CHAPTER II

LITERATURE REVIEWS

Characteristics of *P. pseudomallei*

P. pseudomallei is a gram-negative rod, nonacid-fast, non-spore-bearing, characteristic bipolar staining, and obligatory aerobic bacterium. This organism possesses polar flagella and is capable of oxidizing carbohydrate substrates. The DNA shows the highest molar percentage of guanine plus cytosine (GC,67-70%) of any of pseudomonads (24,25). Colonies are round, smooth, wrinkled and more wrinkled, heaped up appearence on longer incubation. *P. pseudomallei* is capable of producing an insoluble pigment that causes a brown color to colonies and sightly aromatic odor associated with pseudomonads. On blood agar, partially hemolysis is seen after 48-hr growth . The four types of antigen were demonstrated in *P. pseudomallei*; an envelope antigen (K), the soluble antigen, the flagellar antigen and the somatic antigen (O) (6).

Pathogenesis and virulence

The pathogenesis of melioidosis is still a mysterious problem. According to histopathological studies (8), some patients have suppurative lesions and others have granulomatous lesions. The type of reaction may be related to the duration of the illness, the degree

of immunity, the virulence of the organism or the number of infecting organism.

Melioidosis may exist as an inapparent infection , then suddenly emerges at a later time as an acute or chronic infection. Histopathological studies tissue from animals with experimental pulmonary melioidosis (26) showed that animals with acute infection had nodules of caseation and necrosis, with hemorrhage primarily in lungs; while animals with chronic infection had metastatic areas of caseous necrosis and granulation, large progressing abscesses . Tissue necrosis is a regular feature in both acute and chronic melioidosis. The cause of necrosis is not known, but it may be due to either vascular damage or to the direct action of bacterial enzymes (8,26).

The studies of Rappaport *et al.* (9) demonstrated an endotoxic substance from *P. pseudomallei* which was heat-stable and similar to the classical endotoxin in its biological and physiological properties . It elicited a dermal hemorrhagic necrosis in rabbits.

Nigg *et al.* (10) showed that the mice were dead after inoculation with *P. pseudomallei*, but no gross lesions have been found. Moreover, the cell-free filtrate of the organism caused skin necrosis in guinea pig with intradermal inoculation. The causes of death and tissue necrosis were suggested to be from the heat-labile lethal toxin and necrotoxin, respectively (27,28). The necrosis might be caused by proteolytic enzyme, since necrotoxin preparation posessed proteolytic activity , whereas the lethal toxin was separable

from the proteolytic enzyme by differentiation inactivation (11).

Liu (29) demonstrated that culture supernatant with hemolytic activity ,which was prepared by the cellophane plate technique, could kill mice after intraperitoneal injection. Later, Ashdown *et al.*(12) determined the prevalence of virulence factors of *P. pseudomallei* by screening 100 clinical isolates for extracellular enzymes. It was found that 97 strains produced licithinase and hemolysin. Ninety-six and ninety-four strains produced lipase and protease, respectively; but none produced elastase.

In recent years, Yang *et al.* (14,15) studied *P. pseudomallei* siderophore, named malleobactin, which can uptake iron from host proteins such as transferrin and lactoferrin to replicate and maintain infection in host tissue. In addition, resistance to bactericidal action of normal human serum (30) and survival in human phagocyte (31) may be an important determinant in contributing to virulence and pathogenesis.

Hemolysin

Hemolysin is an extracellular substance which lyses red blood cell and differentiated eukaryotic cell (16). Bacterial hemolysins produced by a large variety of gram-positive and gram-negative bacteria are often found associated with clinical isolates. They are considered to be factors contributing to virulence. For example,thiol-activated hemolysin of several gram-positive bacteria include strepto-

lysin O, pneumolysin, listeriolysin O, cerolysin (32). *Actinobacillus pleuropneumoniae* produced heat-labile hemolysin (33). *P. aeruginosa* generated two kinds of hemolysins; one is a heat-stable glycolipid (34), and the others are heat-labile hemolytic phospholipase C (PLC-H) and nonhemolytic phospholipase (PLC-N) functioned cooperatively with alkaline phosphatase under phosphate regulation (21,35-36). The two hemolysin determinants of *Proteus sp.* were demonstrated (37). One is similar to the *E. coli* hemolysin (HlyA) and has a Ca^{2+}-dependent activity. The other, found only in *Proteus* isolates, has a Ca^{2+}-independent activity.

Ashdown *et al.* (12) demonstrated the production of hemolysin from clinical isolates of *P. pseudomallei* and noted that there were at least two kinds of hemolysins. One was heat-stable hemolysin which produced alpha hemolysis (27). It was weakly cytolytic and seen only as small hemolytic zones around heavy growth on sheep blood agar. The other was heat-labile hemolysin which had strongly cytolytic actvity and was capable of producing alpha-hemolysis around well-separated individual colonies on sheep blood agar. The latter hemolysin was cytolytic against many kinds of erythrocytes, with human erythrocytes being the most susceptible to its activity. The hemolytic actvity was most active in an acid environment (pH 5.5) and inhibited by sterols, particularly cholesterol and 7-dehydrocholesterol. Kongcharoensuntorn (23) determined hemolytic activity on human blood agar of 13 clinical isolates and 2 reference strains of *P. pseudomallei* by cellophane plate technique (29). It was shown that hemolytic activty was ranging from 2 to 256 hemolytic units (HU)/ml .

Mechanisms of action of hemolysin against red blood cells (RBCs)

A mechanism common to several hemolysins of gram-negative bacteria is distruption of the target cell membrane by the formation of defined sized transmembrane pores (38) since they interacted with sugars or lipids which could represent the membrane receptors on target cell membrane (39). These pores allowed dissipation of target cell transmembrane ion gradients but retained cytoplasmic protein within the cell, resulting in increased intracellular colloid-osmotic pressure due to water moving into the cell. Consequently, the RBCs swelled and lysed. For example, *E. coli* hemolysin created transmembrane pores of approximately 3 nm effective diameter estimated by using osmotic protection experiments (40). It induced rapid efflux of cellular K^+ and influx of Ca^{2+} followed by cell swelling and hemolysis. For *Moraxella bovis* hemolysin , transmembrane pores in bovine RBCs, approximately 0.9 nm, occurred by the same mechanism as described above (41).

Factors affecting the action of hemolysin

The acitivity of bacterial pore-forming hemolysin is affected by divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}. For example, *E. coli* hemolysin required Ca^{2+} for induction of hemolysis (42). Additionally, Ca^{2+} can enhance cytolysis in *M. bovis* hemolysin action (41).

The hemolytic activity also depends on temperature. Temperatures ranging from 25°c to 37°c are optimal to produce the hemolysis, probably because these temperatures contribute to the stability of the lytic components (39). Moreover, it appeared that pH (12), assay time and hemolysin concentration affected on its action (43). Under various conditions, the pore sizes with diameter ranging from 0.6 to 1.2 nm due to the action of *E. coli* hemolysin could be inferred (43).

In addition, hemolysin is sensitive to some substances. For example, *E. coli* hemolysin is sensitive to lipase such as phospholipase A_2 and phospholipase C. They inactivated hemolysin without affecting the size of hemolysin protein (16). Hemolysins of gram-positive bacteria are usually activated by thiol-reducing agents and inactivated by sterols (32).

Role(s) of hemolysin in pathogenesis

Bacterial hemolysins have been studied and it was found that they play a significant role in pathogenesis although its mechanism remains unclear. For example, *E. coli* strains were isolated from all sites of extraintestinal infection are frequently hemolytic (ranging from 35 to 59%) (44,45). In contrast, the incidence of hemolytic *E. coli* in the feces of normal persons has been estimated to be from 8 to 18% (45,47). Similarly, *Vibrio parahemolyticus* (48) as well as *Aeromonas hydrophila* (49) causing the infection were mostly hemolytic strains.

Welch *et al.* (17) transformed plasmid carrying hemolysin gene into avirulent non-hemolytic faecal isolates of *E. coli*. The high mortality of rat was observed after receiving intraperitoneal injection of this strain. On the other hand, no rat death was found when non-hemolytic *E. coli* which had plasmid carrying mutated hemolysin gene was injected. It was shown that hemolysin can enhance the virulence of extraintestinal *E. coli* infection (17). Moreover, Hacker *et al.* (50) determined different levels of toxicity in mice. It was found that hemolysin was also associated with high levels of toxicity for mice after intraperitoneal injection.

In the study of correlation between the hemolytic activity of *P. mirabilis* and the invasive ability suggested that the bacterial hemolysin may be involved in the invasion process (19). From determination of 50% lethal dose (LD50) in mice, the aerolysin-negative derivative had full toxicity when it was reintroduced the wild-type *aer* determinant. Additionally, this organism was isolated from the spleens and livers of the dead mice as well as the detection of antibody to aerolysin (16). *L. monocytogenes* expressing listeriolysin O and *Clostridium perfringens* expressing perfrinolysin O can disrupt the phagosomal membrane and grow within macrophage to escape from host immune system using *Bacillus sultilis* as a model in study (20,51). The PLC fraction from *P. aeruginosa* an cause paralysis, dermonecrosis, vascular permeability, and death in mice (52).

Molecular study of bacterial hemolysins

The introduction of molecular techniques is useful for detailed studies of hemolysin synthesis and transport in a number of bacteria. The isolation of the genes and their gene products, in conjuction with the study of animal models, has helped to elucidate the contribution of hemolysin to the virulence of hemolytic strains. The studies of hemolysin have been performed in the followings:

Hemolysin from *E. coli*

Hemolysin (*hly*) determinants of *E. coli* are located either on the chromosome or on the plasmid (45). The plasmid-encoded *hly* determinant is flanked by insertion sequence (IS), whereas the chromosomal-encoded *hly* determinant may be located on larger chromosomal inserts that carry short, directly repeated sequences at their ends. Both determinants are highly homologous (53) and consist of four cistrons, designted as *hly*C, *hly*A, *hly*B, and *hly*D, respectively (16,32,54,55). These four *hly* genes are controlled by at least two promoters: one promoter is located proximal to *hly*C for the transcription of *hly*C, *hly*A and *hly*B. A second promotor initiates the transcription of *hly*D. By using transposon mutagenesis, it can be noted that HlyC and HlyA are essential for the synthesis and the transport of active hemolysin across the cytoplasmic membrane, whereas HlyB, HlyD determine the transport of hemolysin across the outer membrane (32,56). Active hemolysin is 58 kilodaltons (kDa) derived from the 106 kDa precursor in transportation. During analysis of plasmid-

encoded hemolysin, the 600-bp sequence *hly*R was characterized. It is located upstream of *hly*C and essential for the enhancement of synthesis and transport of hemolysin (57). Similarly, the *hha* gene was identified by mutation in the *E. coli* chromosome which resulted in high expression of hemolysin (58,59). However, the regulation of hemolysin gene expression in *E. coli* are unclear and required for further study.

Hemolysin from *P. mirabilis*

Welch (60) identified two different hemolysin determinants which were molecularly cloned . One (*hpm*A) is similar to *E. coli* hemolysin (HlyA) and has a Ca^{2+}-dependent activity. The second hemolysin (*hpm*B), Ca^{2+}-independent hemolysin, only found in *Proteus* sp. This gene was cloned from *P. mirabilis* strain 477-12 into pUC19 (37). The DNA sequence of a 7,191-bp region was determined by using subclones of recombinant plasmid harbored *hpm*B and *hpm*A. It revealed two open reading frames (ORFs) encoding polypeptides of 63 kDa (HpmB) and 166 kDa (HpmA) by using *in vitro* transcription-translation and immunoblotting. In addition, it was shown that HpmB is necessary for the extracellular secretion and hemolytic activity of the structural hemolysin HpmA.

Hemolysin from *Serratia marcescens*

A 7300-bp fragment of DNA encoding hemolytic actvity was identified and sequenced by using chain termination method (61).

It consisted of *shl*A ORF and *shl*B ORF encoding polypeptides of 165 kDa and 62 kDa, respectively. Both were required for hemolytic activity. Uphoff and Welch (37) revealed significant nucleotide identity (52.1%) was seen between *shl* and *hpm* hemolysin gene sequences of *P. mirabilis*. The predicted amino acid sequences of ShlB and ShlA are also similar to those of HpmB and HpmA, the respective sequence identities being 55.4% and 46.7%.

Hemolysin from *Streptococcus pneumoniae*

Walker *et al.* (62) isolated a recombinant lambda bacteriophage which carried pneumolysin gene from *S. pneumoniae* serotype II (NCTC 7466) and this insert of 1650 bp was subcloned and sequenced by using the dideoxy chain termination method. The sequence revealed two ORFs which encoded two polypeptides of 56 kDa and 53 kDa observed in the *in vitro* transcription-translation system. The predicted amino acid sequence of the polypeptide revealed a single cysteine residues located at 44 residues from the C-terminus.

Hemolysin from *P. aeruginosa*

Vasil *et al.* (35) had cloned and mapped phospholipase C (heat-labile hemolysin) gene of *P. aeruginosa* PAO1 in pBR322. This gene encoding 78-kDa hemolytic phospholipase C (PLC-H) was subsequently sequenced by Pritchard and Vasil (63). By analysis of an insertional mutation, Ostroff *et al.* (36) found a 77-kDa non-hemolytic phospholipase C (PLC-N) of which the amino acid sequence

was 40% identical to that of PLC-H. For the predicted isoelectric points (pIs) of these two proteins (64), PLC-H is an acidic protein (pI 5.5) whereas PLC-N is a basic protein (pI 8.8).

Hemolysin from *P. cepacia*

In the study of *P. cepacia* hemolysin, a hemolytic PLC gene was identified by using a gene specific fragment from the *plc*-H gene of *P. aeruginosa* as probe (65). It was found that two closely linked genes encoding a 77-kDa and a 22-kDa protein. These two proteins were required for expression of hemolytic PLC activity in *E. coli*.

Hemolysin from *P. pseudomallei*

The gene encoding the hemolysin determinant was cloned from *P. pseudomallei* strain K1/88 in pUC18 and screened for hemolytic activity by cellophane plate technique (23). It was found three transformants harbored recombinant plasmids pWC3, pWC7 and pWD1 of which the inserts are approximately 0.9,0.9 and 5.6 kb,respectively. No homology among these three insert DNA was detected by Southern blot analysis.