CHAPTER II

LITERATURE REVIEWS

Pseudomonas pseudomallei

History and geographic distribution

Pseudomonas pseudomallei was first reported by Whitmore and Krishnaswami in 1912, by named of *Bacillus pseudomallei* from autopsy victims in Rangoon, Burma (1). The organism was described under various names as follows : *Actinomyces pseudomallei*, *Pfeifferella whitmori*, *Actinobacillus pseudomallei*, *Loefflerella whitmori* and *Malleomyces pseudomallei* (11,39). This organism is capable of oxidizing glucose and possesses polar flagella which are the characteristics of Pseudomonadaceae. Finally,the nomenclature of *P. pseudomallei* was adopted (40).

P. pseudomallei is a causative agent of melioidosis which is, as the name implied, a glanders-like or melioid disease of man. After the first report by Whitmore, the cases of melioidosis were reported in tropical and subtropical areas (5-7). The endemic areas are Malaysia, Madagascar, Guam, Vietnam, Australia and Thailand. Sporadic cases have been also reported from Korea, Hong Kong, Philippines, Iran, Turkey, England, France, Africa, USSR,

Central and South America. (8-11) In Thailand, Nigg has reported a result of serological examination of 405 sera from Thai population, and found that 118 (29%) were positive with infection of *P. pseudomallei* (12).

In fact,the geographic distribution of *P. pseudomallei* is relatively limited in 20° N to 20° S. This organism is a free living bacterium found in the environment such as water, soil and rice paddies (2,3). Melioidosis has been dubbed " the time-bomb disease or the Vietnam bomb disease ". It has been reported that the persons in U.S. military, who had engaged in the endemic areas had serologically positive sera and some of them developed pulmonary melioidosis and septicemia after several years of returning (4).

Bacteriological aspects

P. pseudomallei is a gram-negative rod, obligately aerobic, non-acid fast, non-spore forming rod that shows bipolar staining. Capsule has not been demonstrated . The organism possesses polar flagella. Colonies vary from round smooth , round 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 slightly aromatic odor associated with pseudomonads. On blood agar, partially hemolysis is seen after growth is more than 48-hour incubation.

P.pseudomallei is biochemically distinguished from *P.mallei* in motility and growth 42° C. Both organisms contain GC 68 % that was the highest GC contents of any other pseudomonads.

The antigen of *P. pseudomallei* has been delineated to 4 types : an envelope antigen (K),the soluble antigen, the flagellar antigen and the O-somatic antigen. The latter antigen cross-reacts with O-somatic antigen of Enterobacteriaceae. However, sonic aqueous extracted antigen was used in complement fixation test. It had specificity in animal infected *P. pseudomallei* , but not in human (12,41,42). Polysaccharide antigen was coated on red blood cells for haemagglutination test. It had specificity in both animal and human for serodiagnosis (5,7,43).

Clinical manifestation

Melioidosis can occur in a wide spectrum of clinical features, ranging from asymptomatic infection through acute septicemia. In acute septicemia melioidosis ends fatally unless a proper antibiotic therapy is used. The organism can be obscured in lung or other organs for several years without any symptom. However, the clinical manifestations could resemble those of pulmonary tuberculosis, or mycoses which could be self-limited or progressive to septicemia. Host status might be very important for melioidosis , for instance, a report from Suwangool, et.al, that out of 19 cases with melioidosis, 13 were from pateints with underlying diseases (44).

The followings are clinical classifications of melioidosis: (4,11,13).

1. **Septicemic melioidosis** : This type is defined by isolating of bacteria from a blood stream. Septicemia form are quite severe disease and fatality rate is high despite promptly antibiotic therapy. This type can be further classified by several clinical pictures :

1.1 Disseminated septicemia : The organism was isolated from a blood stream and almost every viceral organs ; such as lung,liver, spleen, kidney and lymph node are involved.

1.2 Non-disseminated septicemia: The organism could be isolated from blood, but only one single organ was involved such as lung or liver or lymph nodes or subcutaneous tissue which implied that the organism does not spread to the other organs.

1.3 Benign bacteremia : The organism could be isolated from blood of an asymptomatic patient .

2. **Localized melioidosis** : This type is defined by isolation of the organism from some foci such as wound and abscess but not in blood stream. The pulmonary infection resemble tuberculosis could also be classified in this type.

3. **Subclinical melioidosis:** This type is classified via serological test , either in a symptomatic or asymptomatic persons. In addition, a person can or cannot progress to a chronic or acute melioidosis.

Melioidosis is quite difficult to diagnose by clinical picture. It is a "great immitator" of various acute and chronic infectious diseases, for instance , generally it likes gram negative septicemia, staphylococcus septicemia, also tuberculosis and mycotic infections. Thus bacteriologically diagnosis is essential and provable for identifying melioidosis.

Histopathological studies in melioidosis

Histopathology of melioidosis has been studied by Piggot and Hochholer (14) from sixteen bacteriologically confirmed cases of melioidosis. Ten cases of acute melioidosis, later died of septicemia gave positive results of all viceral organs, mostly in lung, liver and spleen. In lungs, small lesions were often surrounded by a narrow hemorrhagic zone, in contrast to liver and spleen the lesions are not hemorrhagic. Necrosis is a constant feature in melioidosis. In chronic melioidosis ; six cases were studied ; the lungs and lymph node are frequently involved. Histopathologic finding microscopically showed a combination of necrosis and granulomatous inflammation. The central necrosis had the caseation necrosis ; the histopathologic picture is similar to that seen in tuberculosis.

Histopathologically, melioidosis has the same appearance in animal as it does in man(45).

Immunity

Both cellular and humoral immune response plays significant role in *P. pseudomallei* infection. Immunization of a sublethal dose or avirulent strains of *P. pseudomallei* or attenuated live vaccine in mice can cease or protect the progression of disease (46 ,47). Kishimoto and Evenland (48) found that both normal and immune macrophages were phagocytic and higher efficient in immuned than normal macrophages. Moreover, the enhanced phagocytic activity of immune macrophage might be due to the presence of an opsonic antibody.

P. pseudomallei is able to provoke a delayed hypersensitivity which is analogous to that seen in tuberculosis or in mycoses. The materials which gave a rise of this tuberculin-type is called "Whitmorin" which is a crude extract from several weeks culture of *P. pseudomallei* (4). However the dermal reaction was difficult to distinguish from direct dermonecrotic effect (due to toxic substance) from actual delayed hypersensitivity of the tuberculin type.

Pathogenesis and virulence factors

The pathogenesis of melioidosis is still a mysterious problem. According to histopathological studies (14) , 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. Tissue necrosis is a regular feature in both acute and chronic melioidosis. The cause of necrosis is not known, but it has been attributed to either vascular damage or to the direct action of bacterial toxin (14,47).

The studies of Dannanberg and Scott (46,47) on pathogenesis of melioidosis in mice indicated that toxins, particularly the necrotoxin was a significant factor in producing the observed lesions.

The virulence of *P. pseudomallei* could be enhanced by serial passage in the host tissue (49). Miller and Clinger (50) studied an acute infection in rabbits by inoculating organisms in an ear chamber, resulting in vascular damage as the suppuration developed. This suggested the importance of vasculitis in the evolution of the lesion. The studies of Nigg et.al and liu (17,21) demonstrated that the experimental animals 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 when intradermally inoculated. The causes of death and tissue necrosis were suggested to be from the heat labile lethal toxin and necrotoxin respectively (22,51). The necrosis might be caused by

proteolytic activity (24,25) , since necrotoxin preparation possessed proteolytic activity (24). Recently, the *P. pseudomallei* lethal exotoxin has been partially purified and used to develop a competitive immunosorbent assay for detection of the exotoxin (23). Another virulence factor has been reported by Lusby and Levine (19,20). It is a thermostable intracellular material called "mortality enhancing polypeptide " since it enhanced mortality rate when injected together with live organism comparing to that without it.

Rappaport (18) reported an endotoxic substance from *P. pseudomallei* which was similar to the classical endotoxin in its biological and physiological properties , for example, it elicited a dermal haemorrhagic necrosis in animal but this material was heat stable , which is different from heat-labile necrotoxin reported by Heckly (22). In addition, it is believed that an envelop antigen, slime antigen, may associate with the virulence of the organism .

Microbial proteases and their classification

Microbial proteases are proteolytic enzymes which microorganism produces. The first microbial protease, an alkaline proteinase from *Bacillus subtilis* and an alkaline proteinase from *Aspergillus oryzae*, were reported in 1950-1952 (52). Microbial proteases are almost an extracellular proteolytic enzyme which is unbiquitous in micro-organisms and synthesized in the high yeild .

Since the decades of 1960, some medical important microorganisms have been studied for their proteases, extensively in their virulence factors. These are genera of *Pseudomonas sp.*, *Serratia sp.*, *Vibrio sp.*, etc. including yeasts and molds (30,32,33,53).

Microbial proteases can be classified into 4 groups according to Hartley, (54) based on mechanism of action , rather than origin, specificity or physiological action . There are 1.) serine protease, 2.) thiol protease, 3.) metalloprotease and 4.) acid protease according to their actions and inhibitors and optimal pH.

1. **Serine protease**

Serine proteases characteristically possess a serine residue at the active site of the molecule. They are active at neutral to alkaline pH. Serine proteases are sensitive to di-isopropylfluorophosphate (DFP) and phenylmethylsulfonylfluoride (PMSF) but not to chelating agents . Generally, they resemble the animal enzyme trypsin. The first microbial serine protease was derived from *Bacillus subtilis* , subtilisin, and latter found that serine protease from *Bacillus lichenifomis* was identical to subtilisin.

2. **Metalloproteases**

This group of protease contains an essential metal atom, usually zinc. They are active at neutral pH and Ca^{2+} is essential for

stability, so the molecule therefore inactivated by chelating agent such as EDTA, phenanthroline (PHE). Metallo proteases are widespread and are synthesized by several bacilli; e.g. an alkaline metallo proteases from *Serratia macescens* (27), *Pseudomonas aeruginosa* (26,55), *Vibrio vulnificus* (28).

3. **Thiol protease**

This group of enzyme is activated by reducing agents such as HCN or cystein but sensitive to sulhydryl reagents for instance HMB (para-chloromerbenzoate). It has optimal activity at neutral pH. Clostripain from *Clostridium hemolyticus*, and streptococcal proteases from *Streptococcus pyogenase* are examples of their group.

4. **Acid protease**

These enzymes are characterized by their optimal activity at acid pH and almost predominate in fungi. The molecule invariably contains an aspartic acid residue at the active site so unaffected by chelating agents, thiol-group reagents or by serine protease inhibitors. Therefore, acid proteases are inhibited by diazoacetyl norleucine methyl ester (in the presence of copper ions). Proteases which are produced by *Aspergillus orzae*, *Aspergillus niger*, *Penicillium notatum* , *Mucor pusillus* and also *Candida albicans* represent the enzyme of this group (52).

These are roughly classifications , however, microbial proteases are rather difficult to classified in definite groups. These are due to their broaden specificities and complex structure of enzyme. Morihara (52) classified the microbial protease by their primary specificity, a kind of amino acid residues of the splitting point that specific for the enzyme.

Moreover, a comparative study of the kinetics of hydrolysis of a small molecular synthetic substrate is made and can be classified in a relatively action on their "broad" or "narrow" specificity against protein substrates. So microbial proteases can be divided into subgroups in each of the above classification. Microbial proteases sometimes are also classified according to their biological activities, for example, collagenase-like enzyme, elastase or elastase-like enzyme, and keratinase.

Collagenase

Collagenase is a group of enzymes, originally described for microorganisms but it is now also being detected in animal tissue. Generally the microorganism producing collagenase is host invasive and presumably the enzyme contributed to pathogenicity by allowing the organisms to penetrate a connective tissue barrier. The examples of collagenase containing microorganisms are the Clostridia, *M. tuberculosis* and certain fungi. Its function, generally is accompanied by elaboration of other enzymes capable of acting on

different connective tissue components or membrane structures. Thus collagenases may act together with elastase, specific peptidases, hyaluronidases, and phosphatase (56).

Collagenases was defined as a group of enzymes that catalyze the hydrolytic cleavage of undenatured collagen. The basic collagen units consist of three chain (α1, α2, and a second α1 or an α3), each of approximately 95,000 MW. These are intertwined into the characteristic triple stranded collagen helix. Collagenases may cause proteolytic scissions in a single α-chain without causing collapse of the whole collagen structure.The terminal portions of α-chain are differ from those of the main chain portions. Trypsin, chymotrypsin, pepsin and other common proteases can promote a limited digestion of these ends without disrupting the main collagen structure. In this, they could also be classified as collagenase. Substrates are gelatin, azocoll and a synthetic peptides. Schoellman and Fisher (57), studied collagenase from *P. aeruginosa* used azocoll and a synthetic peptides for its assay system. The same as Smyth and Arbuthnott (58) purified collagenase form *Clostridium perfringens* used azocoll as substrate. However, Seifter and Happer (56) prefered that collagenase should be attack the native collagen.

Elastase

Elastase usually is present in all mammals, including man. Porcine pancreatic elastase is now a commercially available. Elastase hydrolyze peptide on the c-terminal of amino acids bearing

charged nonaromatic side chain (59). Elastase is an enzyme that capable digesting " elastic " fibrous protein of connective tissue. The most convenient assay is the colorimetric determination of the dye liberated from dyed elastin by elastase. Congo red elastin, and orcein-elastin have also been used as substrate for elastase assays (60). In additon the activity of elastase toward synthetic substrate; N-benzoyl-L-alanine methyl ester (BAEE), N-acetyl-L-tyrosin ethyl ester (ATEE) are also used for the assay. Microorganism such as *P.aeruginosa* can produce an elastase like enzyme, and were proved to have roles in pathogenesis (61,62)

Role(s) of microbial proteases in pathogenesis

Recently, many microbial proteases from yeast, fungi and some medically important bacteria have been studied and found that they play a significant role in pathogenesis. The action of purified proteases can be concise in the following aspects: tissue damages, enhanced vascular permeability, hemorrhage, cytotoxicity and hemagglutination for instance.

In the experimental models, purified proteases from *P. aeruginosa* caused lung damage with hemorrhage and necrosis of alveolar septal cells, indicating a role of proteases in pseudomonas pneumonia (62,63). In addition *P.aeruginosa* proteases damage corneal proteoglycan ground substance caused keratitis (64,65). Similarly, the serratial proteases were liquefactive necrosis of cornea (66), caused corneal ulcer.

In cutaneous lesions, *Vibrio vulnificus* which caused wound infection and septicemia, its proteases have elastase and collagenase activity, also play a role in pathogenesis by enhancing vascular permeability and forming a hemorrhagic lesion on rat and guinea pig skin (33,67).

A protease isolated from *Legionella pneumophila* was cytolytic and caused dermal ulcerative activity as well (29). In addition, Conlan et.al.(68) had demonstrated that this protease caused lesions in lung of guinea pig which is like those of legionnaires' disease in human.

Both *Vibrio cholerae* 01 and non-01 produce a protease which shows proteolytic activity and hemagglutination as well (36). The proteolytic activity can cleavage fibronectin, mucin and nick choleragen related to heat labile *E. coli* enterotoxin also possesses hemagglutination. Finkelstein et.al.and Crewther et.al. (35-37) demonstrated that this protease played a role in bacterial attachment to gut epithelium.These experiments might be assumed that this protease may play a role or at least a part in pathogenesis of *V. cholerae* gastroenteritis which a major cause is enterotoxin.

Riepe et. al.(38) demonstrated that protease(s) secreted by some enteric *Bacteroides sp*, may cause destruction of maltase and sucrase activity in human brush border, concluded that in bacterial overgrowth syndromes, may due to protease(s) secreted by *Bacteroides sp*.