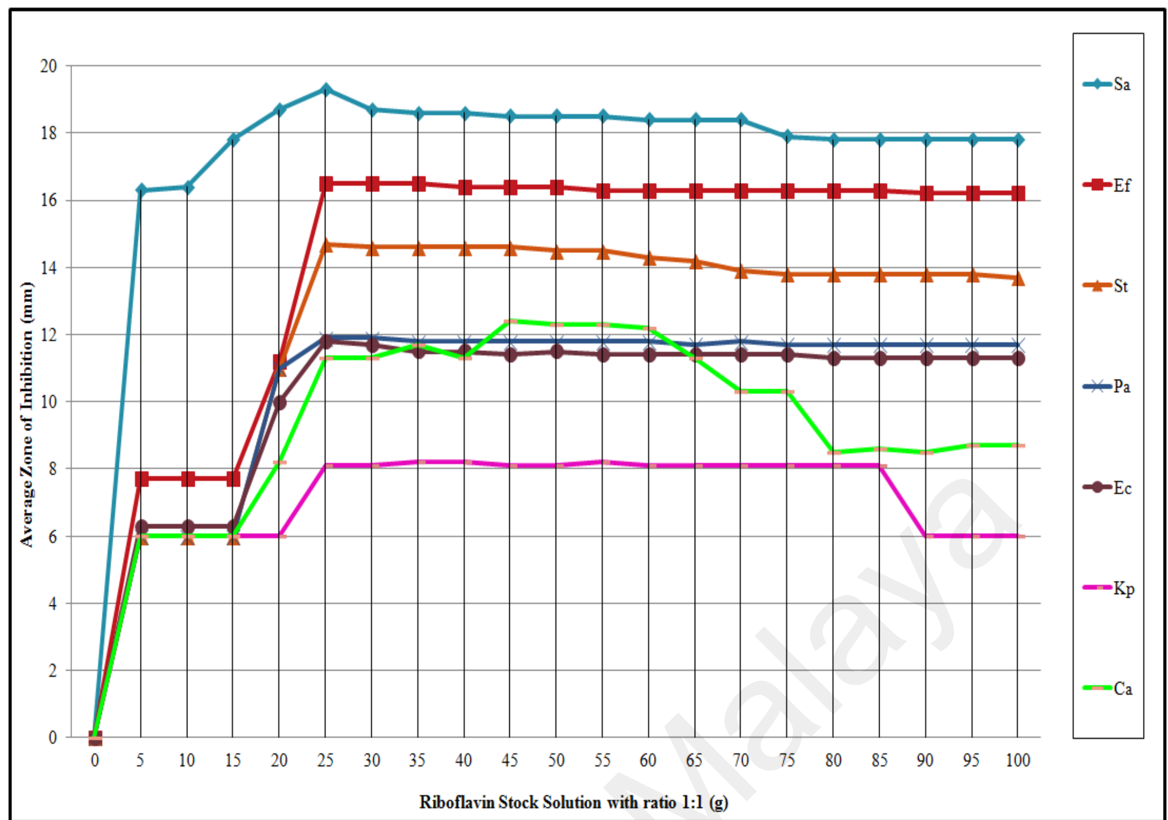

Figure 4.14: The minimum and maximum zone of inhibitions of pathogens against stock solutions such as SA, EF, ST, PA, EC, KP, and CA.

The line graph in Figure 4.14 shows the relationship between riboflavin stock solution and the average zone of inhibition of the selected pathogens. All pathogens were exposed to 20 different riboflavin stock solutions with a ratio 1:1 within the range of 0.0 g/ml to 100.0 g/ml (Table 4.2). According to the graphs, the very first concentration of 5.0 g of riboflavin with 5.0 ml of PBS itself demonstrates positive results against all pathogen especially towards SA, which managed to produce a diameter of 16.3 mm as the initial zone of inhibition. This observation encouraged for continuous test with an increase on the stock solution 5.0 ml of PBS by 5.0 g of riboflavin, which represents the ratio 1:1 g/ml, respectively. The following two tests with 10.0 g/ml and 15.0 g/ml did not cause any changes to the zones of inhibition for 6 out of 7 pathogens. Only SA was affected by these increased concentration and shows ascending zones of inhibition values. At this stage, the resistance level of the pathogens

towards the concentration is high. Therefore, the test continued with higher concentrations for more susceptible results.

Surprisingly, the next two riboflavin stock solutions manage to break through the barriers and reached improved diameters such as 18.7 mm to 19.3 mm for SA, 11.2 mm to 16.5 mm for EF, 11.0 mm to 14.7 mm for ST, 11.0 mm to 11.9 mm for PA, 9.8 mm to 11.8 mm for EC, and 8.2 mm to 11.3 mm for CA. Only KP shows smaller readings with 6.0 mm to 8.1 mm. Assuming this pattern will continue, the test was carried-on with other levels of concentrations. It was observed that contrary to the assumption, the readings started to fluctuate slightly. At this point of observation, the test was decided to be done with a concentration up to 100.0 g/100.0 ml of riboflavin stock solution to study the overall effectiveness of the concentration towards the blood borne pathogens.

As shown in Figure 4.14, SA was the most affected pathogen with bigger areas of inhibition compared to all the other pathogens. This showed that riboflavin is capable of inhibiting SA, especially with 25.0 g/25.0 ml concentration as it reached the peak of the zone of inhibition (19.3 mm) during the test. This particular concentration is also very much effective towards EF, ST, PA and EC. The highest zone of inhibition achieved by this concentrations for the other four pathogens are as follow; 16.5 mm for EF, 14.7 mm for ST, 11.9 mm for PA and 11.8 mm for EC. Meanwhile, from Figure 4.4, we can notice that KP and CA are more resistant than the other selected pathogens. Even so, the biggest zone of inhibition 12.4 mm by CA was produced with 45.0 g/45.0 ml of riboflavin stock solution. For KP, this was achieved by the application of 35.0 g/35.0 ml solution. Even though CA required higher level of concentration for effective inhibition, it was KP which is the most resistant blood borne pathogen according to the collected data.

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Overall, riboflavin stock solution at 25.0 g/25.0 ml was observed to be the best active concentration towards inhibiting SA, EF and ST. Whereas for PA, EC and CA, moderate inhibition was observed. KP meanwhile may require further investigation for effective inhibition as they remain the most resistant pathogen towards all levels of concentrations.

4.6 Antimicrobial Effectiveness of Riboflavin in Combination with UVA Light Sources at 365 nm Wavelength in the Treatment of Blood Borne Pathogens

Table 4.3: Average zone of inhibition of blood borne pathogens against activated and non-activated riboflavin solutions.

Groups	Pathogens	Zone of inhibition (mm) (mean ± SD)			
		Negative Control	Standard Drug	Riboflavin (1.0 g/ml)	R+UVA (1.0 g/ml + 365nm
Gram Positive	SA	0.0 ± 0.0	19.0 ± 0.0	20.0 ± 1.0	17.7 ± 1.5
	EF	0.0 ± 0.0	18.0 ± 0.0	17.7 ± 0.6	14.3 ± 0.6
Gram Negative	ST	0.0 ± 0.0	22.0 ± 0.0	17.3 ± 0.6	14.0 ± 1.0
	PA	0.0 ± 0.0	18.0 ± 0.0	15.7 ± 0.6	11.0 ± 1.0
	EC	0.0 ± 0.0	17.0 ± 0.0	11.7 ± 0.6	11.0 ± 1.0
	KP	0.0 ± 0.0	16.0 ± 0.0	7.7 ± 0.6	7.7 ± 0.6
Fungus	CA	0.0 ± 0.0	18.0 ± 0.0	11.7 ± 0.6	11.0 ± 1.0

The labels in Figure 4.15 refer as following; A-*Staphylococcus aureus,* B-*Enterococcus faecalis,* C-*Salmonella typhi,* D-*Pseudomonas aeruginosa,* E-*Escherichia coli*, F-*Klebsiella pneumoniae* and G-*Candida albicans,* R-Riboflavin, R+UVA-Riboflavin with UVA, SD-Standard drug, and C-Control. Zones of inhibition were measured in millimetre (mm) (Figure 4.15).

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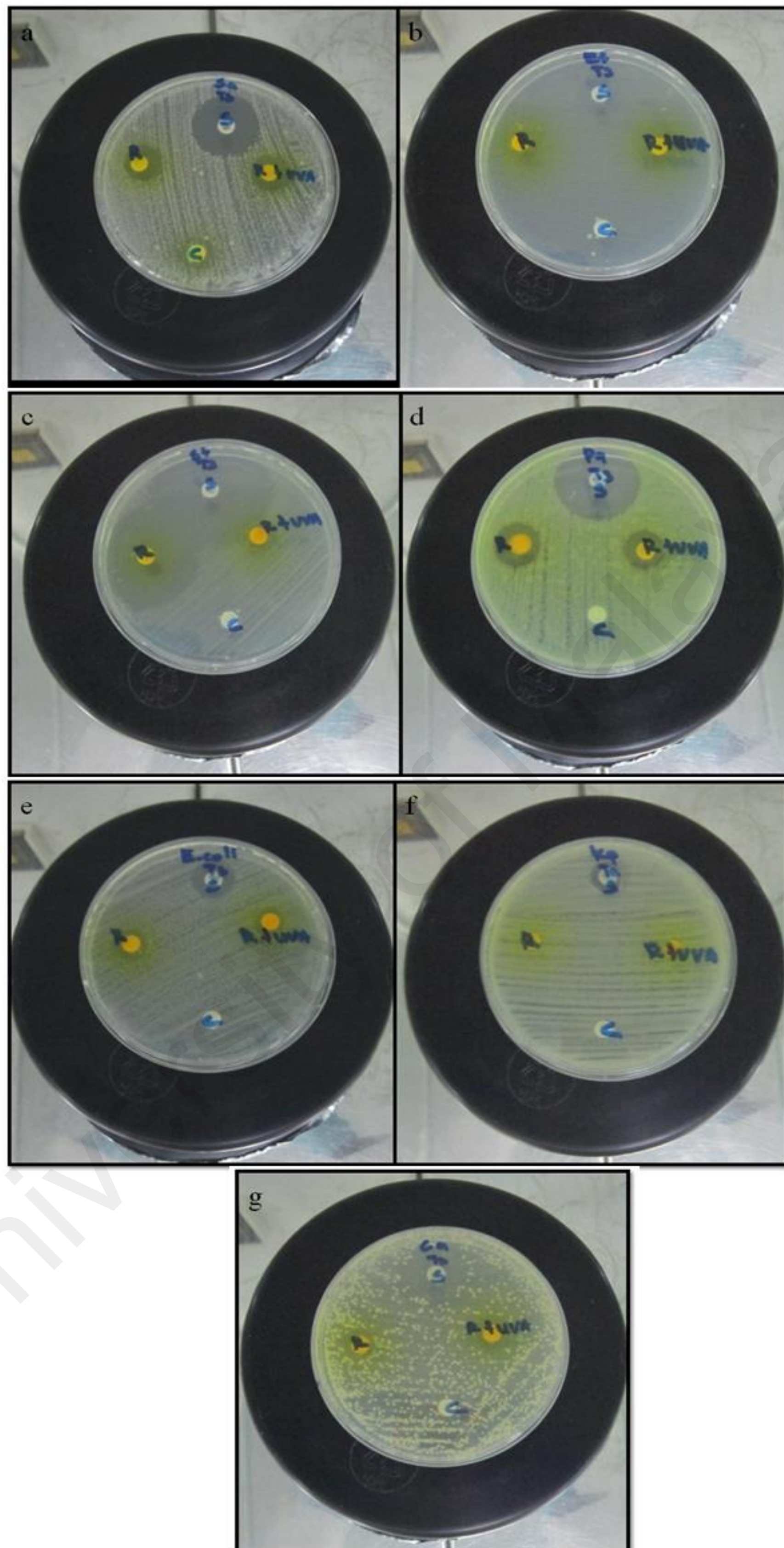

Figure 4.15: Testing the susceptibility of microorganisms to antibiotics by the Kirby-Bauer disc diffusion method. The labels above refer as following; A - *Staphylococcus aureus,* B - *Enterococcus faecalis,* C - *Salmonella typhi,* D - *Pseudomonas aeruginosa,* E - *Escherichia coli*, F - *Klebsiella pneumoniae* and G - *Candida albicans,* R - Riboflavin, R+UVA - Riboflavin with Ultraviolet A 365 nm, and C - Control.

Zones of inhibition of Group 1 (Gram positive bacteria) were significantly greater when compared with Group 2 (Gram negative bacteria), Group 3 (fungus) and control. Riboflavin on its own was found to be quite effective and thus able to inhibit the pathogens when diffused at a concentration of 1.00 g/ml (Figure 4.15). Moreover SA, EF, ST and PA were susceptible towards non-activated riboflavin treatment. Meanwhile Table 4.3 shows the measurement of zone of inhibition of each pathogen and its standard deviation (SD). Zones of inhibitions were observed for non-activated and activated riboflavin, SA showed the highest value at (20.0 ± 1.0) mm and (17.6 ± 1.53 mm), EF with (17.6 ± 0.6) mm and (14.3 ± 0.6) mm, ST with (17.3 ± 0.6) and (14.0 ± 1.0) mm, and PA with a value of (15.7 ± 0.6) and (11.0 ± 1.0) mm. It was found to be intermediately effective against EC with (11.7 ± 0.6) and (11.0 ± 1.0), and CA with (11.7 ± 0.6) and (11.0 ± 1.0) mm. Finally, KP exhibited the same area of (7.7 ± 0.6) mm for both types of treatment showing the smallest zone of inhibition. Results of our in-vitro experiments clearly show that photo-activation of riboflavin using UVA achieves eradication of all six out of seven tested microorganisms (Figure 4.16).

The work outlined here is directed to the development of riboflavin as a novel method of treatment of blood borne infections, which is a cause of significant morbidity worldwide and can cause rapid and devastating death among infected people. Blood infections continue to be difficult to treat despite the use of various topical and systemic antibacterial and antifungal agents and adjuvant surgery, such as blood transfusion and organs removal to remove the infected organs. Medical therapy has been boosted by the use of various antibiotics available commercially, given topically or by other routes. However, the antimicrobials in use are sometimes challenging because of their toxic effects on the blood system and more important, the emerging and increasing patterns of resistance.

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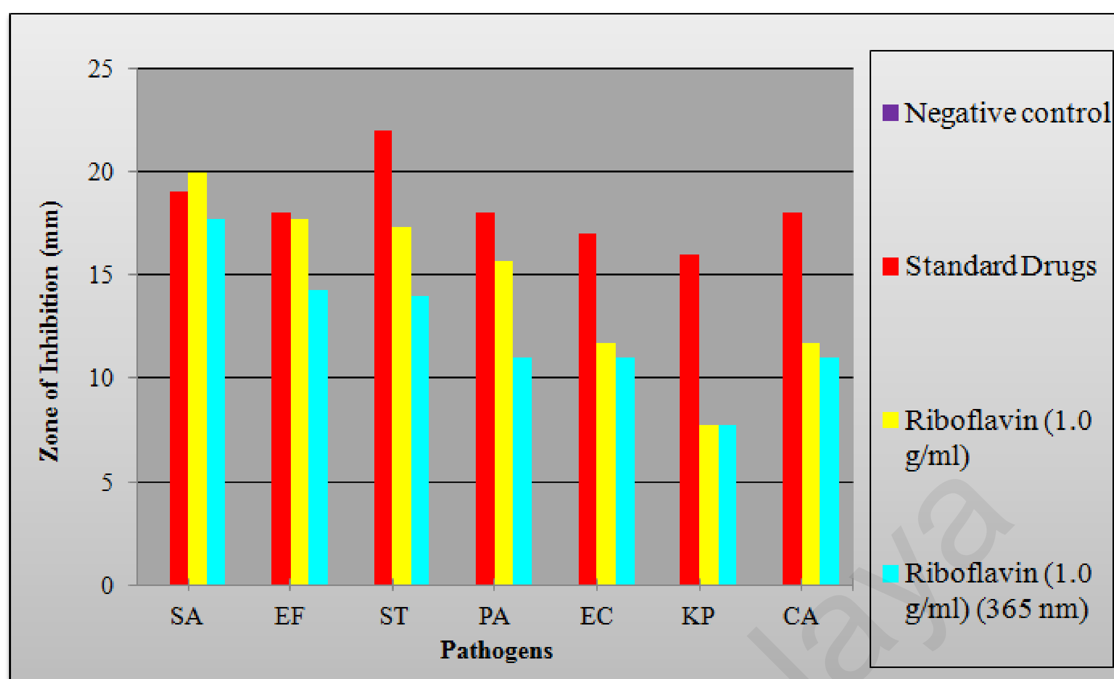

Figure 4.16: Average zones of inhibition of selected blood borne pathogens by activated and non-activated riboflavin solutions.

Recently, there have been studies proving that riboflavin/UVA treatment has been confirmed to induce collagen cross-linking in corneal and blood products (Tsugita *et al*., 1965; Martins *et al*., 2008; Schrier *et al*., 2009; Makdoumi, 2011) and lead to dose-dependent damage that can be expected in human being. Coincidentally, this approach has also been researched for pathogens inactivation via the by-products of riboflavin after UVA exposure. This mechanism affects a large list of pathogens, including bacteria, fungus and virus. Riboflavin and UVA at 280-370 nm may both damage nucleic acids by direct electron transfer, production of single oxygen and generation of hydrogen peroxide with the formation of hydroxyl radicals. Pathogenic DNA/RNA may be affected in the absence of oxygen in this case (Sauer *et al*., 2010).

On the other hand, in our research, we used stand-alone riboflavin and were still able to comparably inactivate the growth of pathogens through disc diffusion method. The theory was same as with the other studies (riboflavin/UVA) except that this current study did not employ UVA to photo-irradiate the riboflavin solutions (Table 4.3).

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There is possibility that riboflavin which is already present in the human body may serve as a natural antimicrobial mechanism (Sauer *et al*., 2010). However, riboflavin concentration in the blood is not enough to produce antimicrobial effects against blood borne pathogens. Since riboflavin is photosensitive, it is more likely that within the part of human body exposed to sunlight, it will be depleted of riboflavin. The data selected in this study reflect a recent effort to demonstrate a potential new treatment for blood infectious agents.

Cytotoxic effect of riboflavin by UVA induced photosensitization may be attributed to the production of singlet oxygen, superoxide ions and hydroxyl radicals resulting in pathogenic cellular death (Sato *et al*., 1995; Corbin, 2002). DNA damage or cell wall lysis may also be instrumental towards inactivation of the pathogens in this case. Several other factors could also be significant for the different zones of inhibition obtained. Length of cell cycle could be of significance, since a shorter cell cycle would increase the possibility of exposure during cell division (Thakuri *et al*., 2011). Cell wall structure, intracellular transport systems and metabolic pathways are other factors that may influence sensitivity to oxidative stress (Thakuri *et al*., 2011).

However, the results observed in this work might suggest a higher antimicrobial contribution from stand-alone riboflavin activity. In the case of SA, EF, ST and PA; decreased efficacies were actually shown when treated in combination with UVA (Figure 4.16). Higher stand-alone riboflavin activity here may suggest that these microbes might be more susceptible to riboflavin diffusion through cell walls intercepting their DNAs. However, it is premature to conclude non-effective UVA combination even though there was no improvement (in the zones of inhibitions) among any of the pathogens tested with UVA-riboflavin. Still, the same assumptions can be applied to the other microbes as DNA inactivation through binding with riboflavin molecules might still occur depending on microbial resistance. Further in-depth studies

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involving a variety of microbes need to be carried-out to understand riboflavin-microbe interaction before attempting to answer UVA and non-UVA assisted riboflavin efficacy.

Furthermore, the lack of penetration and a strong dependence on the distance from the source also causes limitations to the application of UVA, which may result in non-homogeneous microbial inactivation. This presents an obstacle for researches dealing with the treatment of blood borne infections deep within biological tissues. In addition, the negative effects of UVA ionization on the biological tissues also deter advances in in-vivo investigations. Therefore, the need for optimizing the strength and efficacy of riboflavin without UVA combination has become quite necessary.

Being an essential nutrient naturally occurring in both plants and animals, compounds containing antibacterial, antifungal and anticancer properties could also be isolated from riboflavin. Antibiotics presently available are produced from many sources but may not be fully effective in inhibiting pathogens. Therefore, the antimicrobial capacity of biologically active substances that is a derivative of the heterocyclic compound isoalloxazine attached to ribitol, a multi-atomic alcohol from riboflavin proves to be an exciting field of research to be explored (Hohmann & Stahmann, 2010; Abbas & Sibirny, 2011). Furthermore safer combinational treatments of standard drugs with riboflavin could be used to inhibit the growth of pathogens. SA, EF, ST and PA were observed to be susceptible towards riboflavin treatment while EC and CA showed intermediate reaction towards riboflavin (Figure 4.15). Meanwhile KP, which is a Gram negative bacterium, was found to be resistant to riboflavin treatment unlike ST, PA and other Gram negative bacteria, which demonstrated biggest zone of inhibition (Figure 4.15). This could be due to the bacterial cell firmly covered by apolysaccharide outer layer (Lee *et al*., 2002; Jakobsen & Jonsdottir, 2003; Cress *et al*., 2014). The outer layer acts to protect the bacteria from phagocytes and immune response. KP can produce extended-spectrum beta-lactamases (ESBL), which causes

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these bacteria to be resistant to many classes of antibiotics (Llobet *et al*., 2008; Cress *et al*., 2014). Furthermore, presence of various peptides in mucosal surfaces might also protect the encapsulated bacteria (Llobet *et al*., 2008). These may cause the encapsulated bacteria to act as a shield against riboflavin activity. Still, by increasing the concentration of riboflavin, the possibility of inhibiting the pathogens may still be possible.

Variability in zones of inhibition was observed among the tested drugs. The difference between groups of non-activated riboflavin was found to be significant at $p=0.001$ against activated riboflavin. Therefore, riboflavin alone was concluded to be more effective compared to activated riboflavin since $p<0.05$.

The current work successfully demonstrated stand-alone riboflavin efficacy for the first time. It is expected that without the need for UVA photosensitization, application of riboflavin to inactivate pathogens would allow low-cost and distance independent processes to be developed. Minus the negative implications of the harmful UVA wavelengths, riboflavin can be investigated and applied especially in the treatment of topical infections.

4.7 Riboflavin Treatment on Selected Pathogens as an Additional Food Supplement

The culture plates were pre-treated with standard drugs for 5 minutes and then with riboflavin for overnight. Gram positive bacteria were treated with Vancomycin while Gram negative bacteria were treated with Gentamicin. The labels refer as following; A-*Staphylococcus aureus,* B-*Enterococcus faecalis,* C-*Salmonella typhi,* D-*Pseudomonas aeruginosa,* and C-Control. Zones of inhibition were measured in millimetre (mm) as shown in Figure 4.17.

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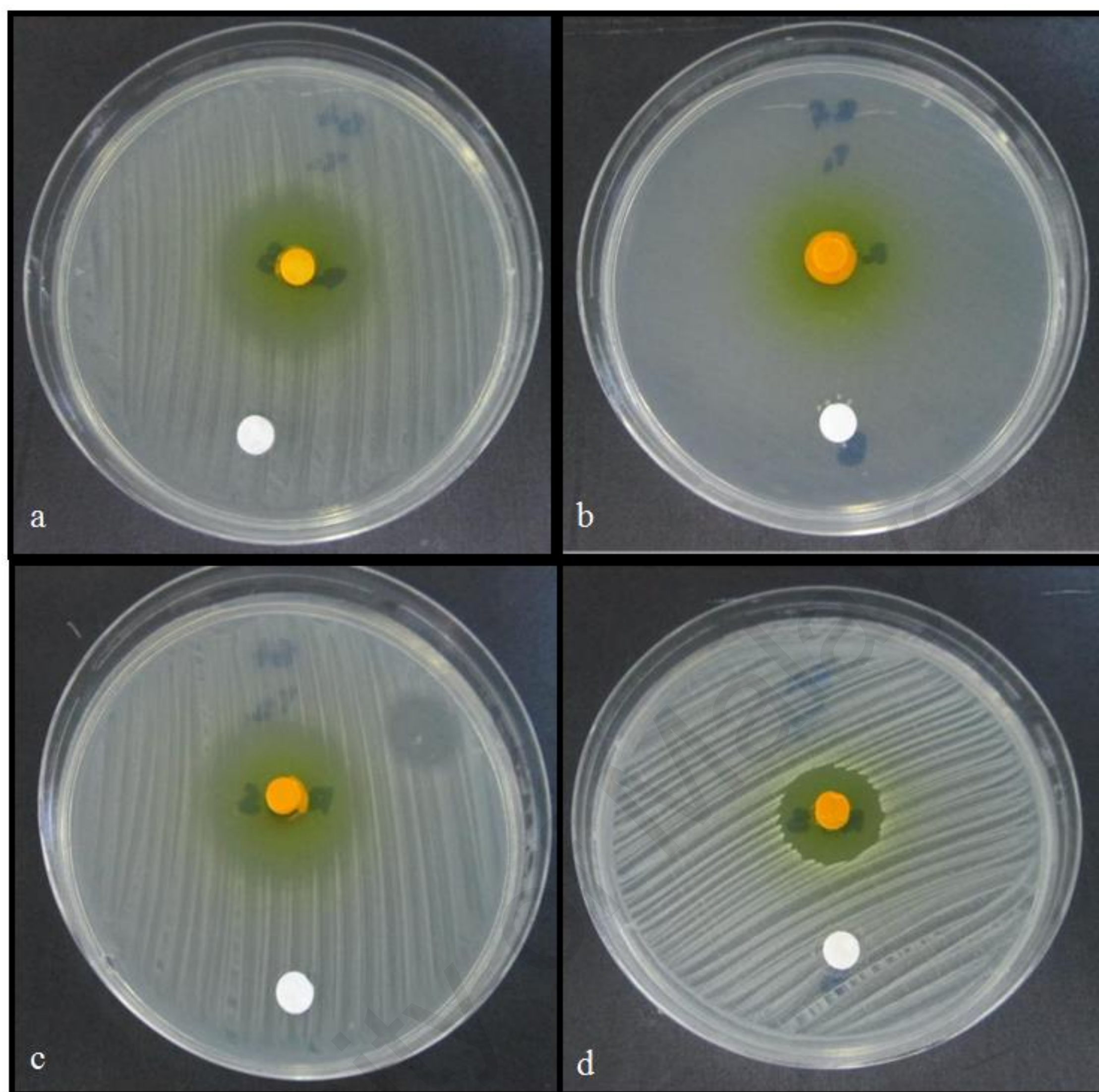

Figure 4.17: Combinational drug studies of standard drugs and riboflavin. The labels above refer as following a - *Staphylococcus aureus,* b - *Enterococcus faecalis,* c - *Salmonella typhi,* and d - *Pseudomonas aeruginosa.*

In another set of experiments, pathogens treated with standard drugs; SA and EF with Vancomycin, ST with Imipenem and PA with Gentamicin show bigger zones of inhibition compared to non-activated riboflavin (Figure 4.17). Gram positive bacteria SA and EF showed (25.1± 0.6) mm and (24.4 ± 0.1) mm, respectively. Gram negative bacteria ST and PA meanwhile showed (30.2 ± 0.8) mm and (25.1 ± 0.6) mm (Figure 4.17), respectively,

The study at this point describe the development of riboflavin as an alternative medicine complementing the conventional antibiotics supported by the fact that besides

showing comparable efficacy, riboflavin also does not cause any toxicity or adverse effects to human being. Riboflavin alone was found to successfully inhibit the growth of pathogen (including fungus) and when coupled together with conventional drugs (Table 4.4), improved efficacies were observed. As discussed earlier, there is a possibility that the riboflavin already present in the blood may serve as a natural antimicrobial mechanism (Martins *et al*., 2008). However, riboflavin concentration in blood is not enough to kill foreign pathogens. The treatment proposed based on the results observed in these works, may increases the concentration of riboflavin in blood, boosting up the immune system. Even upon intake of a higher than necessary dose of riboflavin, the excess will be safely excreted easily through urine.

In this current study, pathogens were selected randomly and subjected to combinational studies consisting of standard drugs coupled with riboflavin (stand-alone riboflavin and UVA irradiated riboflavin) as shown in Table 4.4 and Figure 4.17. The results demonstrate extremely better inhibitions compared to riboflavin alone. Vancomycin, Gentamicin and Imipenem are standard drugs that are used to treat infections of pathogens. However, these drugs are also known to cause severe effects such as kidney damage, hearing loss, redness on skin and others (Mattew & Thomas 2009). Most of the standard drugs generally inhibit pathogens by means of cell wall synthesis of the pathogens (Harold & Thomas, 1996). However, it also damages the bacterial cell membrane and interferes with bacterial RNA/DNA synthesis (Harold & Thomas, 1996). Application of standard drugs for five minutes and then with riboflavin treatment kills pathogens much more effectively as demonstrated by the wider zones of inhibition observed on the cultured agar plates (Figure 4.17). Riboflavin may enter the bacteria by passing through trans-membrane channels developed by the standard drugs (Sauer *et al*., 2010).

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Table 4.4: Average zone of inhibition of selected blood borne pathogens.

Pathogens	Zone of inhibition (mm) (mean ± SD)		
	Negative control	Standard drug	Riboflavin (50 mg/ml)
SA	0.0	21.8 ± 0.4	25.1 ± 0.6
EF	0.0	21. 9 ± 0.7	24.4 ± 0.1
ST	0.0	28.4 ± 1.4	30.2 ± 0.8
PA	0.0	18.3 ± 0.0	25.1 ± 0.6

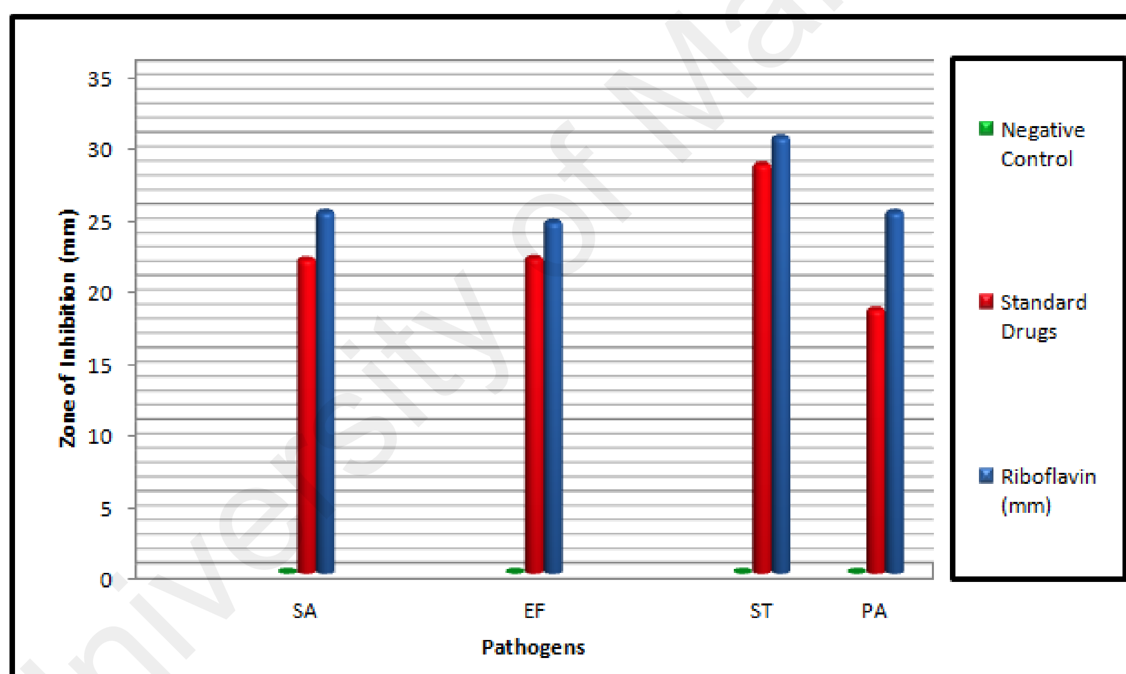

Figure 4.18: Average zone of inhibition of selected blood borne pathogens.

Riboflavin diffuses into impermeable membranes of bacteria and fungus killing Gram positive and negative bacteria effectively (Sauer *et al*., 2010). Standard drugs treatment may allow better diffusion of riboflavin in a horizontal direction, reducing its vertical diffusion into the agar, leading to an overall higher surface concentration of the agent (Sauer *et al*., 2010). However, the mechanisms of action may be more complex and multifaceted as expected. Furthermore, in approximately 80% cases of blood

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infections, deep bacterial and fungal penetration develops, often leading to dissemination systemically. The riboflavin effects can be found only down to approximately 50.0 µl in each disc with 50.0 mg of riboflavin, which means that it can give certain impact on deep blood borne pathogen infection.

In these studies, we were able to express in-vitro activity of riboflavin with standard drugs against selected Gram positive and Gram negative bacteria (Figure 4.18). This treatment is considered safe when all recognized criteria's for the treatment were fulfilled. In conclusion, riboflavin was found to severely affect the development and function of the pathogens when used in combination with the standard drugs (Figure 4.18). The concentrations of riboflavin used in this study as an antimicrobial drug were considered safe to use for treatment with human patients suffering from septicaemia without toxic and adverse effects (Select Committee on Gras Substances, 1979). We suggest that with further improvements on the drug delivery method, it may be possible to treat blood borne pathogens using this approach.

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CHAPTER 5

CONCLUSIONS AND FUTURE STUDIES

5.1 Conclusions

Riboflavin efficacy without UVA irradiation resulting in inhibition of growth of Gram positive, Gram negative and fungus was first ever observed. Moreover, we also demonstrated that riboflavin combined with UVA irradiation exhibited similar zones of inhibition as compared to application of riboflavin alone. The findings showed that *S. aureus*, *E. faecalis*, *S. typhi*, and *P. aeruginosa* at a range of 20.0 ± 1.0, 17.7 ± 0.6, 17.3 ± 0.6, and 15.7 ± 0.6 respectively. Intermediate zones of inhibition were observed for *E. coli* and *C. albicans* in the range of 11.7 ± 0.6 and 11.7 ± 0.6 respectively. *K. pneumoniae* was resistant with a range of 7.7 ± 0.6 mm. The results observed for pathogens pre-treated with standard drugs and then with riboflavin solution; SA and EF showed 25.1 ± 0.6 mm and 24.4 ± 0.1 mm, respectively. Gram negative bacteria ST and PA meanwhile showed 30.2 ± 0.8 mm and 25.1 ± 0.6 mm respectively. UVA is known for its harmful side effects to human body and could not be used within drug delivery deep within the body. Despite the slightly improved riboflavin efficacy in combination with UVA irradiation, stand-alone riboflavin efficacy due to its non-toxicity may provide alternative or complementary solutions to the current spectrum of conventional antibiotics. In addition to previous treatments with standard drugs and then with riboflavin application effectively inhibited the growth of the selected pathogens.

These initial results are promising but animal studies are further needed to examine whether riboflavin might be an effective adjunct treatment for blood borne pathogens and as supplement to treat more than one disease in future. Combined and complementary treatments might be used widely in future for enhanced treatment of blood borne pathogens.

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5.2 Future Studies

This research has furnished some valuable findings about riboflavin containing natural antimicrobial properties useful to the medical field. Furthermore, this information could provide the necessary understanding for the development of riboflavin as an alternative medicine besides the more conventional antibiotics as it does not have any toxicity or adverse effects towards biological cells. The current stand-alone riboflavin efficacy should be diversified to treat other diseases or coupled with other drugs. Efforts should also be taken to study its anticancer or antioxidant properties before establishing optimized understanding of the mechanisms of riboflavin in the context of medicinal natural food.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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2. Zhijian Chan, Aarthi Ahgilan, Vikineswary Sabaratnam, Yee Shin Tan and Vengadesh Periasamy, (2015). Rectification of DNA films self-assembled in the presence of electric field. *Applied Physics Express*, 8 (4): 047002-1-047002-4.
3. Aarthi Ahgilan, Vengadesh Periasamy, Vikineswary Sabaratnam. Novel investigation of Antimicrobial Properties of vitamin B2. International Conference on Food Properties (ICFP 1), Kuala Lumpur, Malaysia, 24-26 January 2014 (the paper received one of the best papers recognition).

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Antimicrobial Properties of Vitamin B2

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Rectification of DNA films self-assembled in the presence of electric field

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We report rectification phenomena in electric field induced self-assembled thin films of DNA that were employed in the development of an ITO/DNA/Al sensor. The prototype sensor was subjected to electrical characterization involving the acquisition of current–voltage graphs. Electric field aligned DNA films exhibited reduced potential barrier of 0.780 eV while the potential barrier for non-aligned films was 0.796 eV. Similar reduction was also observed for the measured ideality factor and series resistance. This enhanced rectification following electric field induced self-assembly of DNA films may prove beneficial for generating accurate and rapid response in DNA-based devices.
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Molecular electronics has attracted significant attention during the past two decades. Amongst the biomaterials of interest, DNA has attracted most attention owing to its low cost and wide availability. Various groups have published numerous reports regarding the usage of DNA as rectifiers and sensors. However, proper understanding of its functionality and properties, especially when the DNA molecules are assembled into films, remains incomplete. As such, charge transfer mechanisms in DNA molecules are being exhaustively investigated, because this phenomenon provides insights on various biological processes and cell repair mechanisms. Semiconductor characteristics of DNA were first reported in 2000 when Porath et al. demonstrated large band gap semiconductor behavior, while Lintao, Hitoshi and Tomoji demonstrated similar properties of DNA on a mica surface.[1,2] Various other groups[3–7] have also reported rectification in DNA using various modifications and parameters. More recently, one research group investigated the current density–voltage characteristics of an Ag/DNA/p-InP device and reported rectification in this device.[8] Meanwhile, Wang et al.[9] illustrated DNA utilization as a template for fabricating nanowires exhibiting rectification. Finally, Gupta et al.[10] used spin-coating technique for constructing an Ag/DNA/p-Si/Al device with photodiode properties.

The double helix structure of DNA is composed of two strands of DNA wound around each other. The strands are composed of repeating sugar and phosphate groups attached to the bases. DNA strands accommodate four bases: guanine (G), cytosine (C), adenine (A) and thymine (T). These bases are the building blocks of the genetic code. In a double-stranded DNA, every two bases on the opposing strands are coupled by hydrogen bonds. The unique characteristic of these bonds is that adenine can only pair with thymine, while guanine can only pair with cytosine.[11] Previous works[12] have determined that holes (positive charges) are more stable on G–C pairs compared with A–T pairs. It has also been found that the energy difference between the pairs is large compared with the thermal energy of charge carriers, resulting in charge localization on G–C pairs.[12] In addition, energy levels in A–T pairs are higher, acting as a barrier. Various conduction mechanisms have been suggested for describing the electrical properties of DNA molecules, including hopping and tunneling.[1,13,14] The former phenomenon is similar to semiconductor electronics where charges "hop" from a G–C pair to an A–T pair. The latter phenomenon is similar to the quantum tunneling between potential barriers, which only happens at relatively short distances. These explanations provide a simple picture of charge transfer mechanism in DNA molecules. However, a proper understanding of the actual process remains incomplete. To date, little research has addressed the phenomena occurring in thin films of DNA, which is an important and promising field of study. In addition, another interesting property of DNA, the self-assembly ability,[2] allows for manufacturing low cost and high precision nanostructured materials.

Here, we demonstrate rectification in a self-assembled thin film of DNA on an indium tin oxide (ITO) coated glass with Al as a counter electrode. The DNA film exhibits a comparatively low threshold voltage in the forward bias orientation. Applying the Schottky's rule and the current–voltage (I–V) curve, the potential barrier between DNA and Al was 0.780 eV when the sample was dried in the presence of electric field. Without the electric field alignment, the calculated value was 0.796 eV. Field emission scanning electron microscopy (FESEM) images confirmed the formation of highly aligned DNA film on the substrate's surface. These results are likely to assist in developing DNA sensors and detectors for biomedical applications.

DNA molecules were extracted from *Mimosa pudica* by using a genomic DNA mini-plant kit from Yeastern Biotech. UV spectrophotometric analysis indicated that OD260/OD280 was 1.9 and the DNA concentration was 74.1 ng/μL. The ITO-coated glass was purchased from KINTEC, while the Al wire was purchased from Diamond. Deionized water was obtained using an in-house available Barnstead (Nanopure II) water deionizing system.

The DNA solution was diluted in deionized water with a weight ratio of 1 : 100. Using a micro syringe, the solution was cast on the ITO-coated glass substrate and allowed to air-dry overnight. The circuit was completed by attaching an Al foil as a counter electrode atop the DNA. After obtaining the I–V measurements, the sample was wetted using a few drops of deionized water and dried in the presence of electric field ($120\,\mathrm{V\,m^{-1}}$) that was utilized for aligning the DNA strands parallel to the field lines. The electric field helped to hold the molecules in place during the drying process. Owing to their electrodynamic characteristics, the DNA strands realigned

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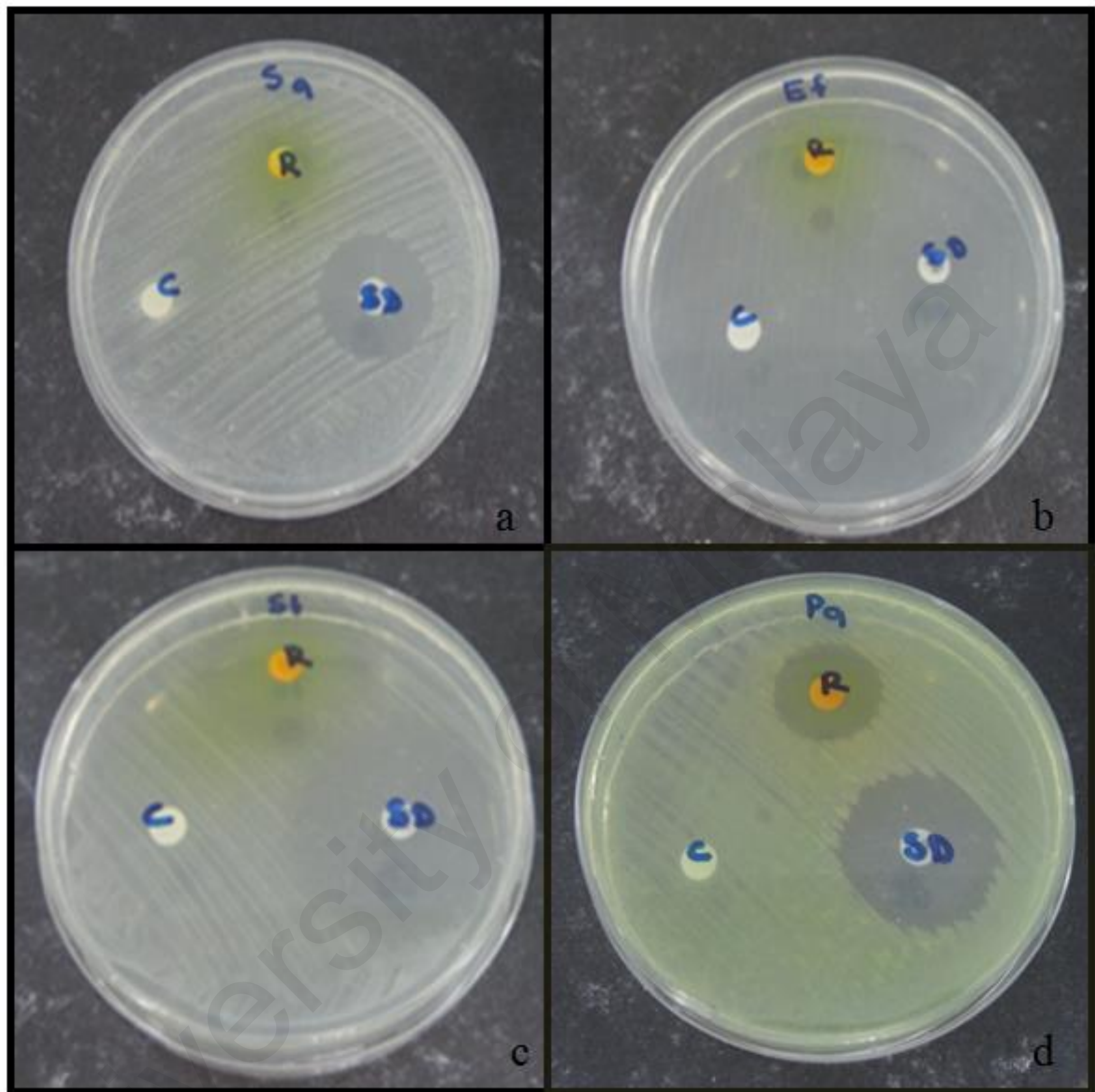
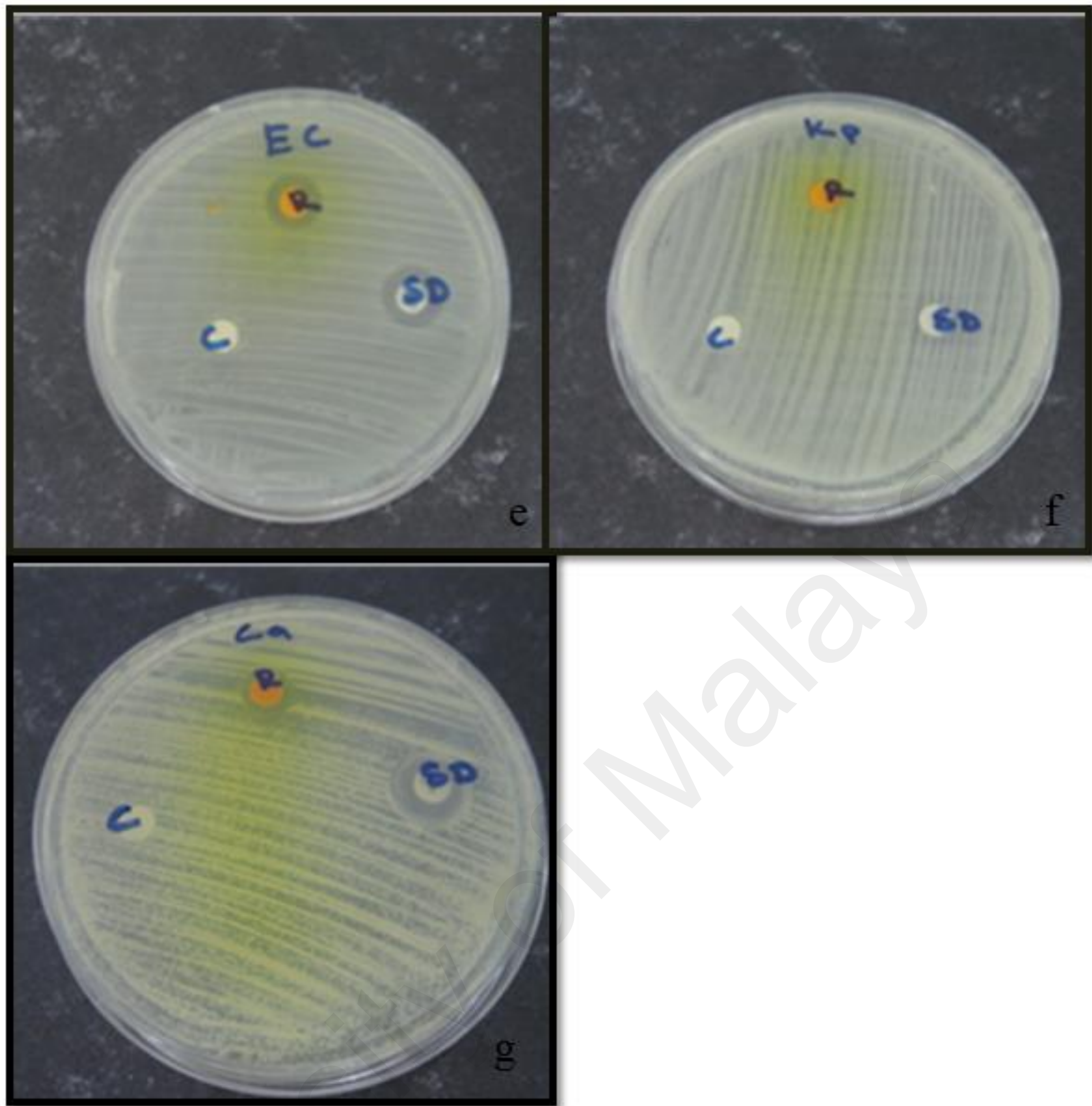
APPENDICES

Appendix A : Testing the susceptibility of microorganisms to antibiotics by the Kirby-Bauer disc diffusion method.





The labels above refer as following; a - *Staphylococcus aureus,* b - *Enterococcus faecalis,* c - *Salmonella typhi,* d - *Pseudomonas aeruginosa,* e - *Escherichia coli*, F - *Klebsiella pneumoniae* and g - *Candida albicans,* R - Riboflavin, SD - Standard drug, and C – Control.

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Appendix B : The minimum and maximum zone of inhibition on selected riboflavin concentration for SA, EF, ST, PA, EC, KP, and CA.

Pathogen	Trials	Stock Solution of Riboflavin at ratio 1:1 (g/ml)																			
		5.0	10.0	15.0	20.0	25.0	30.0	35.0	40.0	45.0	50.0	55.0	60.0	65.0	70.0	75.0	80.0	85.0	90.0	95.0	100.0
SA	Test 1	15.00	15.30	17.20	18.40	19.50	18.80	18.60	18.50	18.60	18.60	18.50	18.50	18.40	18.30	18.10	18.10	18.10	17.90	17.80	17.70
	Test 2	16.00	16.00	18.00	18.80	18.90	18.70	18.60	18.60	18.40	18.50	18.40	18.30	18.40	18.50	17.80	17.70	17.80	17.70	17.90	17.80
	Test 3	18.00	18.00	18.10	18.90	19.40	18.50	18.90	18.60	18.50	18.40	18.60	18.40	18.30	18.40	17.70	17.70	17.60	17.80	17.80	17.80
	Ave	16.3±1.5	16.4±1.4	17.8±0.5	18.7±0.3	19.3±0.3	18.7±0.2	18.6±0.1	18.6±0.1	18.5±0.1	18.5±0.1	18.5±0.1	18.4±0.1	18.4±0.1	18.4±0.1	17.9±0.2	17.8±0.2	17.8±0.3	17.8±0.1	17.8±0.1	17.8±0.1
EF	Test 1	7.00	7.00	7.00	11.20	16.40	16.60	16.60	16.50	16.40	16.50	16.30	16.40	16.40	16.50	16.40	16.30	16.30	16.30	16.20	16.10
	Test 2	8.00	8.00	8.00	11.30	16.50	16.50	16.50	16.30	16.40	16.40	16.20	16.50	16.20	16.30	16.40	16.20	16.20	16.20	16.10	16.20
	Test 3	8.00	8.00	8.00	11.20	16.50	16.40	16.70	16.30	16.40	16.40	16.30	16.20	16.20	16.20	16.20	16.40	16.30	16.20	16.30	16.40
	Ave	7.7±0.6	7.7±0.6	7.7±0.6	11.2±0.1	16.5±0.1	16.5±0.1	16.6±0.1	16.4±0.1	16.4±0.0	16.4±0.1	16.3±0.1	16.4±0.2	16.3±0.1	16.3±0.2	16.3±0.1	16.3±0.1	16.3±0.1	16.2±0.1	16.2±0.1	16.2±0.2
ST	Test 1	6.00	6.00	6.00	11.20	14.70	14.60	14.40	14.70	14.80	14.50	14.50	14.30	14.20	14.10	14.10	14.20	14.00	14.20	14.10	14.00
	Test 2	6.00	6.00	6.00	10.90	14.50	14.80	14.90	14.60	14.50	14.50	14.60	14.20	14.20	13.80	13.20	13.50	13.80	13.60	13..7	13.60
	Test 3	6.00	6.00	6.00	11.10	14.80	14.30	14.40	14.60	14.60	14.40	14.40	14.40	14.20	13.70	14.10	13.70	13.60	13.70	13.70	13.40
	Ave	6.0±0.0	6.0±0.0	6.0±0.0	11.0±0.1	14.7±0.2	14.6±0.3	14.6±0.3	14.6±0.1	14.6±0.2	14.5±0.1	14.5±0.1	14.3±0.1	14.2±0.0	13.9±0.2	13.8±0.5	13.8±0.2	13.8±0.2	13.8±0.3	13.8±0.2	13.7±0.3
PA	Test 1	6.00	6.00	6.00	11.20	11.90	12.10	11.80	11.80	11.70	12.00	11.90	12.10	11.60	11.70	11.80	11.90	12.00	11.80	11.80	11.90
	Test 2	6.00	6.00	6.00	11.10	11.90	11.90	11.90	11.80	11.80	11.80	11.80	11.60	11.70	11.60	11.70	11.70	11.80	11.70	11.70	11.70
	Test 3	6.00	6.00	6.00	11.10	11.80	11.80	11.70	11.90	11.90	11.60	11.70	11.70	11.80	11.90	11.60	11.60	11.60	11.50	11.60	11.60
	Ave	6.0±0.0	6.0±0.0	6.0±0.0	11.1±0.1	11.9±0.1	11.9±0.2	11.8±0.1	11.8±0.1	11.8±0.1	11.8±0.2	11.8±0.1	11.8±0.3	11.7±0.1	11.7±0.2	11.7±0.1	11.7±0.2	11.8±0.2	11.7±0.2	11.7±0.1	11.7±0.2
EC	Test 1	7.00	7.00	7.00	9.80	11.80	11.60	11.60	11.60	11.30	11.60	11.30	11.40	11.30	11.40	11.40	11.20	11.20	11.30	11.30	11.40
	Test 2	6.00	6.00	6.00	9.90	11.90	11.70	11.40	11.50	11.40	11.50	11.50	11.40	11.20	11.30	11.30	11.40	11.40	11.30	11.40	11.20
	Test 3	6.00	6.00	6.00	9.80	11.70	11.80	11.40	11.30	11.40	11.50	11.30	11.50	11.30	11.30	11.30	11.30	11.40	11.40	11.20	11.20
	Ave	6.3±0.6	6.3±0.6	6.3±0.6	9.8±0.1	11.8±0.1	11.7±0.1	11.5±0.1	11.5±0.2	11.4±0.1	11.5±0.1	11.4±0.1	11.4±0.1	11.3±0.1	11.3±0.1	11.3±0.1	11.3±0.1	11.3±0.1	11.3±0.1	11.3±0.1	11.3±0.1
KP	Test 1	6.00	6.00	6.00	6.00	8.10	8.20	8.20	8.30	8.10	8.20	8.20	8.20	8.10	8.10	8.20	8.20	8.10	6.00	6.00	6.00
	Test 2	6.00	6.00	6.00	6.00	8.20	8.10	8.20	8.10	8.20	8.10	8.10	8.00	8.10	8.10	8.10	8.20	8.20	6.00	6.00	6.00
	Test 3	6.00	6.00	6.00	6.00	8.10	8.10	8.10	8.10	8.10	8.10	8.10	8.20	8.20	8.20	8.20	8.10	8.10	6.00	6.00	6.00
	Ave	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	8.1±0.1	8.1±0.1	8.2±0.1	8.2±0.1	8.1±0.1	8.1±0.1	8.1±0.1	8.1±0.1	8.1±0.1	8.1±0.1	8.2±0.1	8.2±0.1	8.1±0.1	6.0±0.0	6.0±0.0	6.0±0.0
CA	Test 1	6.00	6.00	6.00	8.10	11.40	11.40	11.80	11.30	12.50	12.40	12.10	12.20	11.40	10.40	10.30	8.40	8.40	8.40	8.50	8.80
	Test 2	6.00	6.00	6.00	8.20	11.30	11.10	11.70	11.20	12.40	12.40	12.50	12.30	11.20	10.30	10.30	8.60	8.50	8.60	8.80	8.60
	Test 3	6.00	6.00	6.00	8.20	11.30	11.40	11.60	11.40	12.30	12.20	12.40	12.20	11.30	10.10	10.40	8.60	8.50	8.60	8.70	8.70
	Ave	6.00	6.00	6.00	8.2±0.1	11.3±0.1	11.3±0.2	11.7±.1	11.3±0.1	12.4±0.1	12.3±0.1	12.3±0.2	12.2±0.1	11.3±0.1	10.3±0.2	10.3±0.1	8.5±0.1	8.5±0.1	8.5±0.1	8.7±0.2	8.7±0.1

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Appendix C : Identification Test Results of Pathogens

ERIC™ *Electronic RapID Compendium*

Laboratory: My Laboratory
User: KIKI

Ref No: 12.0000389
Report Date: 27/10/2012

RapID STR **Identification Report**

Microcode: 71603

+ ARG	+ SBL	- GAL	- PO4	+ LYS
+ ESC	- RAF	+ GLU	- TYR	+ PYR
+ MNL	- INU	+ NAG	- HPR	- HEM

IDENTIFICATION = E. faecalis

Choice(s)	Probability	Bioscore	Contraindicated Tests
E. faecalis	>99.9%	1/2	None

Probability Level: Implicit BioFrequency: Typical

Group D enterococcus. CDC group II. Normal inhabitant of the skin and gastrointestinal tract. May cause a variety of infections. Vancomycin resistant strains have been encountered.

RapID ™ STR Report Form

Reference # / No. de référence / Referenz-Nr. / Riferimento N. / N° de referencia ENFA
Date / Date / Datum / Data / Fecha 27/10/2012
Tech / Tech / Techn. / Tech / Tec
Source / Source / Quelle / Origine / Origen
Gram Stain / Souche Gram / Gram Färbung / Colorazione di Gram / Tinción de Gram
Lancefield Group / Groupe Lancefield / Lancefield-Gruppe / Gruppo di Lancefield / Grupo de Lancefield

Reagent / Réactif / Reagenz / Reagente / Reactivo	None / Aucun / Keine / Nessuno / Ninguno										RapID STR Reagent /Réactif RapID STR RapID STR Reagens /Reagente RapID STR / Reactivo RapID STR				None
Positive Reactions / Réactions positives / Positive Reaktionen / Reazioni positive / Reacciones positivas	Red or Dark Orange / Rouge ou orange foncé / Rot oder dunkelorange / Rosso o arancione scuro / Rojo o naranja oscuro	Black / Noir / Schwarz / Nero / Negro	Yellow or Yellow-orange / Jaune ou Jaune orangé / Gelb oder gelb-orange / Giallo o giallo-arancio / Amarillo o amarillo-naranja			Yellow, yellow-orange or orange / Jaune, jaune orangé ou orange / Gelb, gelb-orange oder orange / Giallo, giallo-arancio o arancio / Amarillo, amarillo-naranja o naranja	Yellow / Jaune / Gelb / Giallo / Amarillo				Light Purple or purple / Violacé léger ou franc / Helles Violett oder Violett / Porpora chiaro o Porpora / Morado claro o morado		Very Dark Purple / Violacé très foncé / Sehr dunkles Violett / Porpora molto scuro / Morado muy oscuro		Beta / Beta / Beta / Beta / Beta
Cavity # / No. cavité / Kammer-Nr./CavitàN./ N° de cavidad	1	2	3	4	5	6	7	8	9	10	7	8	9	10	
Test Code / Code du test / Testcode / Codice esame / Código de prueba	ARG	ESC	MNL	SBL	RAF	INU	GAL	GLU	NAG	PO4	TYR	HPR	LYS	PYR	HEM
Value / Valeur / Wert / Valore / Valor	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
Result / Résultat / Ergebnis / Risultato / Resultado	+	+	+	+	-	- / +	-	+	+	+	-	-	+	±	±
Value Total / Total des valeurs / Gesamtwert / Valore totale / Valor total	7			5/1			6			1			7/5		

IDENTIFICATION / IDENTIFICATION / IDENTIFIZIERUNG / IDENTIFICAZIONE / IDENTIFICACIÓN 71667

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Printed on: Sabtu Oktober 27 2012

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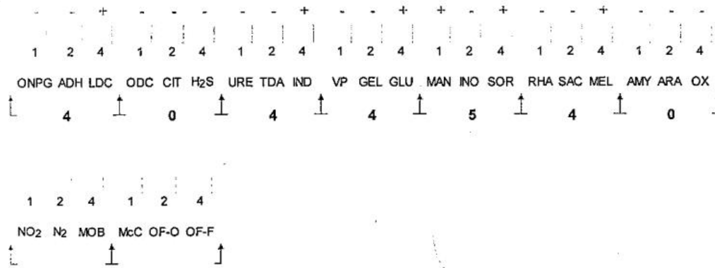
API 20 E V4.0



REFERENCE	DATE
SATY	10/27/12

COMMENT

DOUBTFUL PROFILE

Strip	API 20 E V4.0
Profile	4 0 4 4 5 4 0
Note	CONFIRM BY SEROLOGICAL TESTS

Significant taxa	% ID	T	Tests against
Salmonella typhi	71.7	0.5	IND 0%
Escherichia coli 2	25.4	0.65	MEL 3%
Escherichia coli 1	2.6	0.4	ONPG90% RHA 82% ARA 99%

Next taxon	% ID	T	Tests against
Shigella spp	0.1	0.15	LDC 0% SOR 7% MEL 20%

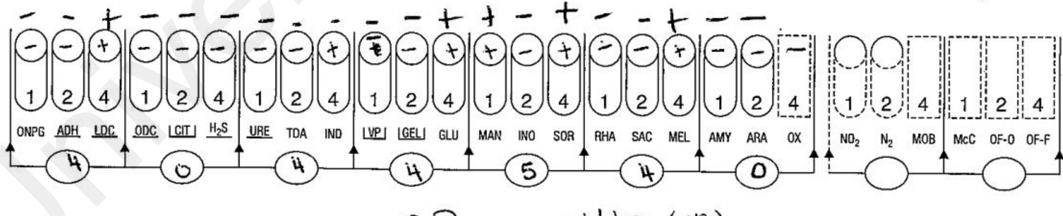
Complementary test(s)	LACTOSEac
Escherichia coli	60%
Salmonella typhi	2%

Close Print

api® 20 E 07223 C

REF.: Salmonella typhi 2012/10/27

Origine / Source / Herkunft /
Origen / Origem / Προέλευση /
Ursprung / Oprindelse / Pochodzenie :



oxidatase -(-ve)

Autres tests / Other tests / Andere Tests /
Otras pruebas / Altri test / Outros testes /
Άλλες εξετάσεις / Andra tester /
Andre tests / Inne testy :

O, H, Vi, Di } +ve.

Ident. / Ταυτοποίηση :

API 20 NE V6.0

-	-	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	-	+
1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
NO3	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
	1			3			5			5			5			7			5	

REFERENCE DATE
PSAE 10/27/12
COMMENT

VERY GOOD IDENTIFICATION

Strip API 20 NE V6.0
Profile 1 3 5 5 5 7 5
Note

Significant taxa	% ID	T	Tests against
Pseudomonas aeruginosa	99.8	0.58	URE 20% ARAa 1%

Next taxon	% ID	T	Tests against
Pseudomonas fluorescens	0.1	0.18	URE 1% MNEa 97% ADIa 10%

Close Print

api® 20 NE 07224 B

REF.: P. aeruginosa 2012/10/28

Origine / Source / Herkunft /
Origen / Origem / Προέλευση /
Ursprung / Oprindelse / Pochodzenie :

BIOMÉRIEUX

	NO3 1	TRP 2	GLU 4	ADH 1	URE 2	ESC 4	GEL 1	PNG 2	GLU 4	ARA 1	MNE 2	MAN 4	NAG 1	MAL 2	GNT 4	CAP 1	ADI 2	MLT 4	CIT 1	PAC 2	OX 4
24 h	- +	+	-	+	+	+	+	-	+	-	-	+	+	-	+	+	+	+	+	-	+
48 h						-	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+
24 h		ϕ			5			5			4			5			7			5	
48 h		1			3			5			5			5			7			5	

Autres tests / Other tests / Andere Tests /
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Άλλες εξετάσεις / Andra tester /
Andre tests / Inne testy :

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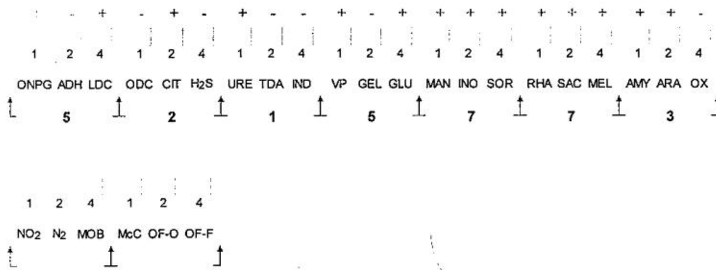

REFERENCE	DATE
KLPN	10/27/12

COMMENT

GOOD IDENTIFICATION

Strip	API 20 E V4.0
Profile	5 2 1 5 7 7 3
Note	POSSIBILITY OF Klebsiella planticola

Significant taxa	% ID	T	Tests against
Klebsiella pneumoniae ssp pneumoniae	97.6	1.0	

Next taxon	% ID	T	Tests against
Klebsiella oxytoca	2.1	0.72	IND 99%

Complementary test(s)	5KG	RM/MR
Klebsiella planticola	98%	100%
Klebsiella pneumoniae ssp pneumoniae	2%	9%

Close Print

api® 20 E CE 07223 C

REF.: KLPN 2012/10/27

Origine / Source / Herkunft /
Origen / Origem / Προέλευση /
Ursprung / Oprindelse / Pochodzenie :

BIOMÉRIEUX

+	-	+	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX

7 5 | 3 2 | 5 1 | 5 5 | 7 7 | 7 7 | 3 3

1	2	4	1	2	4
NO_2	N_2	MOB	McC	OF-O	OF-F

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Autres tests / Other tests / Andere Tests /
Otras pruebas / Altri test / Outros testes /
Άλλες εξετάσεις / Andra tester /
Andre tests / Inne testy : OX → (-ve)

Ident. / Ταυτοποίηση :

University of Malaya

API 20 C AUX V3.0

	O	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	H/PH*
Result	-	+	-	+	-	+	+	+	+	-	+	+	+	-	-	+	+	+	-	-	+
Value	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4

2 5 7 6 1 7 4

REFERENCE CAAL
DATE 10/27/12
COMMENT

GOOD IDENTIFICATION

Strip API 20 C AUX V3.0
Profile 2 5 7 6 1 7 4
Note

Significant taxa	% ID	T	Tests against
Candida albicans 1	97.3	1.0	

Next taxon	% ID	T	Tests against
Candida tropicalis	2.6	0.79	XLT 12%

Close Print

07221 C

REF.: Aarthi CAAL 20/10/12

Origine / Source / Herkunft / Origen / Origem / Προέλευση / Ursprung / Oprindelse / Pochodzenie :

BIOMÉRIEUX

	O	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	Hyphae/ Pseudo-Hyphae
48 h	-	+	-	+	-	+	+	-	+	-	+	+	+	-	-	+	+	+	-	-	+
72 h	-	+	-	+	-	+	+	- +	+	-	+	+	+	-	-	+	+	+	-	-	
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
	2			5			5 7			6			1			7			4		

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Autres tests / Other tests / Andere Tests / Otras pruebas / Altri test / Outros testes / Άλλες εξετάσεις / Andra tester / Andre tests / Inne testy :

Ident. / Ταυτοποίηση :

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