CHAPTER IV
RESULTS

1. Kinetic of *B. pseudomallei* in blood of acute infected BALB/c mice.

1.1 The bacteremia of acute *B. pseudomallei* infection as determined by culture.

Forty BALB/c mice were infected with $12LD_{50}$ (230 CFU) of *B. pseudomallei* (A2) by intraperitoneal (IP) injection for acute infection. The twenty remaining infected BALB/c mice were used to monitored their blood samples by spread plate technique on Ashdown's selective medium at various time points (12, 24, 48, 60 and 72 h) after infection. The results showed that the bacteria could be detected in blood starting from 12 h after infection (mean±SE = 8±8 CFU/ml). The number of bacteria were increased and peaked at 60 h post infection with very high bacterial count (mean±SE = $(1.1\pm0.85)\times10^4$ CFU/ml of blood) (Table 1 and Figure 8). Control mice that received PBS and monitoring of bacterial loads in blood at the same time points as infected mice showed no detectable bacteria in their blood samples (Table 1).

Based on the culture method, bacterial load of blood from acute infected mice could be detectable at earliest time point examined (12 h post infection) with 20% positive (1/5). The positive rate was increased and all blood samples of acute infected animals could be detectable after 24 h infection (100% positive) (Table 1 and Figure 9).

Table 1 The presence of *B. pseudomallei* in acute infected BALB/c mice. The bacterial loads in the blood, lungs, livers and spleens of infected mice at different time points post infection by intraperitoneal (IP) injection with $12LD_{50}$ (230 CFU) of virulent *B. pseudomallei* (A2) as determined by culture.

Time post of infection	Mice	Culture detection			
		Mean number of bacteria ± SE (%positive)			
		CFU/ml	CFU/organ		
		Blood	Lung	Spleen	Liver
12	Infected	8±8 (20)	NG* (0)	6±3.67 (60)	18±11 (60)
	PBS	NG (0)	NG (0)	NG (0)	NG (0)
24	Infected	(60±24.90) (100)	$(3.35\pm3.16)\times10^2$ (80)	$(7.37\pm1.36)\times10^2$ (100)	$(5.29\pm2.09)\times10^2$ (100)
48	Infected	$(3.3\pm1.20)\times10^2$ (100)	$(6.36\pm3.55)\times10^4$ (100)	$(>1\pm0.30)\times10^5$ (100)	$(2.59\pm0.92)\times10^4$ (100)
60	Infected	$(1.1\pm0.85)\times10^4$ (100)	$(>1\pm0.63)\times10^5$ (100)	$(>1\pm0.11)\times10^5$ (100)	$(3.37\pm0.64)\times10^3$ (100)
72	Infected	$(1.08\pm0.83)\times10^3$ (100)	$(1.38\pm0.83)\times10^3$ (100)	$(>1\pm0)\times10^5$ (100)	$(3.7\pm0.70)\times10^3$ (100)
	PBS	NG (0)	NG (0)	NG (0)	NG (0)

*NG = No growth

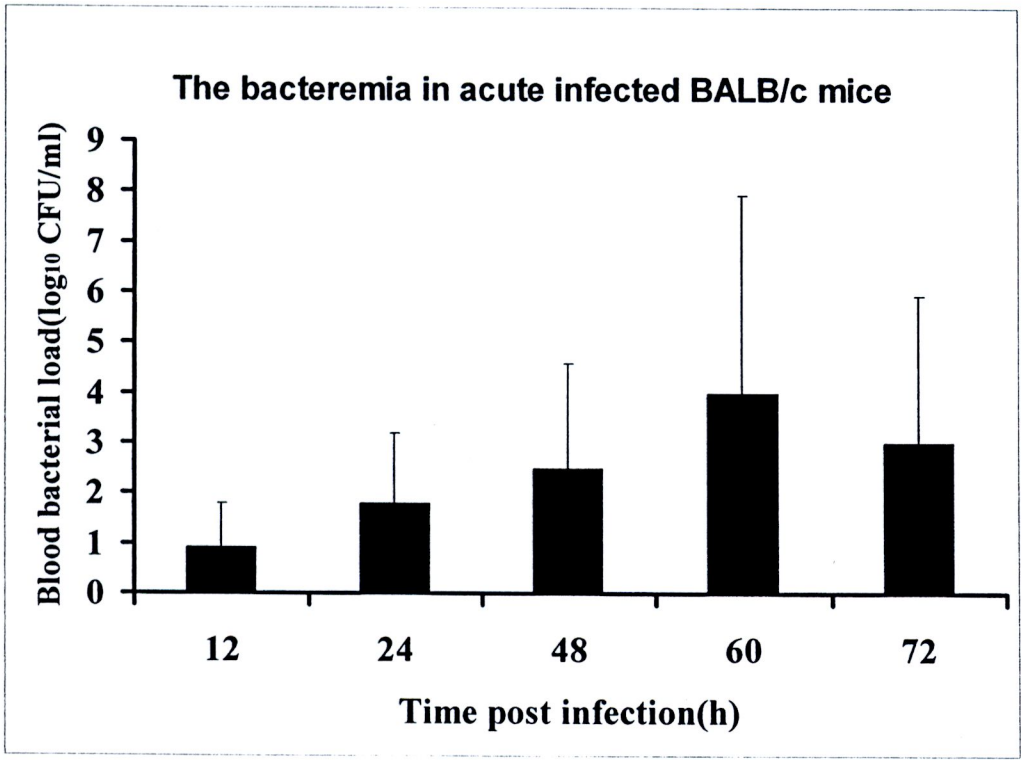

Figure 8 Kinetic of bacterial in blood of acute *B. pseudomallei* infected mice. Twenty BALB/c mice infected with $12LD_{50}$ (230 CFU) of *B. pseudomallei* (A2) by intraperitoneal (IP) injection and monitored their blood samples by culture on Ashdown's selective medium at various time points post infection (12, 24, 48, 60 and 72 h). Each bar represents the logarithm of the mean number±SE of bacterial loads (log_{10} CFU/ml) was plotted against time. No bacteria could be detected in blood samples of control group.

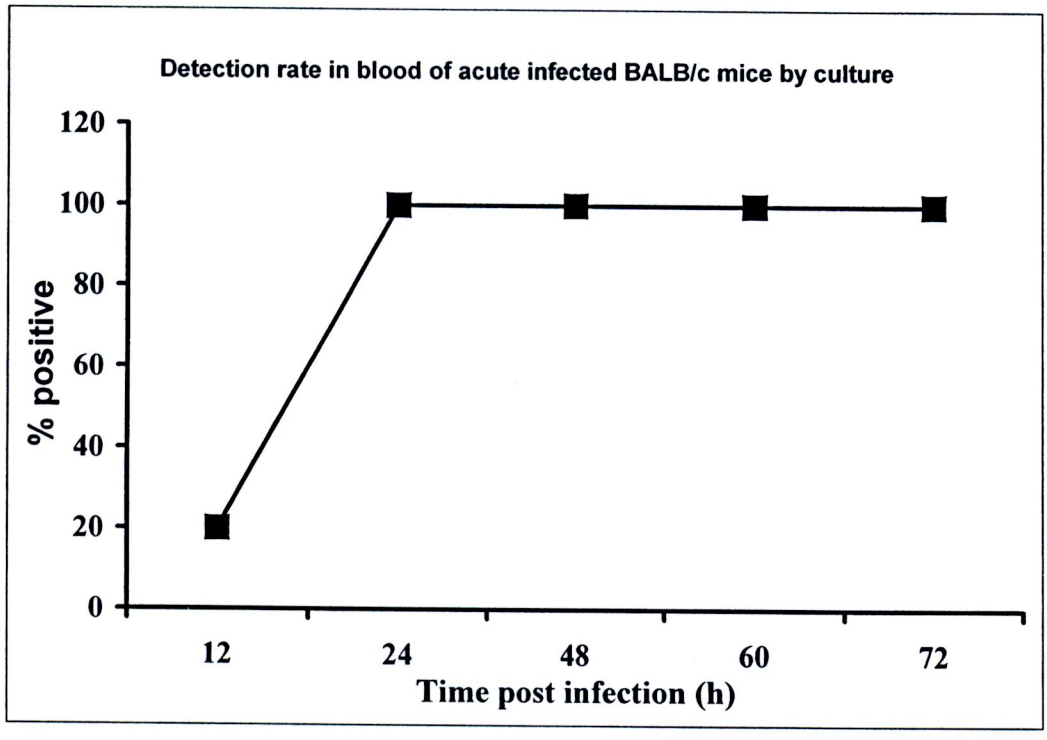

Figure 9 The percent positive in blood by culture method at various time points after infection in acute *B. pseudomallei* infected mice. The bacterial loads were determined in blood of acute infected mice by culture on Ashdown's selective medium at various time points post infection (12, 24, 48, 60 and 72 h). The percent positive is plotted against time.

1.2 *B. pseudomallei* gene detection in blood sample of acute infected mice as determined by PCR.

1.2.1 The sensitivity of *B. pseudomallei* by PCR.

The kinetic sensitivity of *B. pseudomallei* PCR detection in blood of infected mice was compared with culture method. When various DNA concentrations ranging from 1 pg to 10^3 pg/μl were tested, the 1 pg/μl of DNA could be detected (Figure 10, lane 6), whereas the lowest number of *B. pseudomallei* viable cells that could be detected by PCR was 5 CFU (Figure 10, lane 11).

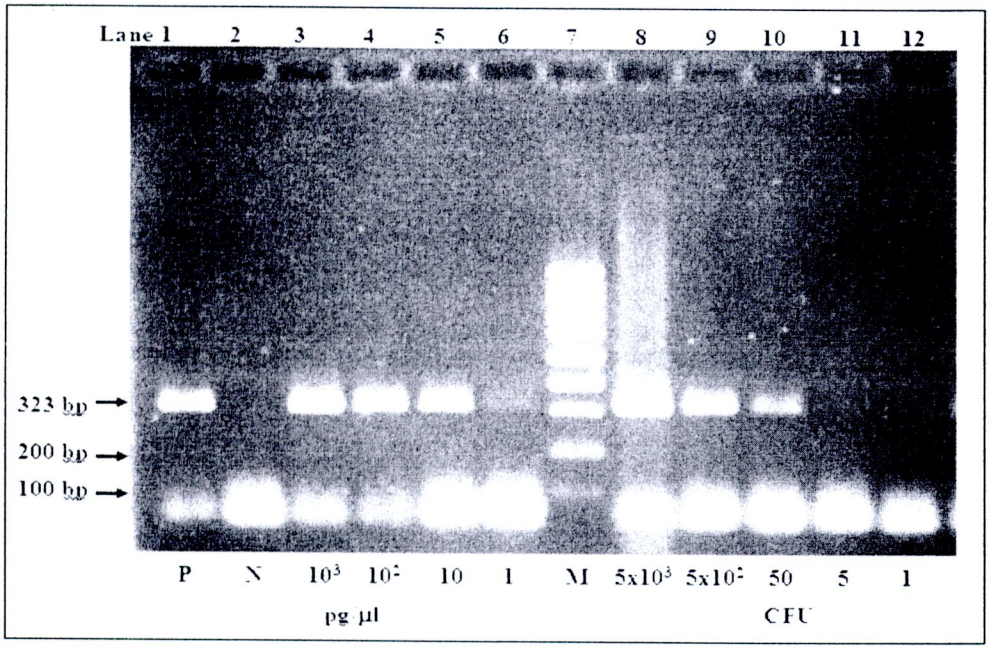

Figure 10 Sensitivity of the PCR for detection of *B. pseudomallei.* Ethidium bromide stained patterns of the PCR products (323-bp fragment of *B. pseudomallei* capsule gene) on a 1.5% agarose gel electrophoresis. The PCR products were amplified from 10^3 (lane 3), 10^2 (lane 4) , 10 (lane 5) and 1 pg/μl (lane 6) of *B. pseudomallei* DNA or from $5x10^3$, $5x10^2$, 50, 5 and 1 CFU of viable *B. pseudomallei* (lanes 8-12 respectively). Lane 7 (100 bp standard markers), lanes 1 and 2 are positive and negative controls containing $3x10^2$ pg/ml of *B. pseudomallei* DNA and no DNA, respectively.

1.2.2 Bacterial DNA in blood of acute infected mice determined by PCR.

Detection of *B. pseudomallei* DNA in blood sample of acute infected BALB/c mice were performed by PCR amplification at various time points after infection (12 , 24 , 48 , 60 and 72 h). PCR product was amplified by using wcbG_for and wcbG_rev as primers, which amplified a 323-bp fragment of *B. pseudomallei* capsule gene. The results showed that *B. pseudomallei* DNA could be detected in 1/5 (20%) blood of infected mice at 12 h after infection. Then positive rate was increased and all blood samples had bacterial capsule genes at 60 h and 72 h post infection (100% (3/3), 100% (2/2), respectively). No PCR products were detected in blood of PBS injected mice (Table 2 and Figure 11).

Table 2 The presence of *B. pseudomallei* DNA in acute infected BALB/c mice. The bacterial DNA in the blood, lungs, livers and spleens of infected mice at different time points post infection by intraperitoneal (IP) injection with 12LD50 (230 CFU) of virulent *B. pseudomallei* (A2) as determined by PCR

Time post of infection (h)	Mice	PCR detection			
		No positive/total (%positive)			
		Blood	Lung	Spleen	Liver
12	Infected	1/5 (20)	0/5 (0)	0/5 (0)	1/5 (20)
	PBS	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
24	Infected	2/5 (40)	1/5 (20)	4/5 (80)	1/5 (20)
48	Infected	4/5 (80)	5/5 (100)	5/5 (100)	2/5 (40)
60	Infected	3/3 (100)	3/3 (100)	3/3 (100)	2/3 (67)
72	Infected	2/2 (100)	1/2 (50)	2/2 (100)	2/2 (100)
	PBS mice	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)

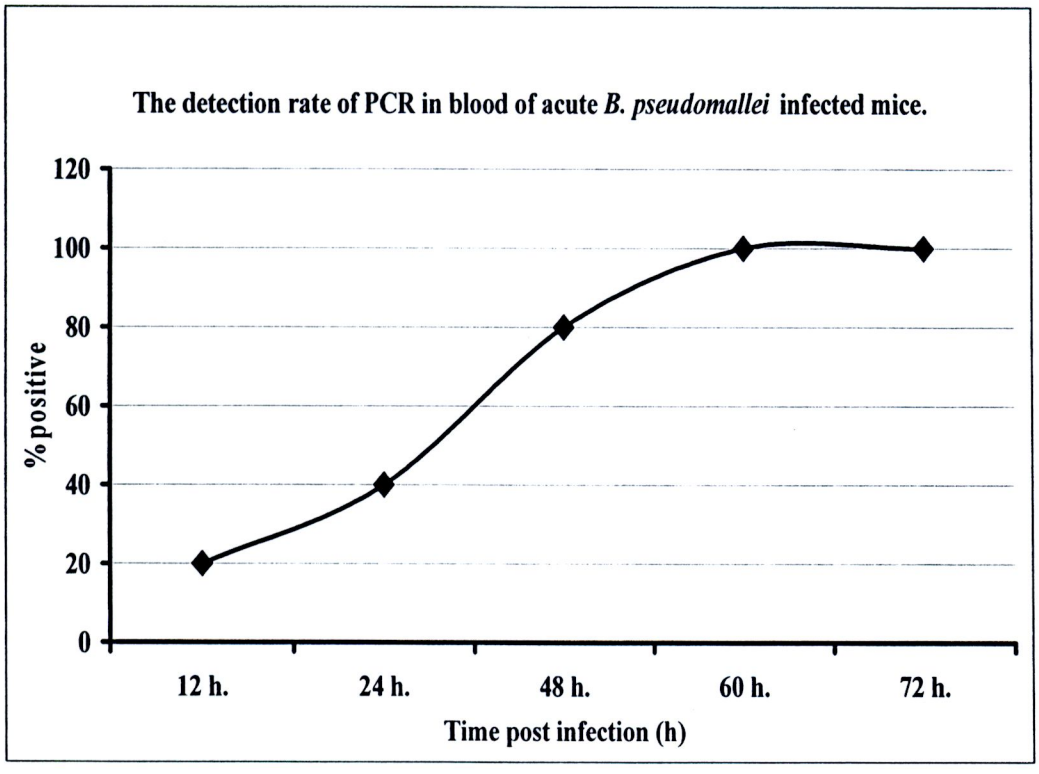

Figure 11 The detection rate of PCR in blood of acute *B. pseudomallei* infected mice. The bacterial DNA in blood of acute *B. pseudomallei* infected mice were determined by PCR at various time points post infection (12 , 24 , 48 , 60 and 72 h). *B. pseudomallei* DNAs were amplified as described in methods which detected a 323-bp fragment of *B. pseudomallei* capsule gene. The percent positives are plotted against time.

2. Kinetic of *B. pseudomallei* in various organs of acute infected BALB/c mice.

2.1 The bacterial load in organs of acute *B. pseudomallei* infection mice.

Assessment of bacterial loads in lungs, livers and spleens of *B. pseudomallei* infected animals after received high doses of *B. pseudomallei* ($12LD_{50}$). At various times post infection, the remaining infected animals were killed and then their lungs, livers and spleens were aseptically excised at 12, 24, 48, 60 and 72 h post infection. The number of viable *B. pseudomallei* presented in various organs was determined. Table 1 and Figure 12 showed bacterial counts in lungs, livers and spleens of acute infected mice. Very few bacteria could be detected in livers and spleens in the first 12 h post infection (mean±SE = 18±11 and 6±3.67 CFU/organ, respectively) whereas no bacteria were detected in the lungs of infected mice. Assessment of bacteria in organs of infected animals demonstrated a rapid increase in numbers and the bacterial loads in livers and spleens which were peaked at 48 h after infections (mean±SE $=(2.59\pm0.92)\times10^4$ and $(>1\pm0.30)\times10^5$ respectively. In the lung, the bacteria was found at 24 h post infection and the mean±SE of bacterial counts was $(3.35\pm3.16)\times10^2$ CFU/organ, it was then increased to more than 10^5 CFU/ml at 60 h post infection and then declined. Interestingly, not only the bacterial load found in the spleens was more than any other organs but they also persisted longer. In contrast, the control group received PBS were negative for any viable of bacteria in their organs (Table 1).

The recovery rate of bacteria shown in Figure 13, in both spleen and liver demonstrated 60% (3/5) positive in earliest time post infection whereas in the lung, no bacteria were detected. The positive rate was increased and all infected animals presence bacterial in both liver and spleen (100% of positive) at 24 h post infection whereas in the lung, all infected animals could be detected by culture at 48 h post infection (Figure 13).

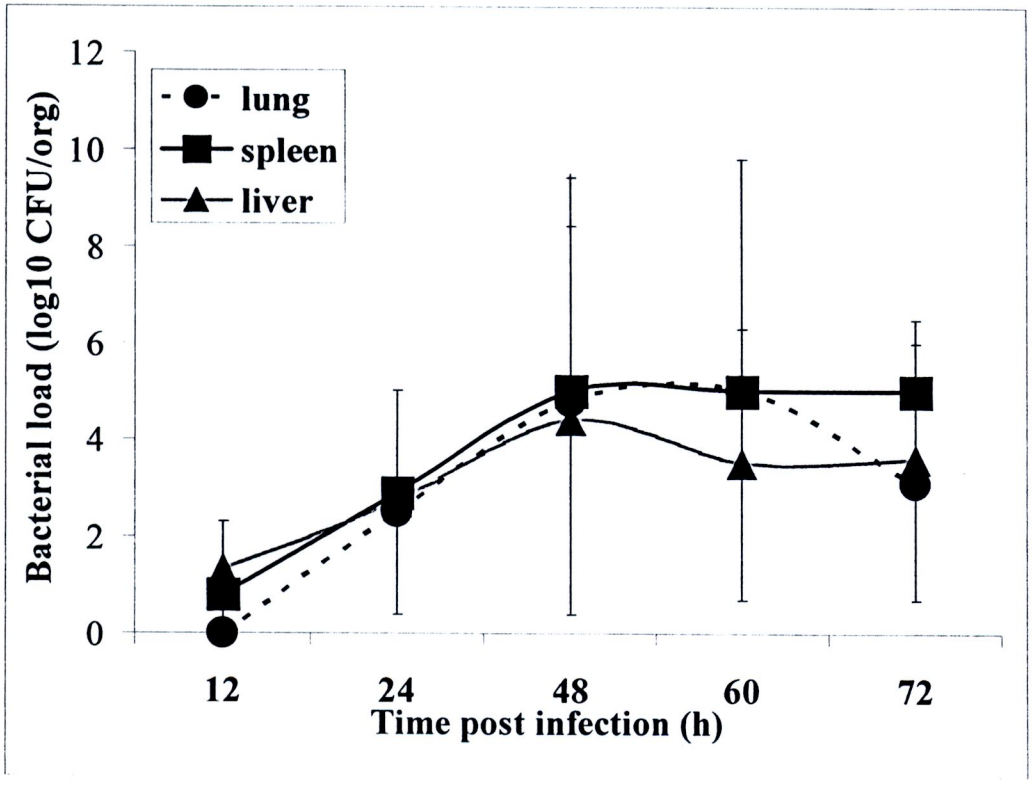

Figure 12 The bacterial loads in various organs of acute *B. pseudomallei* infected mice as determined by culture method. Assessment of bacterial loads in (-●-)lungs, (-■-)spleens and (-▲-) livers of BALB/c mice after intraperitoneal injection with $12LD_{50}$ (230 CFU) of *B. pseudomallei*. Data is presented as individual histograms for the mean ±SE number of bacterial loads (log_{10} CFU/organ). No bacteria in any organs of PBS injected mice.

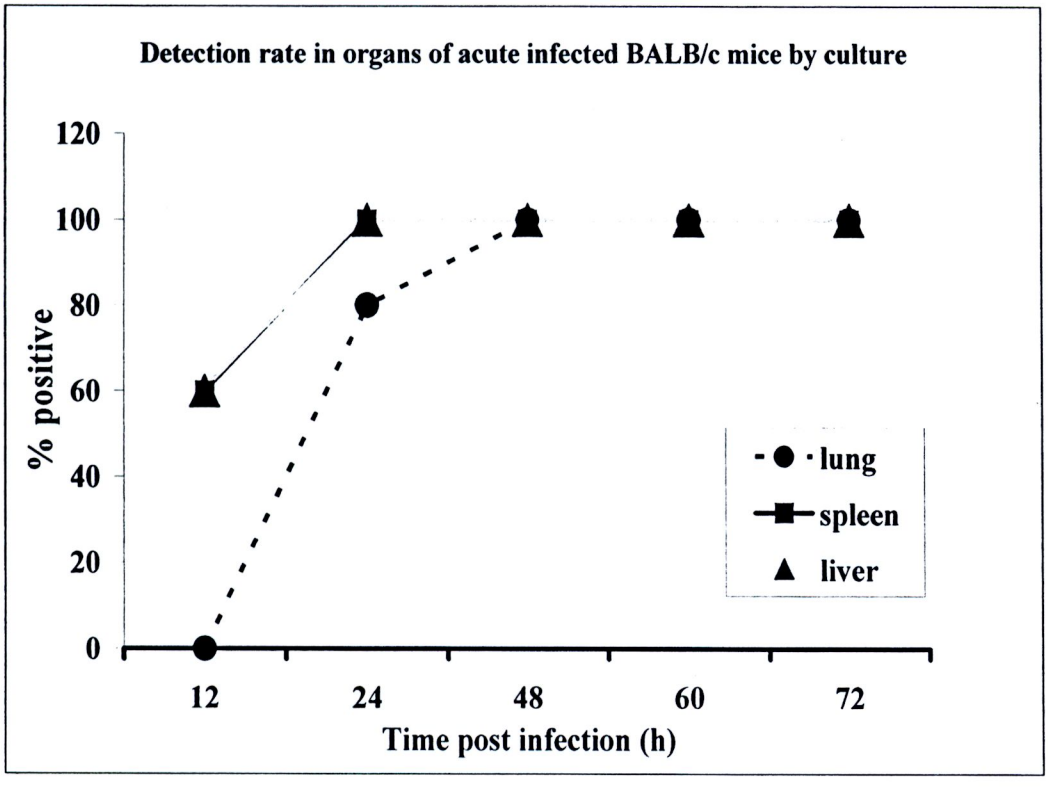

Figure 13 The percent positive by culture method at various time points after infection in various organs of acute *B. pseudomallei* infected mice. The bacterial load were determined in lungs (-●-), spleens (-■-) and livers (-▲-) of acute infected mice by culture on Ashdown's selective medium at various time points post infection (12, 24, 48, 60 and 72 h). The percent positive is plotted against time.

2.2 The bacterial DNA in organs of acute infected mice detected by PCR.

Based on the detection of *B. pseudomallei* DNA in lungs, spleens and livers, the results demonstrated that *B. pseudomallei* DNA in livers could be detected in the first 12 h post infection (20% positive (1/5)) whereas they were negative in lungs and spleens. All organs (lungs, spleens and livers) gave positive in bacterial capsule gene at 24h post infection with percent positive were 20% (1/5), 80% (4/5) and 20% (1/5), respectively. The detection rate was increased, at 48 h post infection and all infected mice gave positive (100%) of bacterial DNA in both their lungs and spleens. In the livers, all infected mice gave positive bacterial DNA at 72 h after infection. At the same time all control mice were negative (Table 2 and Figure 14).

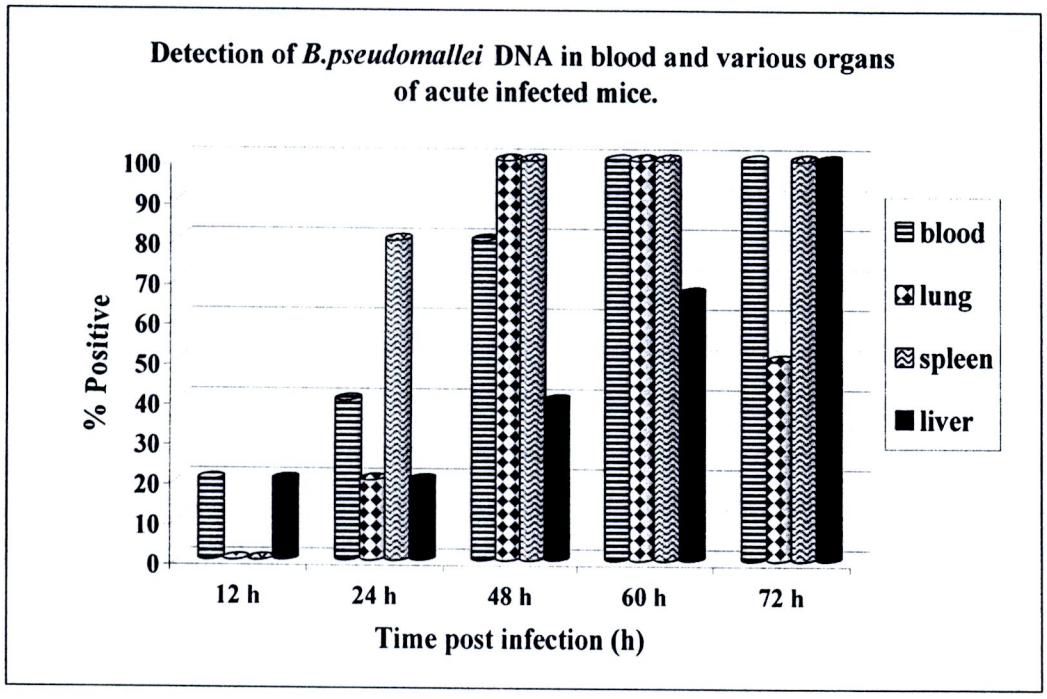

Figure 14 Detection of *B. pseudomallei* DNA in blood and various organs of acute infected mice. To determined bacterial DNA in blood, lungs, spleens and livers of BALB/c mice after intraperitoneal injection with $12LD_{50}$ (230 CFU) of *B. pseudomallei*, samples on time points indicated were collected and detections were performed by PCR. The PCR products were amplified by using wcbG_for and wcbG_rev as primers, which amplified a 323-bp fragment of *B. pseudomallei* capsule gene. Each bar represents the percent positive. All control mice gave negative results.

3. Kinetic of *B. pseudomallei* antibody of acute infected BALB/c mice.

The specific antibody response was investigated in the twenty remaining BALB/c mice infected with of $12LD_{50}$ (230cfu) of *B. pseudomallei* by intraperitoneal (IP) injection. The specific Ig levels were examined in plasma samples at various time points post infection (12 , 24 , 48 , 60 and 72 h) by enzyme-linked immunosorbent assay (ELISA) using culture-filtrated antigen (CF) as an antigen as described in materials and methods. Most plasma of the acute infected mice showed low levels of specific antibodies except two mice were positive at 12 and 24 h after infection (Table 3). The results shown in Figure 15 demonstrated that the mean levels of specific immunoglobulin against *B. pseudomallei* in plasma of acute infected mice during 12-72 h after infection were low and were not significantly different when compared to PBS control group ($P > 0.05$). These data demonstrated that *B. pseudomallei* antibody levels were low and could not be detected in early time of *B. pseudomallei* infection.

Taken together, our result demonstrated that antibody detection was not appropriate for diagnosis especially in the case of acute infection because all mice died before specific antibodies were developed. The culture or PCR were all positive after 60 h of infection although the culture method seems to be able to detect the bacteria in low amount as early as 12h after infection. These 2 methods were therefore recommended for use in diagnosis of acute infection.

Table 3 The detection of antibodies to *B. pseudomallei* in plasma samples of infected BALB/c mice at various time points after infection by ELISA using CF.

mice		No positive*/Total (%)									
		12 h	**24h**	**48h**	**60h**	**72h**	**Day5**	**Day7**	**Day14**	**Day21**	**Day28**
Acute	**Infected**	1/5 (20)	1/5 (20)	0/5 (0)	0/3 (0)	0/2 (0)	ND	ND	ND	ND	ND
	Control	0/3 (0)	ND	ND	ND	0/3 (0)	ND	ND	ND	ND	ND
Chronic	**Infected**	ND	0/10 (0)	ND	ND	3/10 (30)	7/10 (70)	7/10 (70)	10/10 (100)	7/10 (70)	6/10 (60)
	Control	ND	0/5 (0)	ND	ND	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

ND = Not determined

*Positive result is samples giving OD values that were greater than the mean OD+2SD of the normal controls.

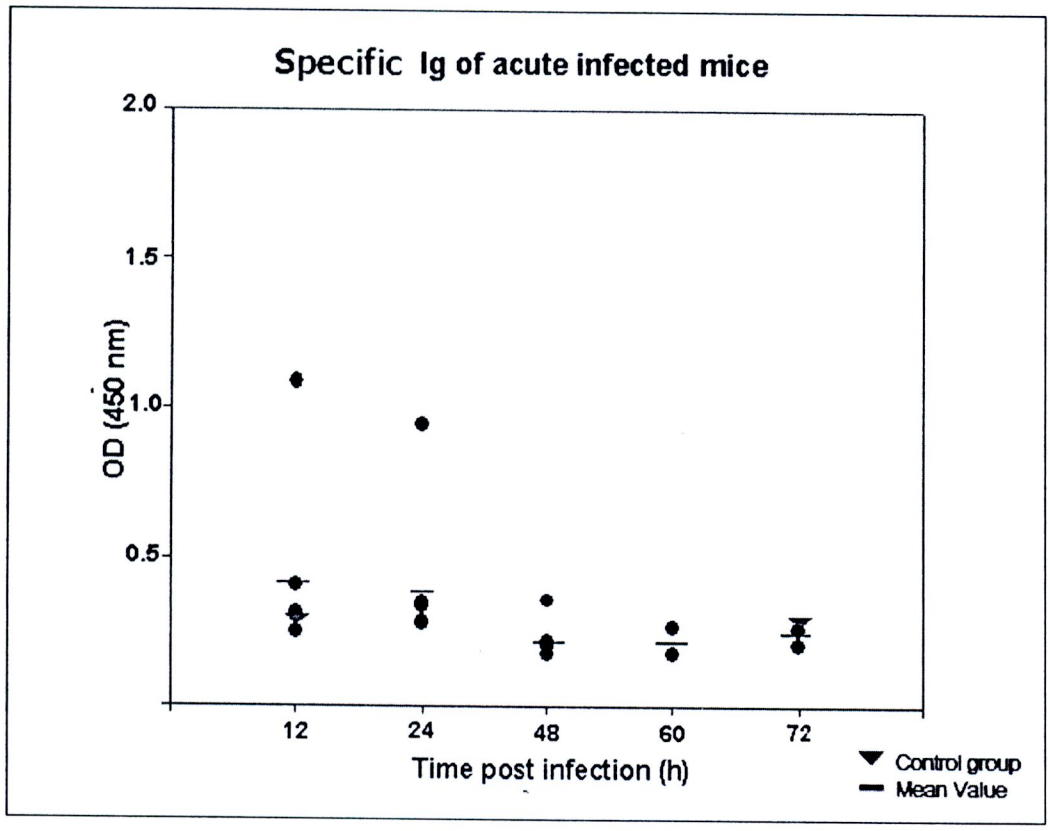

Figure 15 ELISA analysis of specific antibody responses during acute *B. pseudomallei* infected mice. BALB/c mice were infected with $12LD_{50}$ (230 CFU) of *B. pseudomallei* by intraperitoneal injection. Plasma samples on days indicated were collected and the specific antibodies were measured by ELISA. Each symbol represents a 1:100 dilution of plasma at indicated time after infection (12, 24, 48, 60 and 72 h). Results represent means of duplicates OD (optical density). Antibody levels were also measured in control mice (PBS injected mice). No significant difference was found in mean of the specific Ig levels between acute infection and PBS injected group (P =0.430 (12 h) and P=0.244 (72 h).

4. Kinetic of *B. pseudomallei* in blood of chronic infected BALB/c mice.

Fifteen BALB/c mice were infected with $0.3LD_{50}$ (6 CFU) of *B. pseudomallei* (A2) by intraperitoneal injection. Another five mice were injected with PBS for control group. The bacterial numbers in blood were detected at 1st, 3rd, 5th, 7th, 14th, 21st and 28th days post-infection by culture on Ashdown's selective medium. The results shown in Table 4 demonstrated that most of infected animals gave negative blood culture (10/15). The few number of bacteria (mean ±SE = 1.82±1.82 – (3.23±3.21)x10^2 CFU/ml) were found in 5 animals between 1st to 5th days after infection. There was the high peak of bacterial count (3.23±3.21)x10^2 CFU/ml) found on day 3 after infection then the number of bacteria decreased and no bacteria were detected after 5th day post infection. Four of five mice died in 1 day later they have bacteremia and another one (1/5) died in 2 day later. The positive rate of chronic *B. pseudomallei* infection in blood as determined by culture method and PCR methods were showed in Table 4. In culture method, positive rate was 13% (2/15), 21% (3/14) and 9% (1/11) on 1st, 3rd and 5th day after infection, respectively, whereas no *B. pseudomallei* DNA could be detected by PCR method (Table 4). These 5 mice were diagnosed as acute infection; therefore they were excluded from chronic infection study. At terminated of chronic study, some survive animals were killed and removed their organs for culture. The result shown in table 4, the infected and control mice gave negative for culture and PCR. This indicates that some infected mice clear all bacteria and might be did not get chronic infection.

Table 4 Kinetic of *B. pseudomallei* in blood of chronic infected BALB/c mice.

Day post infections	mice	PCR detection No positive/total (%positive)		Culture detection Mean±SE (CFU/ml) No positive/total (%positive)	
		Blood	Various organs (Lung, Spleen, Liver)	Blood	Various organs (Lung, Spleen, Liver)
Day 1	Infected	0/15 (0)	ND**	10.67±9.97 2/15(13)	ND
	Control	0/5 (0)	ND	NG* 0/5 (0)	ND
Day 3	Infected	0/14 (0)	ND	(3.23± 3.21) x10^2 3/14 (21)	ND
	Control	0/5 (0)	ND	NG 0/5 (0)	ND
Day 5	Infected	0/11 (0)	ND	1.82±1.82 1/11 (9)	ND
	Control	0/5 (0)	ND	NG 0/5(0)	ND
Day 7	Infected	0/10 (0)	ND	NG 0/10 (0)	ND
	Control	0/5 (0)	ND	NG 0/5 (0)	ND
Day 14	Infected	0/10 (0)	ND	NG 0/10 (0)	ND
	Control	0/5 (0)	ND	NG 0/5 (0)	ND
Day 21	Infected	0/10 (0)	ND	NG 0/10 (0)	ND
	Control	0/5 (0)	ND	NG 0/5 (0)	ND
Day 28	Infected	0/10 (0)	0/3 (0)	NG 0/10 (0)	NG 0/3 (0)
	Control	0/5 (0)	0/1 (0)	NG 0/5 (0)	NG 0/1 (0)

*NG = no growth

**ND= not done

5. Kinetic of *B. pseudomallei* antibodies in chronic infected mice.

The specific antibody response was investigated in 15 BALB/c mice after infected with $0.3LD_{50}$ (6 CFU) of *B. pseudomallei* (chronic infection). Specific Immunoglobulin levels were determined in plasma samples that were collected on 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 14^{th}, 21^{st} and 28^{th} days post-infection by ELISA. The results demonstrated that the mean ± SD of specific Immunoglobulin levels (0.72±0.32) was first detected at 5^{th} days, peaked at 14^{th} days post-infection and significantly different when compared to control mice (P=0.001). Their specific antibodies persisted until 21^{st} day post-infection and then decreased. By day 28 (at the end time point of study) antibody levels were still higher than those observed in PBS injected mice (P=0.05) (Figure 16). By ELISA technique, the specific antibody could be detected early at day 5 with 30% of the chronic infected mice. The percent positive were increased and highest at day 14 post infection (100% of positive), then decreased. This result demonstrated that our infected mice were culture and PCR negative but their specific antibodies to *B. pseudomallei* were positive thus it indicated proprably as chronic infection.

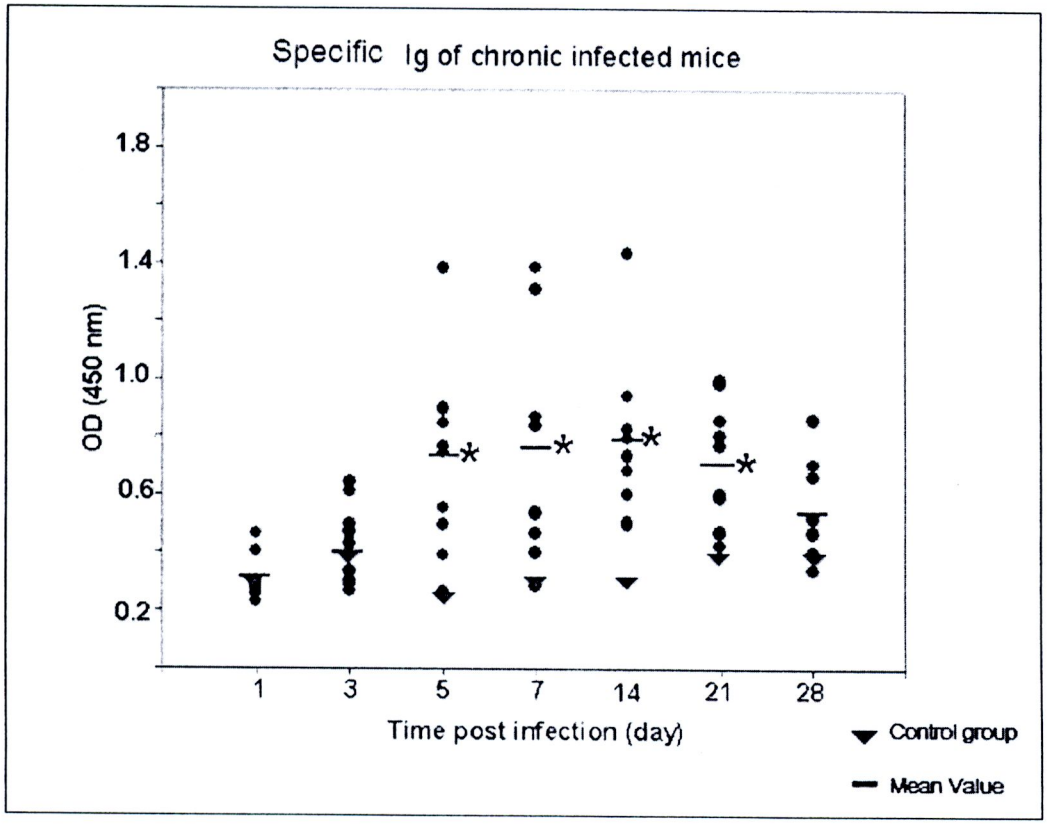

Figure 16 ELISA analysis of specific antibody responses in chronic *B. pseudomallei* infected mice. BALB/c mice were infected with $0.3LD_{50}$ (6 CFU) of *B. pseudomallei* by intraperitoneal injection. Plasma samples on days indicated were collected and measured by ELISA for specific antibodies and expressed as absorbance reading. Antibody levels were also measured in control mice (PBS injected mice). The (—) indicated the mean of each group and asterisks (*) represented the significantly different from control ($P<0.05$).