# CHAPTER 1

# INTRODUCTION

The importance of gram-negative pathogens such as the salmonellae, shigellae, gonococci and meningococci was well known, also several factors led to the emergence of gram-negative bacteria, such as Escherichia coli, Pseudomonas aeruginosa, and Klebsiella spp. as the most important causes of hospital associated bacteremia. It was estimated that such infections were responsible for approximately 1,800 deaths among 33 million hospital admissions in the United States in 1972.[1]

P. aeruginosa infection was a serious problem. The most frequent site of infection was the burn wound. Pseudomonas also in vaded the urinary and lower respiratory tracts of burn patients as well as of medical, general surgical, urologic and gynecologic patients. It was an important pathogen for children with cystic fibrosis. In long-standing infections, P. aeruginosa tended to replace other organisms. A major characteristic of the organism was resistance to nearly all antibiotics. It was responsible for eye infections in people who wear contractlenses, since Pseudomonas could grow in the sterilizing solutions used for overnight storage of the lenses.[2]

In attempts to elucidate the mechanism of pathogenicity of gram-negative infections, attention has been on the role of lipopolysaccharide (LPS) endotoxin. The numerous biological activities of LPS endotoxin included many of featured observed in gram-negative infection of human beings and experimental animals and it was tempted to conclude that the toxin was responsible for the symptoms of the shock which frequently accompanied gram-negative sepsis and sometimes led to death.[1,3]

The main purpose of this research project is to study the pathogenic effect of endotoxin from P. aeruginosa in experimental animals.

### 1.1 Biology of Pseudomonas aeruginosa

P. aeruginosa was found in soil, in fresh water, and in almost every part of the human habitat such as kitchen sink and air conditioner filters. P. aeruginosa was unique among human pathogens in that it infected not only vertebrates, both warm-and cole blooded, but also lower animals, terrestial and aquatic, including insects. It even infected plants, occasionally destroying a tobacco or sugar cane crops.[4]

The clinical approach to the prevention and control of Pseudomonas infections should ultimately rest upon basic understanding of the organism, Therefore, it was worth while to consider some of the highlights of what was now known about

the taxonomy, cellular structure, metabolism, genetics, and pathogenic properties of P. aeruginosa. The pathogenic properties was specially interested for study.[2,4]

### 1.1.1 Taxonomy and Cellular Structure

P. aeruginosa was classified according to Bergey as;[5]

Genus Pseudomonas

Family Pseudomonadaceae

Order Pseudomonadales

Class Schizomycetes

P. aeruginosa is a gram-negative saprophytic rod ranging in size from 0.5 to 0.8 microns ($\mu$) by 1.5 to 3.0. Nearly all strains are motile, with a single polar flagellum, and most have many long, very fine projections called pili or fimbriae, non-sporing and non-capsulated. P. aeruginosa was the only living system in the world known to produce the pigment pyocyanin, which was blue in neutral on alkaline media and red in acid media, although some strains do not produce pyocyanin and others produce the pigment only in certain media. P. aeruginosa grew on a wide variety of laboratory media, formed smooth round colonies with a fluorescent greenish color and a sweetish aromatic oder, P. aeruginosa had a slime, a largely polysaccharide, with some lipid, protein and other substances. It seemed to serve primarily as a protection against phagocytosis, and contained some toxicity.[6,7]

The cell wall structure of P. aeruginosa was similar to that of the Enterobacteriaceae. The inner core of P. aeruginosa LPS contained 2-keto-3-deoxyoctonic acid (KDO), as did the enteric bacilli, but the side-chains of P. aeruginosa LPS contained amino sugar rather than the neutral sugars found in the Enterobacteria LPS. Also, lipid A[1] of P. aeruginosa LPS lacked B-hydroxymyristic acid.

1.1.2 Metabolism

P. aeruginosa was an aerobic organism, in contrast to most bacteria that caused disease in human beings which were facultative anaerobes. The requirement of oxygen for growth might account for the lack of invasiveness of the organism into deep tissue and internal organs after it had infected the skin. Also, since production of various types of toxic substances by this organism required oxygen, the few cells that entered the blood stream from the infectious site in the skin were probably unable to make the toxins with which they might otherwise destroy the phagocytes of the hosts. P. aeruginosa was an extremely adaptable organism that could utilize over 80 different organic compounds for growth, and ammonia could serve as nitrogen source.[8] A temperature of 35 C was optimal for growth, but growth could still occur at 42 C. Clinical isolated growth on blood agar were frequently beta hemolytic. Pyocyanin, the well known pigment produced by P. aeruginosa, accounted for the species name: " aeruginosa " refers to the blue-green colour of oxidized copper or bronze, which the

pigment closely resembles. Various strains of the organism could also produced many other pigments, such as phenazine-alpha-carboxylic acid and chlororaphin and a brown pigment, pyomelamin.[9,10]

Energy from carbohydrates was derived by oxidative rather than fermentative metabolism. Since the acid produced by the oxidative pathway was less than that produced by other organisms using a fermentative pathway.[2,11]

1.1.3 Genetics

Most bacteria that were lysogenic carried only one phage at a time. P. aeruginosa was unusual in that it was often simultaneously lysogenic for three or even four phages. For this reason it was difficult to associate a particular characteristic with one phage. Genetic material was transfered from one bacterium to another by transduction of the phages. Phage was used for typing various strains of bacteria. Related to phage, and also used for typing was pyocin phenomenon.[2]

Of great clinically were the plasmids, or resistant factors, of Pseudomonas the genetic units responsible for much bacterial resistance to antibiotics. As was well known, they could be transfered from one bacterium to another, and even between bacteria of quite different species. In Pseudomonas, resistance factors seemed to be primarily responsible for resistance to streptomycin and other nonpenicillin antibiotics. Exchanges of

genetic information accounted for the great variety of P. aeruginosa strains, but were especially responsible for the inherent mutability of the species.

1.1.4 Pathogenic Properties

The substances that have been implicated in the pathogenicity of P. aeruginosa were as follows :

1.1.4.1.Phytotoxic factor

P. aeruginosa was well known as a plant pathogen. Natural infections in plants could be of considerable economic significance for farmers growing tobacco or sugar cane. The phytotoxic factor of P. aeruginosa was sufficiently small to pass through a cellophane sheet and was heat-stable. The effect of P. aeruginosa on tobacco plants appeared similar to that caused by Pseudomonas tabaci. The phytotoxic factor of the latter organism was an amino acid. Its structur was similar to that of methionine, excepted that the sulfur molecul of methionine was replaced by a carbon. This substance called tabtoxinine, functioned as a metabolic analogue of methionine blocking its metabolism and appeared to be entirely nontoxic to animal tissue. (4,1!)

1.1.4.2 Pigments

P. aeruginosa was known to produce a number of pigments the known were :[2,5,8,12]

- Pyocyanin; a phenazine pigment, bluish material, soluble in chloroform and water.

- Fluorescein; a greenish fluorescent, water soluble (but not chloroform-soluble) material.[6,7]

This organism was formerly named " Pseudomonas pyocyaneous."[8]

Some investigators have proposed that the pigments played a role in inhibiting phagocytosis. The pigments were as important as toxins. For one thing, there were virulent strains with very low pigment production and highly pigmented strains that were not virulent. Injection of the pigments into experimental animals appeared to cause no deleterious effects.

1.1.4.3 Hydrocyanic acid

Production of hydrocyanic acid by P. aeruginosa was interesting phenomenon, but there was no evidence to indicate that sufficient quantity of substances was produced in vivo to account for the symptom of infection. The autopsy finding in human beings animals that died with infections due to P. aeruginosa, were not those of cyanide poisoning.[8]

1.1.4.4 Proteolytic Enzyme

Most strains of P. aeruginosa were highly proteolytic. The materials from P. aeruginosa designated proteases not to be a single entity but rather a mixture of proteolytic enzymes. These proteases generally liquid gelatin, cleared milk, and dissolved elastin and fibrin.[2,11] It appeared that many proteolytic enzymes that were not the classical collagenase could attack terminal

peptides of native collagens and liberated amino acid or peptides. The roteases of P. aeruginosa appered to belong to this group of non-specific collagenases.

Injection of proteases of P. aeruginosa into skin of animal induced hemorrhagic lesions within a few minutes[8] and within 24 hours the lesions became necrotic. The same proteases attacked the cornea of the eye, it could also appearently produce hemorrhagic lesions of the lung and destroyed collagen in the colon and other parts of the body.[2,13,14] Intraperitoneal or intravenous injection proteases produced hemorrhagic lesions of intestines and lungs.[15] However, the effects of proteases were usually quite localized and proteolytic activity was not demonstrated in sera of moribund animals.[16] Furthermore, autopsy finding of human-beings and animals that died of infection due to P. aeruginosa were usually differed from those observed in animals that were killed experimentally by injections of large doses of proteases, therefore, they are not the lethal factor in the usual infections with P. aeruginosa. The 50 % Lethal Dose ($LD_{50}$) of proteases in mice is about 75 micrograms (mcg.).[17] It is not very potent when compared with other protein toxin of bacteria.

1.1.4.5 Hemolytic substances

Strains of P. aeruginosa produced two hemolytic substances.[18]

- A heat-labile and appeared to be a phospholipase C that liberated phosphorylcholine from licithin.[19]

- A heat-resistant glycolipid appeared to function as a detergent in solubilizing phospholipids.

A purified preparation of the glycolipid was not extremely toxic, about 5 milligrams (mg.) was required to killed a mouse.[20] When a phospholipase preparation of P. aeruginosa was injected into the skin of animals, within 24 hours it produced a central abscess surrounded by an area of redness and induration.[15] The skin lesions produced by the injection of live culture of P. aeruginosa resembled in some features the lesions produced by phospholipase that was not the important factor in the lethality due to P. aeruginosa infection."

1.1.4.6 Enterotoxin

P.aeruginosa have been associated with diarrheal conditions, variously described as five-day fever or Shanghai fever. Enterotoxin was produced a necrotizing enteritis and out pouring of fluid and electrolytes into the lumen of intestine.[21] The enterotoxin has not been characterized, but it was heat-labile and probably protein in nature.[22]

1.1.4.7 Surface Slime

The slimes of P. aeruginosa was polysaccharides on the surface of the cells[23] that were probably the functional equivalent of the capsules of many gram-negative bacilli.[24,25] The toxicity of the slime varies considerably from one preparation to another, and completely purified polysaccharide fractions was usually nontoxic.[24,26,27]

1.1.4.8 Exotoxin

Many strains of P. aeruginosa could produced an exotoxin in vitro and probably also in vivo that markedly inhibits protein synthesis and caused tissue necrosis. They were sensitive to heat (60°C for 30 minutes) highly antigenic and usually potent.[28,29]

Heat-labile exotoxin of P. aeruginosa have been purified and concentrated. Studied in mice had shown that the toxin affect protein synthesis in the live within 3 hours of administration, where as inhibition of protein synthesis in other organs occured only during terminal stages. In short-term experiments in vitro, purified exotoxin did not inhibit ingestion or killing of bacteria by human polymorphonuclear leukocytes.

P. aeruginosa was known to elaborate various toxic fraction that were demonstrable in vitro and in experimental animals. A heat-labile exotoxin have been described.[16,29,30] This toxin was lethal for mice [16] and was capabled of eliciting hypotensive shock in stumptial rhesus monkeys and dog.[5]

### 1.1.5 The Characteristic of Endotoxin

An endotoxin of P. aeruginosa was composed of two components separable by electrophoresis.[31]

- Component I : Consisted of a lipopolysaccharide-protein complex with a molecular weight of one million.[32]
- Component II : Consisted of deoxyribonucleic acid, ribonucleic acid and polyribose complex.[33,34]

The mechanisms of pathogenicity of gram-negative infections attention had focused on the role of LPS endotoxin. The constituent of the outer membrane of the gram-negative envelope and consists of heteropolysaccharide chains covalently linked to a lipid known as lipid A (Fig 1 page 17). The polysaccharide component contains two regions, the O-specific side chain and the core. The O-specific side chain was represented by a polymer of oligosaccharide repeating units and carried the antigenic determinant. The core oligosaccharide was made up of a main chain of sugar residues which was substituted at various points by monosaccharides, phosphate, phosphoethanolamine and pyrophosphoethanolamine. This core polysaccharide is covalently linked A which consists of a glucosamine phosphate back bone carrying long chain fatty acids in ester and amide linkages.

The various biological activities of LPS endotoxin are summarised in table 1 page 15. These numerous biological acivities of LPS endotoxin include many of featured observed in gram-negative infections of man and experimental animal. The toxin was responsible for the symptoms of the shock which frequently accompanied gram-negative sepsis and sometimes led to death. The extremely sensitive limulus test (a reaction based on the cloting of a lysate of amoebocytes from the horseshoe crab limulus polyphemus)[35] was useful in the diagnosis of sepsis and was predictive for shock and death.

The comparative properties of two kinds of proteins, one obtained from the cell wall of P. aeruginosa and the other from the the endotoxin in the autolysate. It was proved that the two proteins possessed a common specific antigen. They were found to be the same in their potencies in eliciting the Shwartzman phenomenon and pyrogenic reaction.[34,36,37]

Hydrolysis of the polysaccharide fraction of the endotoxin of P. aeruginosa yield glucose, glucosamine, galactosamine, rhamnose, heptose and ketodeoxyoctonate. Fatty acids identified as methyl esters in the lipid fraction were lauric, palmitic, stearic, oleic, and linolic, capric acid also appeared to be present.[38] Endotoxin of P. aeruginosa isolated from the cell wall was found to possess pyocine activity. In a series of investigation[39,40] was found that the pyocine-netralizing antibodies could be produced in sera

of rabbits immunized with the protein moiety bound with LPS.[41,42] Protein moiety to be a serologically common antigen existing in all strains of *P. aeruginosa* as well as a common infection protective antigen which work against all infections due to *P. aeruginosa* regardless of the variety of serotype.[43]

The active principle and the mechanism of complement activation of original endotoxin protein (OEP) of *P. aeruginosa* the complement consumption of the LPS from OEP was studied in guinea pig serum (GPS). The LPS from OEP had about 5 times higher the activity than OEP in GPS. This value corresponds to the LPS content (about 20 %) in OEP. A property of OEP of *P. aeruginosa*, OEP activated both the classical (CP) and alternative (AP) pathways of complement, without participation of the antibody.[44]

The purified LPS from OEP shown both the interferon inducing and the antitumor activities are restricted to the LPS. The OEP activated complement depending upon the LPS portion in OEP and the activation was operated through both the CP and the AP[45]

It was known that the LPS derived from cells of *p. aeruginosa* was protective only against the infection with the same serotype strain that it was derived from.[46,47] There were many serotypes in *P. aeruginosa* Therefore, several LPSs had to be admixed in order to develope a vaccine for use against all the types of *p. aeruginosa* infection. Both active and passive immunizations with OEP were very

effective in preventing infection with all the types of bacillus[48] OEP hyperimmune serum therapy would be effective in certain cases of *P. aeruginosa* infection such as that of newborns or compromised hosts, that were persons with leukemia or cutaneous burn or ones who were undergoing systemic immunosuppressive therapy.[43]

There were several of reports on the adjuvant effect of endotoxins (LPS) of gram-negative bacteria, it was surmised that OEP (as a fraction of endotoxin) had also the same effect as LPS. The present study were stimulated by the report of the anti-tumor activity of OEP.[49]

Table 1 Biological activities of lipopolysaccharide endotoxin [1]

| |
|---|
| Pyrogenicity (ability to induce a rise in body temperature) |
| Lethal action |
| Depression of blood pressure |
| Activation of complement |
| Intravascular coagulation |
| Leucopaenia and leucocytosis |
| Inhibition of glucose and glycogen synthesis in liver |
| Stimulation of B-lymphocytes |
| Macrophage inhibition |
| Interferon release |
| Induction of prostaglandin synthesis |
| Clotting of a lysate of amoebocytes from the horseshoe crab |

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Table 2 Shwartzman phenomena produced by cell-wall protein A, original endotoxin protein and the lipopolysaccharide of component I*

| Preparatory material | Injection dose (mcg) | Reaction | |
|---|---|---|---|
| | | Grade | Diameter (mm) |
| Cell - wall protein A | 3 | Necrosis | 13 x 13 |
| | 1.5 | Necrosis | 7 x 7 |
| | 0.7 | Necrosis | 7 x 7 |
| | 0.4 | Necrosis | 7 x 7 |
| | 0.2 | Necrosis | 5 x 5 |
| Original endotoxin protein | 3 | Necrosis | 10 x 10 |
| | 1.5 | Necrosis | 8 x 8 |
| | 0.7 | Necrosis | 5 x 5 |
| | 0.4 | Hemorrhage | 10 x 2 |
| | 0.2 | Hemorrhage | 10 x 2 |
| Lipopolysaccharide of component I | 3 | Necrosis | 18 x 18 |
| | 1.5 | Necrosis | 18 x 18 |
| | 0.7 | Necrosis | 18 x 18 |
| | 0.4 | Necrosis | 8 x 8 |
| | 0.2 | Necrosis | 8 x 8 |

* Rabbits used weighed 2.5 kg. As aprovocation, 500 ug/kg of original endotoxin were injected. Each one of the intradermal injection (provocative injection) was given in a dose of 0.1 ml. After 20 hrs., an intravenous injection (provocative injection) was given in a dose of 1 ml/kg of body weight.[34]

Figure 1 Diagram of the structure of LPS. (From review of Reitscher et al. (1975), Microbiology, 307 - 13.)