CHAPTER II

LITERATURE REVIEW

1. *Streptococcus suis (S. suis)*

S. suis is a facultatively anaerobe gram-positive bacteria being rounded-shape with a diameter of 1 micrometer and occurs as a single cell, diplococcus or short-chain. *S. suis* is considered as an alpha-hemolytic streptococci, possessing cell wall antigenic determinants related to Lancefield group D (Gottschalk et al., 1989). *S. suis* is a pathogen responsible for a variety of clinical syndromes including meningitis, septicaemia, endocarditis, arthritis, and septic shock in pigs (Reams et al., 1994; Gottschalk and Segura, 2000). *S. suis* strains are identified by various methods such as the presence of specific epitopes on the polysaccharides capsule. Until now, 35 different serotypes of capsular polysaccharides have been identified which are types 1 through 34 and ½ (Staats et al., 1997; Higgins and Gottschalk, 1999). However serotypes 32 and 34 have been proven to be *Streptococcus orisratti* (Hill et al., 2005). Virulence is different among various strains of *S. suis*, including muramidase-release protein (MRP), extracellular protein (EP), suilysin (Okwumabua, Abdelmagid and Chengappa, 1999; Li et al., 2012), and adhesions (Higgins et al., 1995; Higgins and Gottschalk, 1999; Wei et al., 2009). The protein inhibits the C_3 complements binding. This affects phagocytosis of white blood cells and induces inflammation in meninges and serositis.

Pigs may harbor a variety of *S. suis* strains in their upper respiratory tracts, particularly tonsils and nasal cavities, as well as genital and alimentary tracts (Robertson and Blackmore, 1989; Gottschalk and Segura, 2000). Usually, healthy pigs have been infected by *S. suis* within nasal cavities or tonsils without showing symptoms. However, the stress due to congestion, dirtiness or air pollution may increase the number of *S. suis*

infection and spread from tonsils to lymph nodes and to bloodstream bringing about to brain and, consequently causing meningitis. *S. suis* can spread from the infected pigs to other pigs in the herd. Especially, weak pigs or piglets during the weaning and vaccination are easily infected (8-15 weeks of age) (จันทรา, 2007). Infected pigs usually die from meningitis (Reams et al., 1994; Gottschalk and Segura, 2000).

S. suis type 2 can tolerate various environmental conditions. At 0 °C, It can outlive in dust for 1 month and in faeces for over 3 months. At 25 °C, it can survive in dust for 24 hours and in faeces for 8 days. At 4 °C, this microorganism survives in cold water for 1-2 weeks (Higgins and Gottschalk, 1999). It can subsist in carcasses for 6 weeks at 10°C, 2 hours at 50 °C and 10 minutes at 60 °C. However, 1:799 dilution of 5% bleach can kill *S. suis* type 2 easily (Clifton-Hadley and Enright, 1984).

S. suis type 2 lives in the palatine tonsils of clinically ill and apparently healthy pigs. It most commonly involved diseases in pigs and human beings, and was the most frequently reported serotype worldwide (Arends et al., 1984). Except for two cases, *S. suis* type 1 caused human *S. suis* infection and *S. suis* type 14 caused septicaemia (Watkins et al., 2001; Kopic, Paradižik and Pandak, 2002). All other human *S. suis* infections caused by *S. suis* type 2. It was usually transmitted nasally or orally (Arends et al., 1984). In addition, carriers of *S. suis* can be pathogenesis to other pigs and transmit these bacteria in herds (Reams et al., 1994; Gottschalk and Segura, 2000). It was also found that *S. suis* type 2 was transferred from animals to humans (Tarradas et al., 2001; Papatsiros et al., 2011). Human *S. suis* infection is mainly caused by direct contact with carrier pigs, sick pigs or raw pork contaminated with *S. suis* via wounds on *S. suis* infection includes pig farmers, slaughterhouse workers, meat processing workers and veterinarians (Francois et al., 1998). Ordinarily, incubation period of *S. suis* in host is approximately 1 – 3 days. Symptoms of the patients include high fever, headache, chill, nausea, vomiting and vertigo, hearing loss,

articular pain, blind, meningitis and septicemia, consequently causing death (Lun et al., 2007).

In 1968, the first human *S. suis* infection was reported in Denmark (Perch, Kristjansen and Skadhauge, 1968). Later, most of human infections were reported in several countries in Europe, Asia and America such as the China, Thailand, Netherlands, UK, Germany, Spain, France, Croatia, Denmark, Belgium, Japan, Italy, USA, Argentina, Vietnam, Singapore, Hong Kong, etc (Lun et al., 2007; Ma et al., 2008). In China, there have been reported two outbreaks. In 1998, 25 reported cases in Jiangsu province were found that 14 patients died with septic shock syndrome and meningitis. In July, 2005, 204 patients were reported in Sichuan province and 38 patients died due to lack of proper treatment. In the same year, 10 patients got *S. suis* infected in Hong Kong and Guangdong province was reported 4 patients, 1 deceased (Du, Qian and Xu, 2000; Wang et al., 2005). In Thailand, most patients were in the Northern provinces such as Lamphun, Chiang Mai, Phayao, Kamphaengphet, Phichit and patient death from this infection was reported.

Guidelines for treatment and control of *S. suis* infection in swine farm (พรเพ็ญ และ กิจจา, 2007)

Administration of antibiotics

S. suis is sensitive to several types of penicillin providing the benefit of efficient treatment in any infected pigs with such types of bacteria. In pig, treatment guidelines are different based on clinical manifestation. The infected pig with *S. suis* type 2 will be injected with ampicillin, penicillin, amoxicillin, streptomycin or cefquinome (1 time/day for 3-5 days) as the major treatment along with nursing service. Besides administration via injection, it was also found that administration of penicillin, tiamulin, amoxicillin, ampicillin, sulfa-trimethoprim fosfomycin and cefthiazole by mixing in water or pig's feed is able to reduce *S. suis* type 2 infection. However, it was found that penicillin administration is

unable to totally annihilate mortality caused by the infection and some survived pigs are unable to recover completely, i.e., they are often deaf or blind or have joint pain later (พรเพ็ญ และกิจจา, 2007).

The failure of medical therapy may be caused by administrating penicillin in the group of drug concentration-dependent to kill the bacteria by mixing it with pig's feed. Administration of medicine with the dose equal to the level with effective therapy may hide clinical manifestation of the disease but fail to kill bacteria in pigs (Clifton-Hadley, Enright and Alexander, 1986; Staats et al., 1997). Consequently, this may subsequently cause exacerbation and epidemiology of disease, especially while pigs were pregnant leading to transmission of the bacteria from the pigs to their piglets. Moreover, some medicines (e.g., penicillin) are not suitable for oral administration because they are easily dissolved in water. After fed, they were absorbed in small amount by alimentary canal causing decrease of treatment efficiency (Clifton-Hadley et al., 1986; Gottschalk et al., 1991; Staats et al., 1997).

Vaccination

Vaccine is another alternative for preventing *S. suis* infection in pig. Although there are several types of vaccines, killed vaccine, distributed in market, there is no vaccine with capability to prevent *S. suis* infection efficiently. This is because there are several serotypes of *S. suis*. Mechanism of action of vaccine against each serotype was unclear until virulent factor (the main cause of disease of these bacteria) was found (Elliott, Clifton-Hadley and Tai, 1980; Jacobs et al., 1994; Jacobs, van den Berg and Loeffen, 1996; Zhang et al., 2008).

Management

Currently, control and prevention on epidemiology of *S. suis* are able to be performed by using good management, such as cleaning pig stable and having sufficient resting period of pig stable. In addition, it is also necessary to reduce all factors that may

cause pigs to be in stress conditions leading to the reduction of immune system's function, for example, adjusting ventilation system of pig stable along with mixing penicillin in pig's feed in order to prevent bacterial infection or injection in serious cases and controlling bacteria of other pigs that effect on efficiency of immune system (เพชรรัตน์, 2006; Direksin, Piewkhao and Somon, 2007).

2. Antibacterial agents

Beta-lactam antibiotics provide bactericidal activities and they are in a group of aminopenicillanic acid structure consisting of beta-lactam ring connected with a ring containing sulfur (S). In chemical structure, penicillin contains a five-member ring called thiazolidine ring while cephalosporin contains a six-member ring called dihydrothiazine ring. Mechanism of beta-lactam antibiotics is to inhibit cell wall synthesis. Penicillin couples with enzyme transpeptidase and becomes penicilloyl enzyme. When a beta-lactams is absorbed in bacterial cells, it replaces D-alanyl-D-alanie causing the malfunction of transpeptidase enzyme providing weak cell wall. Moreover, penicillin also kills gram-positive bacteria by reducing murein hydrolase inhibitor; therefore, enzyme murein hydrolase or autolysis enzyme will destroy cell wall. In addition, it also hinders function of other enzymes or proteins that are necessary for cell membrane and cell wall synthesis. These proteins are called penicillin binding proteins (PBPs) such as transpeptidase and carboxypeptidase. If PBPs are disturbed, cell will split or transform to be circle shape or stretch to be in long shape or cell wall formation will not be complete. Resistant mechanisms of bacteria to beta-lactam antibiotics are as follows: the capability of beta-lactam to pass through bacterial cell is reduced; the capability of cell to couple with PBPs is reduced; PBPs may be changed; bacteria may produce beta–lactamase enzyme to destroy beta-lactam by making beta-lactam ring split leading to the loss of efficiency in

inhabiting bacteria or transforming to be penicilloic acid without antibacterial activity (Dipiro et al., 2008; Yotsombut, 2011).

For *S. suis,* there is no report that this type of bacteria is able to produce beta-lactamase enzyme to destroy beta-lactam ring. At the same time, there are some researchers reporting that the capability of penicillin in coupling with PBP 1 and PBP 2 of *S. suis* reduced in the group of *S. suis* with high level of MICs of the medicine against bacteria. In addition, it was also found that there was the change in PBP 3 molecular size by 5kDa approximately. Therefore, resistance of *S. suis* may be caused by changing PBPs of cell wall of this bacteria (Cain et al., 1995).

Aminoglycosides is obtained from *Streptomyces* or *Micromonospora* spp. These antimicrobials provide bactericidal activities to hinder protein production by coupling with 30r ribosome and leading to incorrect code reading. Normally, other antimicrobials inhibiting protein production provide bacteriostatic effect, but aminoglycosides provide bactericidal activities. This group of medicine covers most gram-negative bacilli for both gram-negative aerobic bacteria and facultative anaerobic bacteria including *enterobacteriaceae* and *pseudomonas* spp. For gram-positive bacteria, it is necessary to be used along with cell wall synthesis inhibitor for better activity. Resistance of bacteria against aminoglycosides can be 3 mechanisms including change in uptake, production of modifying enzymes to change medicine structure and change of binding sites of the medicine on ribosome. For all of these three mechanisms, production of modifying enzymes is mostly found (Kumana and Yuen, 1994; อโนชา และนงลักษณ์, 2007).

Aminoglycosides are combined with beta-lactams to benefit synergistic effect against *staphylococci*, *viridans streptococci* and *enterococci.* In addition, they are also able to be used with beta-lactams to treat serious infection caused by gram-negative bacilli except for CNS infection.

Fluoroquinolones is obtained from synthesizing nalidixic acid. Initially, it covers gram-negative bacteria and it is subsequently developed to cover gram-positive bacteria as well. The capabilities of fluoroquinolones are bactericidal activity to inhibit function of DNA gyrase by inhibiting DNA production. The medicines couple with DNA gyrase or topoisomerase IV that is the enzyme transforming circle DNA (relaxed form) to be helix form. Bacteria will resist to fluoroquinolones through 2 mechanisms including change of capability in coupling with DNA gyrase due to mutation at A subunit and direct absorption to bacteria (Lesse, 1995; Hooper, 2001; อโนชา และนงลักษณ์, 2007)

3. Susceptibility testing method

Antimicrobial susceptibility test is important to confirm susceptibility for empirical antimicrobial selection or drug resistant detection in individual bacteria isolated. Moreover, susceptibility test is a test for determining MIC. Many methods have been used for this test as following (Jorgensen and Ferraro, 2009; CLSI, 2011).

3.1 Broth dilution tests are the microbroth, macrobroth or tube-dilution method. Two-fold dilutions of antibiotics are prepared in a liquid growth medium dispensed in test tubes. A standardized bacterial suspension at 1-5 x 10^5 CFU/mL was inserted into antibiotic containing tubes. Turbidity is observed as bacterial growth after overnight incubation at 35 $^{\circ}$C. The lowest concentration of antibiotic preventing bacterial growth represents the MIC. The precision of this method is determined to be plus or minus 1 two-fold concentration. For the microbroth method, plastic microdilution trays are widely used in broth dilution testing each contains 96 well (Figure 1). Each well provides a volume of 0.1 ml. A single tray allows up to 12 antibiotics to be tested in a range of 8 two-fold dilutions. The advantages of the microdilution procedure are the generation of MICs, reproducibility and convenience of the test. There is also assistance in generating

computerized reports if an automated panel reader is used. The disadvantage is some inflexibility of drug selections available in standard commercial panels.

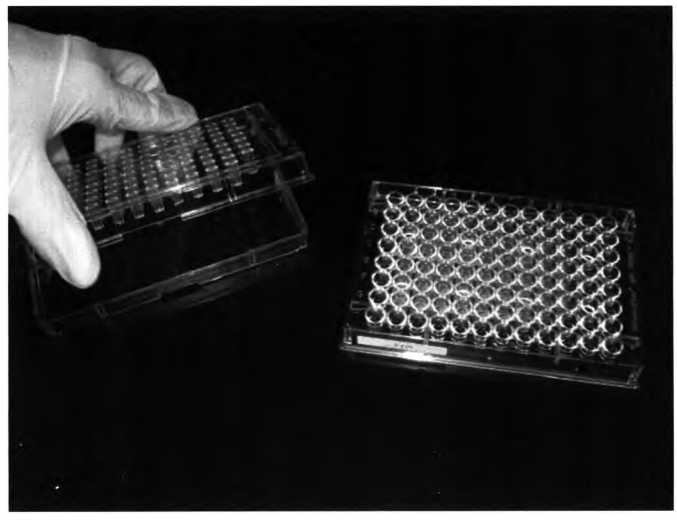

Figure 1 A broth microdilution susceptibility panel containing 98 reagent wells and disposable tray inoculators (Jorgensen and Ferraro, 2009).

3.2 Agar dilution tests can be adapted from the principles of techniques outlined of the broth dilution method. This method generates quantitative results and works properly when a limit number of antibiotics are tested on a large number of bacterial strains. The disadvantages are tedious and manual task to prepare the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test.

Agar dilution method is shown in Figure 2. A two-fold serial dilution of the drug is prepared in agar medium. Bacteria at a certain concentration are placed on the agar surface. The inoculated agar plate is incubated at 37 $^{\circ}$C for 18-24 hours. The MIC is defined at the lowest concentration without visible bacterial growth on the agar surface.

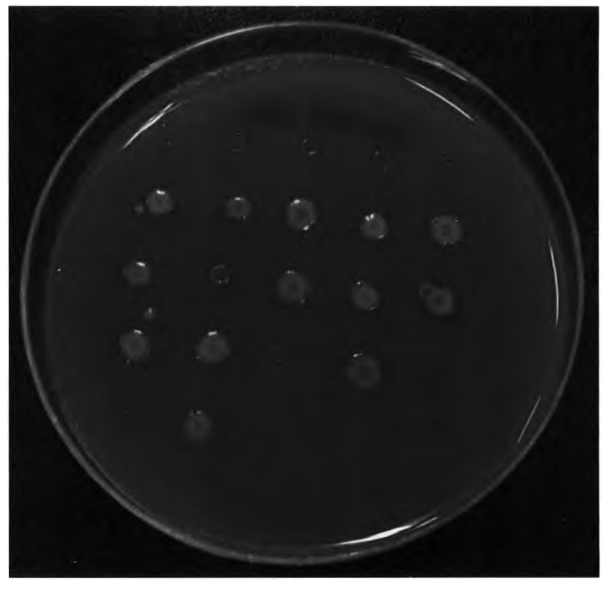

Figure 2 A *S. suis* isolate tested by the agar dilution tests on sheep blood agar.

3.3 Antimicrobial gradient diffusion method is based on an antimicrobial concentration gradient in an agar medium as a means of susceptibility determination. For example, the Etest (bioMe´rieux AB BIODISK) (Figure 3) is thin plastic test strips that are soaked on the bottom by a dried antibiotic concentration gradient. A concentration scale is marked on the upper surface of strips. Five or six strips may be placed on a surface of agar plate with a radial of 150-mm. Agar plate has been inoculated with a standardized organism suspension. The plate is incubated overnight and the result is read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip.

This method is best suited to situations in which MIC of only 1 or 2 drugs are needed or when a fastidious organism requiring enriched medium or special incubation atmosphere is tested, such as penicillin and ceftriaxone with pneumococci. Etest results agree well with MICs generated by broth or agar dilution methods (Baker et al., 1991; Rennie, Turnbull and Brosnikoff, 2008; Jorgensen and Ferraro, 2009).

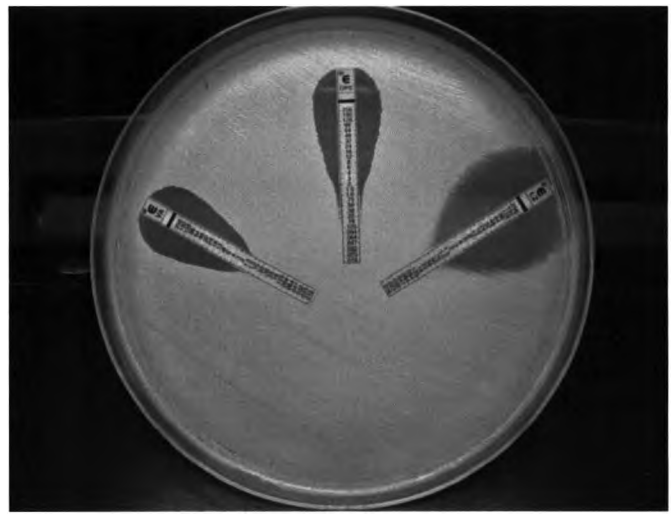

Figure 3 A *Staphylococcus aureus* isolate tested by the Etest gradient diffusion method with vancomycin (VA), daptomycin (DM), and linezolid (LZ) on Mueller-Hinton agar (Jorgensen and Ferraro, 2009).

3.4 Disk diffusion test or disk diffusion susceptibility method is simple and practical and has been well standardized. This test is performed on surface of a large (150 mm. diameter) Mueller-Hinton agar plate applied bacterial inoculums of approximately 1–2 x 10^8 CFU/mL. Paper disks are prepared at fixed antibiotic concentration. The paper antibiotic disks are placed on the inoculated agar surface (Figure 4). The plate is incubated for 16 -24 hours at 35 $^{\circ}$C prior to determination. Zones of growth inhibition around each of the antibiotic disk are measured. Diameters of the zones are correlated to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) or those included in the US Food and Drug Administration (FDA). The results of the disk diffusion test are qualitatively determined (i.e., susceptible, intermediate, or resistant). The advantages of the disk method include the test simplicity, the results easily interpreted by all clinicians and flexibility in disk selection for testing. The disadvantages of the disk test include the lack of automation of the test. Not

slow growing bacteria can be accurately tested by this method. The disk diffusion test has been standardized for testing *streptococci*, *Haemophilus influenzae,* and *Neisseria meningitidis* through the use of specialized media, incubation conditions, and specific zone size interpretive criteria (CLSI, 2011).

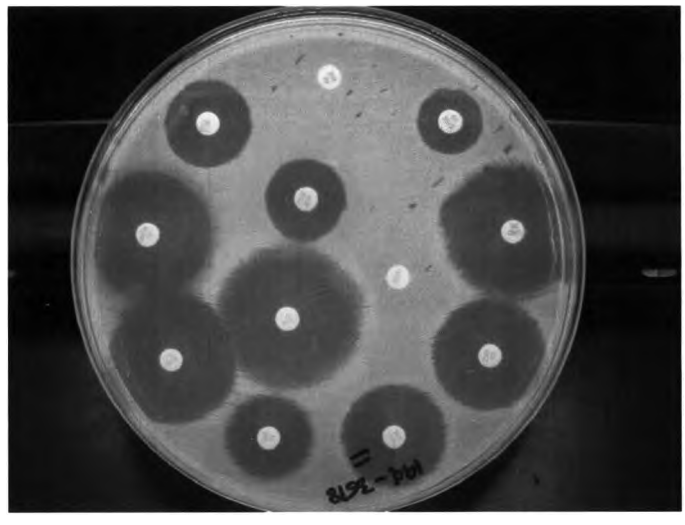

Figure 4 A disk diffusion test with an isolate of *Escherichia coli* from a urine culture (Jorgensen and Ferraro, 2009).

3.5 Automated instrument systems usually produce susceptibility test results in a shorter time period than manual readings because sensitive optical detection systems allow detection of subtle changes in bacterial growth. In the United States, there are 4 automated instruments presently approved by the FDA. MicroScan WalkAway® (Siemens Healthcare Diagnostics), BD Phoenix Automated Microbiology System® (BD Diagnostics) and Vitek 2 System® (bioMe rieux) can generate rapid (3.5–16 hours) susceptibility test results, while Sensititre ARIS 2X® (Trek Diagnostic Systems) is an overnight system. All instruments have been enhanced computer software used to interpret susceptibility results (Figure 5) (Richter and Ferraro, 2007).

Figure 5 Automated instrument systems are (A) BD Phoenix Automated Microbiology System®[a], (B) Vitek 2 System®[b], (C) MicroScan WalkAway®[c], (D) Sensititre ARIS 2X®[d].

[a] http://www.bd.com/aboutbd/global/images/BD_Phoenix.jpg

[b] http://www.biomerieux-usa.com/upload/Susceptibility_Testing1.jpg

[c] http://gurmed.com/TR/wp-content/uploads/2012/08/LabPro-Information Manager.jpg

[d] http://www.trekds.com/products/sensititre/images/arisswin.jpg

4. Methods used to assess the activity of antimicrobial combinations

Synergy is a possible advantage of antimicrobial combination therapy for bacterial infections. Various methods used to investigate in vitro synergy between antibiotics have been explained. Checkerboard and time-kill tests are the most widely used techniques. Furthermore, there are other two methods such as diffusion method and Epsilometer method (Etest). Previous studies have described synergy tests and compared among the time-kill, checkerboard tests and Etest method using trovafloxacin or tobramycin plus cefepime or piperacillin against *Acinetobacter baumanni*. The results showed that synergy

is detected using the time-kill test and checkerboard test but is not detected using Etest method (Bonapace et al., 2000).

4.1 Checkerboard arrays (Pillai, Moellering and Eliopoulos, 2005)

The checkerboard method, a gauge of inhibitory activity, is the most widespread technique for evaluating the synergistic activity of two-drug combinations because it is also easy to calculate and interpret data. Moreover, equipment required for this method is normally available in ordinary microbiology laboratory. The checkerboard method can be done in tubes, microdilution wells or agar plates. Two drugs are diluted and mixed at many ratios and concentrations (upper, lower or equal to the MICs of both drugs). The drugs are commonly diluted in Mueller-Hinton broth or another suitable broth. After that, bacteria are transferred to broth tubes, broth wells or broth plates and then the broths are incubated at 37 $^{\circ}$C for 24 hours. Indicator of synergistic activity is determined as fractional inhibitory concentration (FIC) index which is the sum of the lowest FIC of each individual drug. The FIC index can be calculated as:

$$\text{FIC index} = FIC_A + FIC_B = \frac{(A)}{(MIC_A)} + \frac{(B)}{(MIC_B)}$$

Whereas; (A) = the concentration of drug A

(B) = the concentration of drug B

(MIC_A) = the MIC of drug A alone

(MIC_B) = the MIC of drug B alone

FIC index = the total of fractional inhibitory concentration of drug A and drug B

FIC_A = the fractional inhibitory concentration of drug A

FIC_B = the fractional inhibitory concentration of drug B

If the FIC index is not more than 0.5, the drugs are synergy. The FIC index is higher than 0.5, but not more than 2, those drugs are indifferent or additivity. If the FIC index is higher than 2, they are antagonism. The checkerboard assay has been categorized to be 2 methods including broth dilution method and agar dilution method.

4.1.1 Broth dilution method (Bonapace et al., 2000; Pillai et al., 2005)

Broth dilution method is done in tubes or 96 well plates. The stock solution of each drug is diluted in broth by two-fold serial dilution. The diluted series are dispensed into tubes or wells as two dimensional patterns. The diluted series of drugs are divided into four parts. The first part, the diluted series of drug A, is dispensed to the wells along the y-axis. The second, the diluted series of drug B, is dispensed to the wells along the x-axis. The rest two parts lay between X and Y axes where A and B drug combinations are prepared and filled in the wells. The concentration of each drug in the combinations is presented on the X and Y axes. The inoculum at the final concentration, 2×10^5 CFU/mL, is dispensed into all wells. The plate is covered with lid and incubated at 37 $^{\circ}$C for 24 hours. After that, the bacterial growth is assessed by turbidity of the broth. In some experiments, a bacterial growth indicator is mixed in the broth to enhance the convenience of result detection. If the drugs can inhibit the bacterial growth, characteristic of the wells will not change. In contrast, if the drugs cannot inhibit the growth, the well will become turbid. Finally, the synergistic activities of those drugs are determined using FIC index.

4.1.2 Agar dilution method

Agar dilution method is done in agar plates. Each drug is diluted as two-fold serial dilution to give a desired concentration. After that, the drugs are homogeneously mixed with a Mueller-Hinton broth or another suitable broth and poured into the plates. The prepared inoculums, 4×10^5 CFU, are transferred to the agar surface

using replicating device. The agar plates are incubated at 37 °C for 24 hours. The growth inhibitor can be determined by the spread of colony on the agar surface. Finally, the synergistic activities of those drugs are determined using FIC index. The checkerboard test is a relatively easy test to perform but the interpretation has been limited to the FIC index calculation. The checkerboard method, as usually performed, provides responses only all-or-nothing such as growth or no growth and is thereby incapable of measuring the graded responses in order to define dose-response relationship between an antimicrobial and microorganism. Moreover, the checkerboard method typically provides a static rather than a dynamic, view of antimicrobial interaction, since the results are usually examined only at one point of time. Despite these limitations, the checkerboard technique remains a widely used technique to evaluate antimicrobial combinations.

Checkerboard method also provides the advantages in the sense of convenient detection by observing colonies of bacterial growth in blood agar with naked eyes. Moreover, various strains of bacteria can be detected within the single plate at one time, thereby diminishing cost and time used in an experiment.

4.2 Killing Curves (Time-Kill Curves or Time-Kill Plots) (Pillai et al., 2005)

Killing curve technique is one of the methods for determining the bactericidal activities of antibiotics. Bacteria are cultured in the conditions that contain antibiotics at desired concentrations. Then, the viable cell counts are determined in each period. Finally, the viable cell counts are plotted against time on semi-logarithmic paper. The results are interpreted using the effectiveness of the drug combination to compare with the most active single drug alone. Synergism is defined when the viable cell counts are reduced at least 100-fold at 24 hours. Antagonism is defined when the viable cell counts are increased at least 100-fold at 24 hours.

The disadvantage of the killing-curve technique is that the amount of antimicrobial combinations being tested is earnestly limited by the repetitious sampling essential for each flask and the multiple colony counts required (Zelenitsky et al., 1999).

4.3 Diffusion methods (Pillai et al., 2005)

Diffusion methods are easy to perform. Tested bacteria are cultured at 0.5 McFarland and applied on surface of agar plates by sterile swabs. Filter paper disks or paper strips, soaked with various antimicrobials, are placed on the agar surface and then the plates are incubated at 37 °C for 18 to 24 hours. The interpretation is done by measuring clear zone or inhibition zone around the disks or the strips and also observing shape of the clear zone. The clear zone is generated by the antimicrobial activities of the drugs on the disks or the strips to destroy the bacteria or inhibit bacterial growth. Disk diffusion technique is called when filter paper disks are utilized in the test. Paper strips are used in paper strip diffusion technique. However, cellophane or membrane filter can be used instead of the paper strips. The interpretations of these two techniques are shown in Figure 6 and 7. The results of the antimicrobial combination (drug A and drug B) determined to be 1) additive, indifferent or independent effect 2) synergistic effect and 3) antagonistic effect.

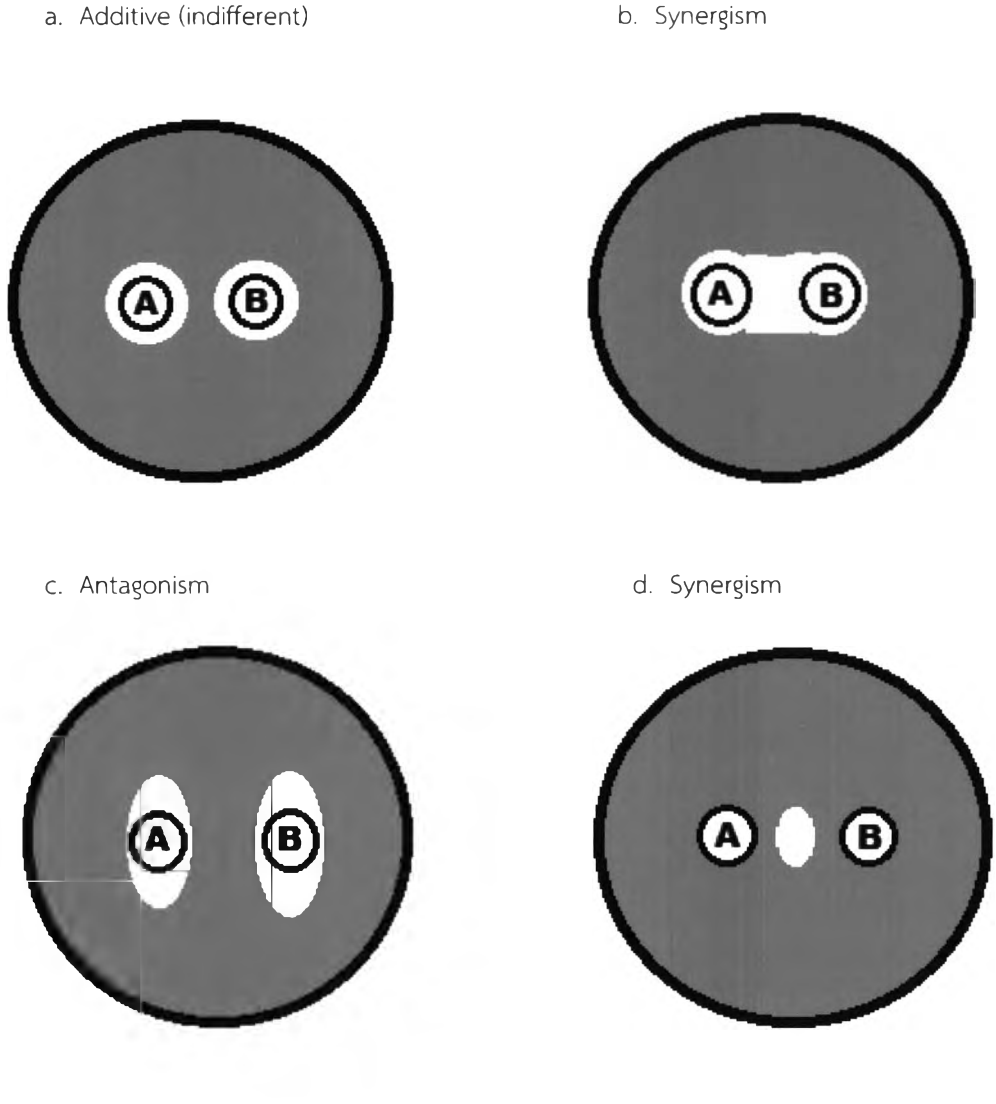

Figure 6 Assessment of antimicrobial combinations with the disk diffusion technique, using disks containing antimicrobial A or B. Red color is bacterial growth and clear area is zone of growth inhibition; a: additive, b and d: synergism, c: antagonism (Pillai et al., 2005).

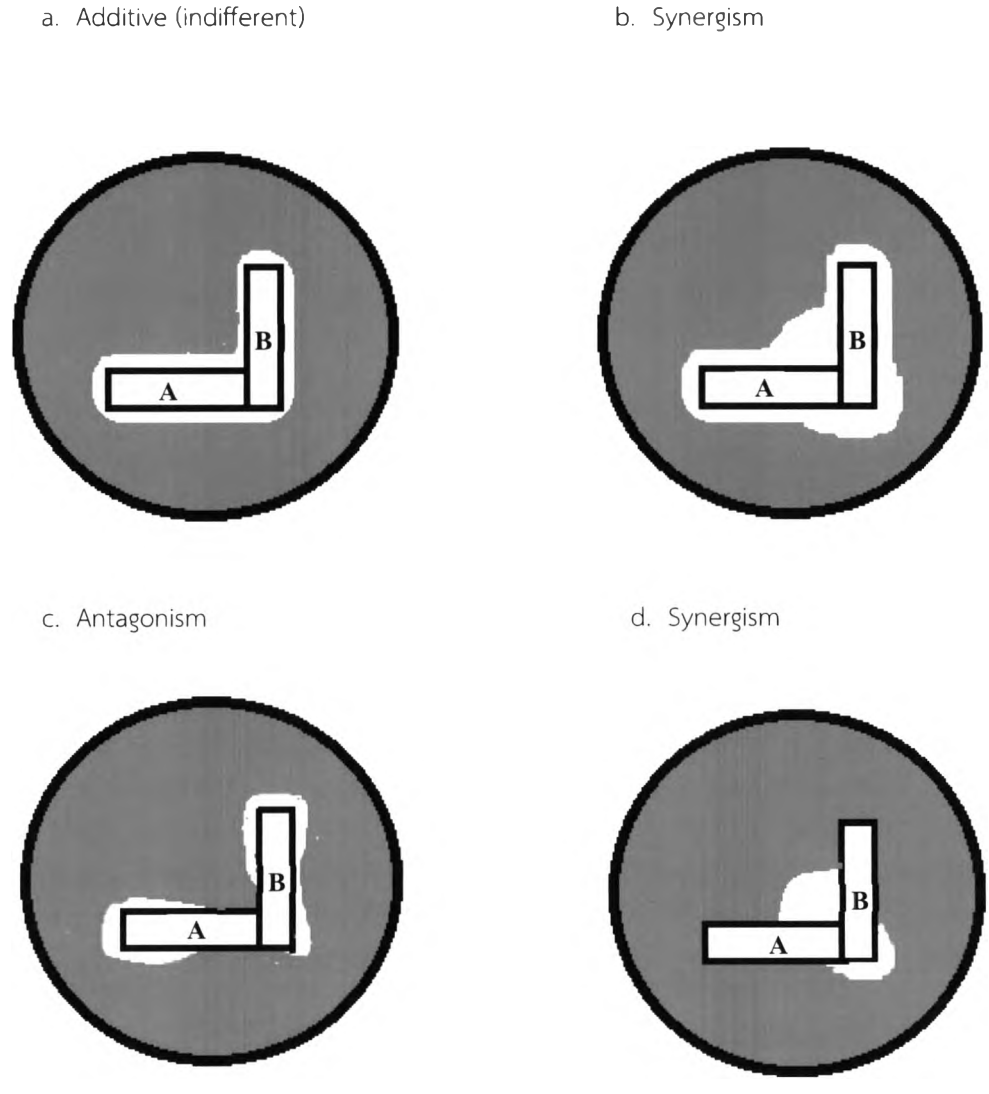

Figure 7 Assessment of antimicrobial combinations with the paper strip diffusion technique, using paper strips containing antimicrobial A or B. Red color is bacterial growth and clear area is zone of growth inhibition; a: additive, b and d: synergism, c: antagonism (Pillai et al., 2005).

5. Suspensions

A pharmaceutical suspension is a coarse dispersion of insoluble solid or drug particles dispersed in liquid medium. Some injectable drugs have been prepared in suspensions. Reasons to prepare in parenteral suspensions are to solve problems of

insoluble drugs which are difficult to prepare in solution dosage form, increase drugs stabilities compared to solution dosage form and control or prolong release of the drugs.

There are factors affecting design of parenteral suspensions. The parenteral agreeable excipients are limited such as vehicles, preservatives and wetting agents. Viscosity affects ease of injection and release of active ingredients. Particle size of the drugs influences stability and dissolution of the preparations.

5.1 Typical excipients used in parenteral suspensions include following

5.1.1 Wetting agents

Wetting agents reduce contact angle between surface of the particles and liquid medium to obtain maximum wetting efficiency. Various nonionic surfactants and non-aqueous solvents including glycerin, alcohol and propylene glycol are types of wetting agents commonly used in injectable suspensions. Certain surfactants have been used in the preparations such as lecithin, polysorbate 20, polysorbate 80, pluronic F-68, and sorbitan trioleate (Span 85). It is found that polysorbate 80 is added in cefazolin sodium suspension, prepared as a non-aqueous suspension in peanut oil. At the concentration of polysorbate 80 greater than 0.17%, the deflocculated suspension, which is difficult to redisperse, is obtained. Microscopic examination reveals extensive agglomeration and crystal growth of cefazolin sodium. Therefore, care should be taken in terms of the amount used; excessive amounts may cause foaming or caking or provide an undesirable odor to the product (Quay and Stucky, 1989; Yotsombut, 2011; Lieberman, Rieger and Banker, 1996).

5.1.2 Suspending agents or viscosity inducing agents

Suspending agents are used to increase the stability of suspensions and prevent caking at the bottom. One of the properties of well-formulated suspensions is that they can be easily resuspended by the use of moderate agitation. There are commonly

used of viscosity inducing agents such as tragacant, sodium carboxymethyl cellulose (SCMC), bentonite, aluminum monstearate, gelatin (nomantigenic), povidone, etc (Yotsombut, 2011). Charges on the viscosity inducing agents are one of criteria to choose. Normally, the charges of suspending agents are the same as that of flocculating agents in case of flocculated suspensions or the same as that of drug particles in case of no flocculating agent added. Non-ionic suspending agents can be used in both cases. Suspending agents are used in injectable suspension formulations in the market. For example, sodium CMC is added in dexamethasone (Decadron®) and triamcinolone acetonide (Kenalog®). PEG 3350 is added in Depo-provera®.

5.1.3 Types of vehicles

Types of vehicles used in parenteral suspension are aqueous and non-aqueous vehicles. Choice of vehicles depends on solubility, stability and desired release characteristics of the drug. Aqueous vehicles are used in most of injectable products due to the physiological compatibility of water with biological tissues.

In parenteral suspensions, non-aqueous vehicles are water-immiscible liquid including fixed oil (ethyl oleate isopropyl myristate and benzyl benzoate) and vegetable oil (sesame, peanut, corn, olive, castor, and cottonseed). Parenteral suspensions in non-aqueous vehicle are usually administered to subcutaneous and intramuscular routes. Excessive unsaturation of oil can lead to tissue irritation. Some patients have exhibited allergic reaction to a certain vegetable oil (Patel, 2010; Lieberman et al., 1996).

5.1.4 Flocculating agents

Flocculating agents allow the formation of floccules or clusters as particles are held loosely together by weak van der Waals forces. Since the particles are combined together only loosely, they will not cake and may be easily redispersed by

shaking the suspension. There are types of flocculating agents, such as hydrophilic colloids, surfactants or electrolytes (Patel, 2010; Yotsombut, 2011).

5.1.5 Tonicity Agents

Tonicity agents are usually added in aqueous parenteral suspensions. Isotonicity of the parenteral preparation for subcutaneous or intramuscular administration is desired to prevent pain, irritation and tissue damage at the site of administration. Tonicity agents used in aqueous parenteral suspensions include dextrose and various electrolytes (Patel, 2010; Lieberman et al., 1996).

5.1.6 Preservatives

Preservatives protect the product from accidental microbial contamination during clinical usage and maintain sterility. Preservatives should be added into preparations packaged in multiple-dose containers unless prohibited by compendia monograph. There are many preservatives used in parenteral suspensions, such as benzyl alcohol (0.9%w/w to 1.5%w/w), methylparaben (0.18%w/w to 0.2%w/w) propylparaben (0.02%w/w) benzalkonium chloride (0.01%w/w to 0.02%w/w), thimerosal (0.001%w/w to 0.01%w/w) and chlorobutanol. Benzyl alcohol is effective against most gram-positive bacteria, yeast and mold, but is less effective against gram-negative bacteria (Smolinske, 1992). Convulsions in neonates are caused by the benzyl alcohol. Therefore, the used benzyl alcohol in certain drug product with neonatal indications should be avoided. Parabens have a broad spectrum of antimicrobial activity at a pH range of 4-8, but are more effective against yeast and mold when compared with bacteria (Haag and Lincrini, 1984). Aqueous solutions of parabens are stable at a pH range of 3-6, but they are degraded by hydrolysis at pH greater than 8 (Smolinske, 1992). Most antimicrobial preservatives and antioxidants are known to volatilize or adsorb to rubber closures. This

can lead to loss of sterility, degradation and potential problems with flocculation and resuspendability of the product (Patel, 2010).

5.1.7 Antioxidants/chelating agents

Antioxidants are added in the preparation to prevent oxidation reactions between the drug and free radicals as a result to increase the stability of the drug and reduce discoloration of the drug product. Antioxidants used are ascorbic acid, cysteine, sodium bisulfate, sodium metabisulfate, tocopherols and monothioglycerol. An alternative way to prevent oxidation of a drug is purging of inert gas (nitrogen) to fill head space (Patel, 2010).

5.2 Evaluation and stability of parenteral suspension

5.2.1 Particle size measurements and variable particle size distribution

The particle size and particle size distribution of suspensions play an important role on their behavior such as physical appearance, stability, syringeability and release rate as well as their efficacy (Akers, Fites and Robison, 1987). Crystal growth in suspension is affected by particle size distribution. Fine particles may dissolve and consequently recrystallize on large particles leading to larger particles. This observation is known as Oswald ripening. Particle size measurement is useful to evaluate aggregation or crystal growth. The particles in suspensions can be measured using several methods such as microscopic determination, Andersen pipette, subsieve sizer, turbidimetry and photon correlation spectroscopy. Microscopic determination is more popular than Andersen pipette or subsieve sizer and turbidimetry. Malvern particle size analyzer (photon correlation spectroscopy) can measure very fine particles (< 10 μm in diameter). Coulter Multisizer and HIAC/Royco particulate counter are used for particle size measurements and size characterization of reconstituted, lyophilized and attenuated *mycobacterium bovis*. For intravenous injections, particle should be less than 1 μm in diameter. For

subcutaneous or intramuscular injections, the particle should preferably be less than 250 μm in diameter. Larger particle sizes can be used for oral formulations. Particle size distribution also affects stability of suspensions. A large particle size distribution accelerates agglomeration and coagulation of the particles that may lead to instability of the formulation (Patel, 2010).

5.2.2 Viscosity explains resistance to flow of a particular system with applied stress. A more viscous system requires greater force or shear stress to make it flow at the same shear rate as a less viscous system. An ideal suspension should present a high viscosity at low shear. The suspension viscosity can be changed due to concentration of active ingredient(s), particle shape, size and size distribution. Many techniques have been used to measure viscosity, such as capillary viscometers, falling ball viscometers, cup-and-bob viscometers and cone-and-plate viscometers. Capillary viscometers and falling ball viscometers are simple instruments for measuring viscosity, but only for Newtonian liquids. Rotational viscometers including to cup-and-bob viscometers and cone-and-plate sensor systems are instruments for measuring Newtonian and non-Newtonian liquids (Leblanc, Secco and Kostic, 1999). Viscosity of parenteral suspensions affects ease of injection (กรีพล, 2008; Patel, 2010).

5.2.3 Redispersibility explains the ease of suspensions to be resuspended by gentle shake after the suspension has stood for sometimes. When a suspension is very well shaken the particles should be evenly dispersed throughout the suspension. If the sediment is dense and difficult or impossible to redisperse, this situation can lead to caking in the suspension (Patel, 2010).

5.2.4 Sedimentation volume is a qualitative term used to explain the amount of settling that has occurred in a suspension. The sedimentation volume of the suspension is determined by measuring the volume of the sediment in the suspension

placed in a measuring cylinder. The sedimentation volume is calculated from the ratio of the final volume of the sediment, Vu, to the total volume of the suspension, V_O (Equation 1). Sedimentation volume is used to evaluate the changes in suspension characteristics with time and also to compare different suspension formulations. Normally, the value of sedimentation volume ranges from 0 to 1. If the sedimentation volume is closer to 1, the suspension becomes more acceptable due to the loose and porous of the sediment. When the sedimentation volume is equal to 1, no sedimentation is observed considered as an ideal suspension.

The sedimentation volume (F) was calculated using the formula

$$F = V_u / V_o \qquad \text{..... Equation 1}$$

Whereas, V_u = final volume of sedimentation and

V_o = total volume of sediment before settling occurred.

Degree of flocculation: It is a qualitative expression of flocculation. Degree of flocculation (β) is expressed as

$$\beta = F / F_\beta \qquad \text{..... Equation 2}$$

Whereas, F = ultimate sedimentation volume in flocculated suspension and

F_β = ultimate sedimentation volume in deflocculated suspension.

High β value indicates that sediment is loosely held together. The minimum value of β is 1; this is the case when the sedimentation volume of the flocculated suspension is equal to the sedimentation volume of deflocculated suspension (กรีพล, 2008; Patel, 2010; Yotsombut, 2011).

5.2.5 Freeze -Thaw cycle test is important for determining the capability of the suspension to resist thermal shock, hinder crystal growth and maintain chemical stability of the active ingredient and overall physical stability. The freeze-thaw cycle test may predict long term storage at room temperature (Patel, 2010).

5.2.6 Crystal Growth The crystal growth in suspension is influenced by the particle size distribution, changes in pH, temperature, freeze-thaw cycles, solvate formation, dissolution and recrystallization of the particles. Crystal growth should be observed by examining changes in particle size over time and comparing that with the initial particle size distribution. The inclination for crystal growth in a suspension can be diminished by using a narrow particle size range, decreasing interfacial tension (to reduce the free energy of particle), increasing the viscosity of dispersion medium (may be difficult for parenteral suspensions as it affects the syringeability and flow), use of hydrophilic gums like polyvinylpyrrolidone, polysorbates (to adsorb at particle surface and retard crystal growth) and choosing a different chemical form of the drug (Patel, 2010).

6. *In vitro* release study

In vitro release methods for parenteral depot formulations have been well reviewed (Gido et al., 1993; D'Souza, Faraj and DeLuca, 2005; D'Souza and DeLuca, 2006; Iyer, Barr and Karnes, 2006; Giteau et al., 2008). Generally, three methods have been used to evaluate *in vitro* release of parenteral depot formulations such as separation methods, flow-through cells and dialysis techniques. Each has certain advantages and limitations. There are currently no regulatory standards for *in vitro* release testing of parenteral depot formulations, and available compendia apparatus has not been designed for this purpose (D'Souza and DeLuca, 2006). Separation technique is the simplest and appears to be the most widely utilized. A dosage form is paced in a vessel along with a specified volume of release medium. The medium is assayed for drug content at specified time points and

fresh medium is returned to the vessel. Flow-through method allows for maintenance of sink conditions, but is more complex and potentially less robust. Dialysis technique may provide the best approximation of the confined environment at the injection site and couple with sink conditions in the bulk release medium. Moreover, the selection of release media is another important aspect of method development. Many reports specified phosphate buffered saline (PBS) at 37 $^{\circ}$C as the release medium representing *in vivo* condition, although in some cases media with different pH, ionic strength or protein content are more appropriate. Media volume is a critical variable, particularly for drugs with solubility limitation where sink conditions may not be achieved. Media should be selected on a case-by-case basis based on the properties of the actives and the formulations (D'Souza and DeLuca, 2006).