SDS-PAGE of *Pseudomonas Pseudomalley* Exotoxins

Jeanne Indriana
Dept. of Animal Diseases and Veterinary Public Health
Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta

ABSTRACT

The protein profiles of *Pseudomonas pseudomallei* exotoxins which derived from *P. pseudomallei* strains of different virulence and origin (J53, AN, C2, X1003 and 1328 strains), have been studied by electrophoretic analysis using SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis).

Each of these *P. pseudomallei* strains is propagated in mucin broth and then incubated statically at 32^0 for 7 days. The exotoxin of each strain has been prepared from that broth culture by centrifugation and followed by ultrafiltration.

The result indicated that all of the strains, regardless of its different virulence and origin, showed one protein band common to all, i.e. at a molecular weight of approximately 35 K.

INTRODUCTION

It was frequently reported that mice and hamsters which died within 1 or 2 days post-infection wtih *P. pseudomallei* showed no gross lesions at *post-mortem* examinations (Dannenberg Jr. and Scott, 1958). This is most likely due to a lethal toxin of *P. pseudomallei* (Nigg *et al.*, 1955; Heckly and Nigg, 1958). Production of a lethal toxin in broth culture filtrates of *P. pseudomallei* has been demonstrated by many workers (Liu, 1957, Colling *et al.*, 1958; Heckly and Nigg, 1958; Heckly, 1964). Nevertheles, the role of this exotoxin in the pathogenesis of melioidosis, an infection caused by *P. pseudomallei,* is still in doubt, as in the experiment studied by Indriana (1992), only concentrated crude exotoxins were lethal for mice, whereas mice which were injected with unconcentrated crude exotoxins remained alive. Moreover, these exotoxins required at least 1 mg of protein to kill the mice, suggesting they were of low lethal toxicity.

The exotoxin of *P. pseudomallei* has been detected by electrophoresis by Heckly (1970) and Ismail *et al.* (1987). The first investigator reported that the molecular weight of this exotoxin was under 50 K and of low lethality, requiring 10 - 1,000 μg of protein to produce an effect, while the second investigators showed that there was only band at a molecular weight of about 31 K. However, both investigators did not describe clearly whether they tested on strains of different virulence or not.

Since *P. pseudomallei* strains have also

been studied to have variations in virulence (Miller *et al.*, 1948; Indriana and Hirst, 1993), then the purpose of this study is to analyse the protein components of the exotoxins derived from *P. pseudomallei* strains of different virulence and origin, using SDS-PAGE.

MATERIALS AND METHODS

Strains of *Pseudomonas pseudomallei*

The *P. pseudomallei* strain used in this study are shown in Table 1. These strains vary in their virulence, showed by their LD50.

millilitre of each broth culture (containing about 10^8 organisms) was inoculated into 100 ml mucin broth (i.e. BHI broth containing 2% glycerol and 1% mucin), and incubated statically at 32ºC for 7 days.

As the pH range for stability of *P. pseudomallei* exotoxin is 6 to 9 (Heckly, 1970), before adding the organism, the pH of each of the mucin broth was checked to confirm that each fell within this range. The sterility of the mucin broth was also checked by streaking it on to MacConkey and blood agars.

After static incubation for 7 days, the pH of each broth culture was again checked, followed by centrifugation at 12,000 RPM

Table 1. Strain of *Pseudomonas pseudomallei* used in this study

Strain	Origin	LD_{50} (log_{10}) *)	Source
J53	Sheep	5.8	Oonoonba Veterinary Laboratory, Townsville, Queensland, Australia
AN	Human	5.4	Townsville General Hospital, Queensland, Australia
C2	Soil	4.3	Oonoonba Veterinary Laboratory, Townsville, Queensland, Australia
X1003	Goat	3.6	- idem -
1328	Pig	0.3	- idem -

* Cited from : Indriana (1991).

Production of the exotoxin

The procedure was a modification of the methods of Colling *et al.*, (1958) and Ismail *et al.* (1987).

The *P. pseudomallei* strains were propagated as follows :

A single colony (about 1 mm in diameter) was suspended in 10 ml sterile physiological saline, mixed well, and 10 μl was transferred into 10 ml BHI (Brain Heart Infusion) broth, followed by mixing it well and incubating at 37ºC for 16 hours. One

for 1 hour at 4ºC, and the supernates (toxins) were filtered through 0.8 μm and further filtration through 0.45 μm pore-size sterile membrane filters to remove any remaining cells. These cell-free culture filtrates were designated as the crude exotoxins, and stored at 4ºC.

The sterility of the crude exotoxins was checked by incubating 1 ml crude exotoxin of each strain in 10 ml BHI broth for a week at 37ºC. Provided the results of the sterility tests were satisfactory, some of the crude exotoxin of each strain was concen-

trated to approximately one-tenth of its original volume in an Amicon Ultrafiltration Unit, at a cut off point of 10,000 MW, with a nitrogen pressure of 250 kPa, then dialysed twice against PBS (pH 7.2) for 24 hours at 4°C. This was followed by further filtration through a 0.2 µm pore-size sterile membrane filter, and stored at -20°C as concentrated crude exotoxins.

SDS-PAGE of *P. pseudomallei* exotoxin

Electrophoresis as described by Laemmli (1970) was performed to separate the proteins contained in the concentrated crude exotoxins of the five strains of *P. pseudomallei* studied. These exotoxins were then adjusted to a protein concentration of 4mg/ml with sterile distilled water, followed by diluting the samples with sample buffer (pH 8.3) in the ratio 3:1, and boiled for 5 minutes.

Gels for electrophoresis were made from stock solutions of acrylamide, upper and lower gel buffers. An 11% separating gel was made using 4.4 ml acrylamide/bis (30%), 3.0 ml separating buffer (pH 8.8), and 4.6 ml distilled water. The gel mixture was de-aerated under vaccum for at least 15 minutes, then 40 µl of freshly made 10% ammonium persulphate and 10 µl NNN'N" - tetramethylethylenediamine (TEMED) were added to polymerise the acrylamide. This gel mixture was quickly poured between acetone-cleaned glass plates (8 cm x 10 cm) clamped together with spacers 0.75 mm thick and overlaid with butanol. Continued by allowing the gel to polymerise for 40 minutes. The butanol overlay was rinsed off completely with distilled water.

A 4% acrylamide gel used as the stacking gel was prepared. It consisted of 0.75 ml acrylamide/bis (30%), 1.25 ml stacking buffer (pH 6.8) and 3.0 ml distilled water. The gel mixture was de-aerated under vaccum for 15 minutes, then 20 µl of f reshly made 10% ammonium persulphate and 10 µl TEMED were added. The stacking gel was then poured onto the separating gel, and a 10 well comb was inserted to form the sample wells. After allowing the gel to polymerise for 30 minutes, the comb was removed by pulling it straight up slowly and gently, then the wells were rinsed completely with distilled water. These gel plates were attached to the inner cooling core, then loaded into the chamber of the Bio-Rad Mini Protean II Cell containing running buffer (pH 8.3).

Low molecular weight standard of Pharmacia, USA which consisted of phosphorylase b (94,000 MW), bovine serum albumin (67,000 MW), ovalbumin (43,000 MW), carbonic anhydrase (30,000 MW), soybean trypsin inhibitor (20,100 MW), and α-lactalbumin (14,400 MW) were used to determine the molecular weights of the exotoxin bands and the complete resolution of their protein separation.

Electrophoresis was conducted at a constant voltage of 200 volts until the tracking dyes were 0.5 cm from the bottom of each gel. The gels were then removed from the glass plates, fixed and stained with Coomassie blue stain overnight at room temperature, then rinsed in distilled water and destained over several hours with 2 or 3 changes of destaining solution.

RESULTS

The protein profiles of the concentrated crude exotoxins derived from *P. pseudomallei* strains of different virulence and origin, are presented in Figure 1. It shows that the exotoxin of human (AN) strain consists of 4 protein bands at approximate molecular weights of 21 K, 23 K, 35 K and 48 K. While the exotoxin of soil (C2) strain has 5 bands of protein at 23 K, 29 K, 35 K, 53 K and 58 K MW. However, the exotoxins of sheep (J53), goat (X1003) and pig (1328) strains show only one band, each at 35 K MW.

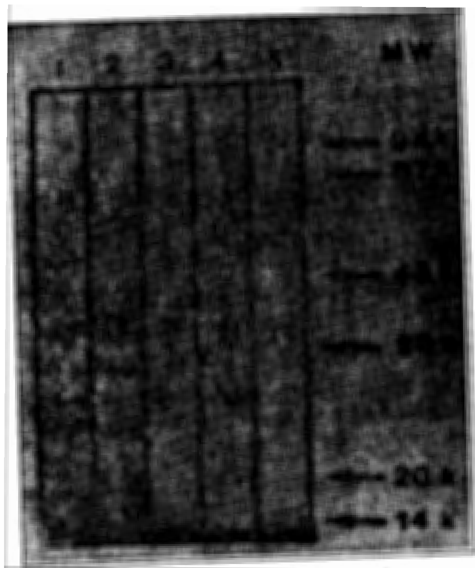

Figure 1. SDS-PAGE of *Pseudomonas pseudomallei* exotoxins obtained from 5 strains on 11% acrylamide gel stained with Coomassie Blue.

1. Human (AN) strain; 2. Soil (C2) strain
3. Sheep (J53) strain; 4. Goat (X1003) strain
5. Pig (1328) strain.

DISCUSSION

From the results of the electrophoretic analysis of the concentrated crude exotoxins of *P. pseudomallei* strains tested, there was one common protein band at a molecular weight of approximately 35 K, suggesting it to be the toxin due to the molecular weight of *P. pseudomallei* exotoxin was reported under 50 K (Heckly, 1970). Moreover, this band is similar in molecular weight to that reported by Ismail *et al.* (1987) who described the *P. pseudomallei* exotoxin as having only one band of 31 K MW.

Since the band at 35 K MW was found in all strains of different virulence, ranging from low to high, this suggests that there is no direct relationship between the toxin and the virulence for each of the strains. This result supports the findings of Nigg *et al.* (1955) and Colling *et. al.* (1958) who showed that no correlation between toxicity and colonial morphology or virulence. Furthermore, it has been demonstrated that the *P. pseudomallei* exotoxins produced by the 5 strains tested were of low lethal toxicity, requiring at least 1 mg of protein to kill mice (Indriana, 1992).

In this present study, the protein profiles of the concentrated crude exotoxins of human and soil strains showed more than one band. This perhaps due to the exotoxin from each strain was not purified, and the bands additional to the 35 K MW are probably oter membrane proteins derived from the bacterial cells during their 7 days culture period. Their toxigenicity is unknown.

SDS - PAGE Dari Eksotoksin *Pseudomonas Pseudomallei*

ABSTRAK

Gambaran protein dari eksotoksin *Pseudomonas pseudomallei* yang berasal dari galur-galur *P. pseudomallei* dengan virulensi dan asal yang berbeda (galur-galur J53, AN, C2, X1003 dan 1328), telah dipelajari secara analisis elektroforetik dengan menggunakan SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis).

Tiap galur *P. pseudomallei* ini dibiakkan dalam kaldu musin dan kemudian diinkubasikan secara statis pada suhu 32°C selama 7 hari. Eksotoksin dari tiap galur disiapkan dari biakan kaldu tersebut secara sentrifugasi dan diikuti dengan ultrafiltrasi.

Hasil studi menunjukkan bahwa semua galur, terlepas dari perbedaan virulensi dan asalnya, memperlihatkan adanya satu pita protein yang sama, yaitu pada berat molekul kira-kira 35 K.

REFERENCES

Colling M., Nigg C. and Heckly R.J. (1958). Toxins of *Pseudomonas pseudomallei.* I. Production *in vitro. Journal of Bacteriology* **76** : 422-426.

Dannenberg Jr. A.M. and Scott E.M. (1958). Melioidosis : Pathogenesis and immunity in mice and hamsters. I. Studies with virulent strains of *Malleomyces pseudomallei. Journal of Experimental Medicine* **107** : 153-166

Heckly R.J. (1964). Differentiation of exotoxin and other biologically active substances in *Pseudomonas pseudomallei* filtrates. *Journal of Bacteriology* **88:** 1730-1736

Heckly R.J. (1970). Toxins of *Pseudomonas.* In: T.C. Montie, S. Kadis and S.J. Ajl (eds.). Microbial Toxins. Vol. III, pp. 473-491. Academic Perss: New York

Heckly R.J. and Nigg C. (1958). Toxins of *Pseudomonas pseudomallei.* II. Characterization. *Journal of Bacteriology* **76:** 427-436

Indriana J. (1991). Studies in *Pseudomonas pseudomallei* Infection in mice. MSc. Thesis. James Cook University of North Queensland, Australia.

Indriana J. (1992). Toxicity of *Pseudomonas pseudomallei* Exotoxins in mice. *Buletin IPKHI* **2:** 2-6

Indriana J. and Hirst R.G. (1993). Virulence studies of *Pseudomonas pseudomallei* in mice. *Bulletin FKH - UGM* **12:** 64-74

Ismail G., Embi M.N., Omar O., Allen J.C. and Smith C.J. (1987). A competitive immunosorbent assay for detection of *Pseudomona pseudomallei* exotoxin. *Journal of Medical Microbiology* **23:** 353-357

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685

Liu P.V. (1557). Survey of hemolysin production among species of pseudomonads. *Journal of Bacteriology* **74:** 718-727

Miller W.R., Pannel L., Cravitz L., Tanner W.A. and Rosebury T. 1948). Studies on certain biological characteristics of *Malleomyces mallei* and *Malleomyces pseudomallei.* II. Virulence and infectivity for animals. *Journal of Bacteriology* **55:** 127-135

Nigg C., Heckly R.J. and Colling M. (1955). Toxin produced by *Malleomyces pseudomallei. Proceedings of the Society for Experimental Biology and Medicine* **89:** 17-20