



CHAPTER IV

RESULTS

4.1 Leptospiral Antibody Titer (Microscopic Agglutination Test).

4.1.1 Production of antisera in rabbits

Antisera derived from rabbits immunized with live leptospire showed high agglutinating activity in MAT. Pooled rabbit anti-whole cell antisera against *L. biflexa* serovar patoc produced agglutination, (titer 1:5,000), as did each pooled rabbit antisera against *L. interrogans* serovar bataviae, autumnalis and icterohaemorrhagiae, (titer 1:3,000) (Table 5A).

4.1.2 Human anti-leptospiral antibody

Sera from 3 patients with Leptospirosis caused agglutination of *L. interrogans* serovar bataviae (titer 1:3,000), autumnalis (1:100) and icterohaemorrhagiae (1:3,000) respectively (Table 5B). No reactivity could be detected in each of the pooled normal rabbit sera before immunization, pooled normal human sera from 8 healthy graduate students and a positive TPHA serum at the dilution of 1:100.

Table 5 : Agglutinating activity of antileptospiral sera as measured by MAT.

Antisera	Leptospiral antibody titer
A. Pooled rabbit antisera against whole cell	
<i>L. biflexa</i> serovar patoc	1:5,000
<i>L. interrogans</i> serovar bataviae	1:3,000
<i>L. interrogans</i> serovar autumnalis	1:3,000
<i>L. interrogans</i> serovar icterohaemorrhagiae	1:3,000
B. Human antiserum against	
<i>L. interrogans</i> serovar bataviae	1:3,000
<i>L. interrogans</i> serovar autumnalis	1:100
<i>L. interrogans</i> serovar icterohaemorrhagiae	1:3,000

4.2 Leptospiral Protein Profiles on SDS-PAGE.

Effect of protein concentration (30, 35 and 40 μg of protein/lane) was demonstrated on the Coomassie Blue R-250 stained SDS-PAGE (Figure 3). It was found that 35 μg of protein/lane of sonic extracted leptospiral antigen appeared to show the best resolution. Therefore this concentration was used throughout the experiments. A complex pattern of protein bands with molecular weights (MW) ranging from 25 to 90 kilodalton (Kd) was readily discernible for each serovar. Comparison of protein profiles disclosed several bands of similar molecular weights among various serovars. However, differences between the pathogenic and non-pathogenic leptospires (*L. biflexa* serovar patoc) were observed.



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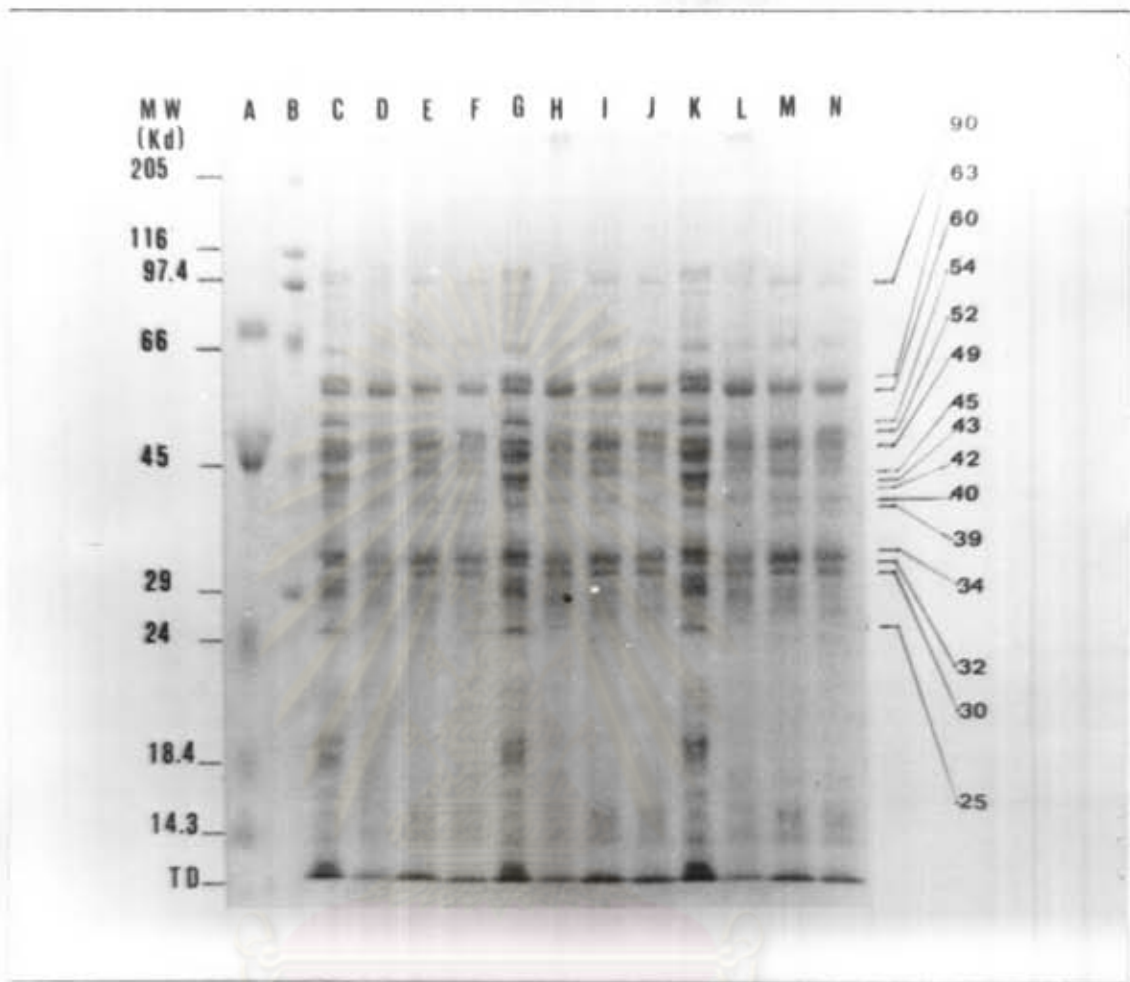


Figure 3 : SDS-PAGE profiles of sonic extracted leptospiral antigens stained with Coomassie blue R-250. Numbers indicate molecular weight in kilodalton (Kd).

- lane A, B : Molecular weight marker proteins.
- lane C, G, K : *L. biflexa* serovar patoc.
- lane D, H, L : *L. interrogans* serovar bataviae.
- lane E, I, M : *L. interrogans* serovar autumnalis.
- lane F, J, N : *L. interrogans* serovar icterohaemorrhagiae.
- lane C-F : Protein concentration 30 μ g/lane.
- lane G-J : Protein concentration 35 μ g/lane.
- lane K-N : Protein concentration 40 μ g/lane.

4.3 Analysis of Sonic Extracted Leptospiral Antigens Immunoblotted against Homologous and Heterologous Rabbit Anti-Leptospiral Antisera.

4.3.1 Immunostaining with pooled rabbit antiserum against *L. biflexa* serovar patoc.

Rabbit anti-patoc antiserum reacted well against homologous leptospiral antigens giving rise to over 20 precipitate bands on immunoblot. The antisera cross reacted with three heterologous serovars, and resulted in 10, 14 and 9 precipitate bands against *L. interrogans* serovar bataviae, autumnalis and icterohaemorrhagiae antigens respectively (Figure 4). Proteins of 33, 49 and 90 kilodaltons (kd) and a 60 kd diffuse band were found in all serovars. Three specific antigens of about 52, 46 and 39 kd were only detected on the homologous extract of serovar patoc. Rabbit antisera to serovar patoc reacted with the 33-34 kd doublet of homologous extract and appeared to cross-react with the 32-33 kd doublet of all 3 heterologous serovars.

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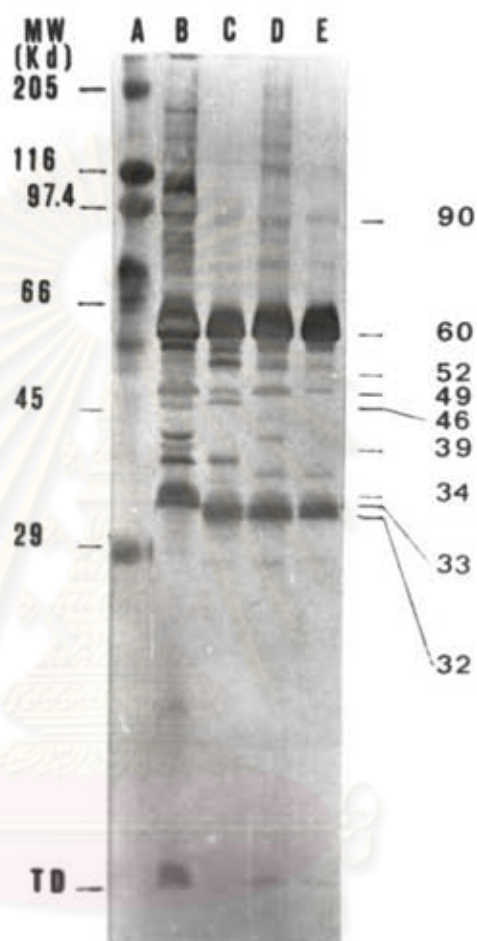


Figure 4 : Immunoblotting of sonic extracted leptospiral antigens with rabbit antiserum against *L. biflexa* serovar patoc.

lane A : Molecular weight marker proteins.

lane B : *L. biflexa* serovar patoc.

lane C : *L. interrogans* serovar bataviae.

lane D : *L. interrogans* serovar autumnalis.

lane E : *L. interrogans* serovar icterohaemorrhagiae.

The numbers indicate molecular weight in kilodalton (kd).

4.3.2 Immunostaining with pooled rabbit antiserum against *L. interrogans* serovar bataviae

Figure 5 shows the immunoblotting pattern of bataviae antiserum against sonicated antigens. There were 20 precipitate bands for the bataviae antigen, in comparison to the 13, 15 and 11 bands for patoc, autumnalis and icterohaemorrhagiae respectively. The common bands found in all sonicates were of 33, 60, 70 and 90 kd. Sonicates of 3 *L. interrogans* serovars tested, showed some common antigens among *interrogans* at 76, 45, 41, 32 and 30 kd. There were cross-reactivity in the sonicates of serovar patoc and all three *L. interrogans* as doublet bands on 33-34 and 32-33 kd respectively.



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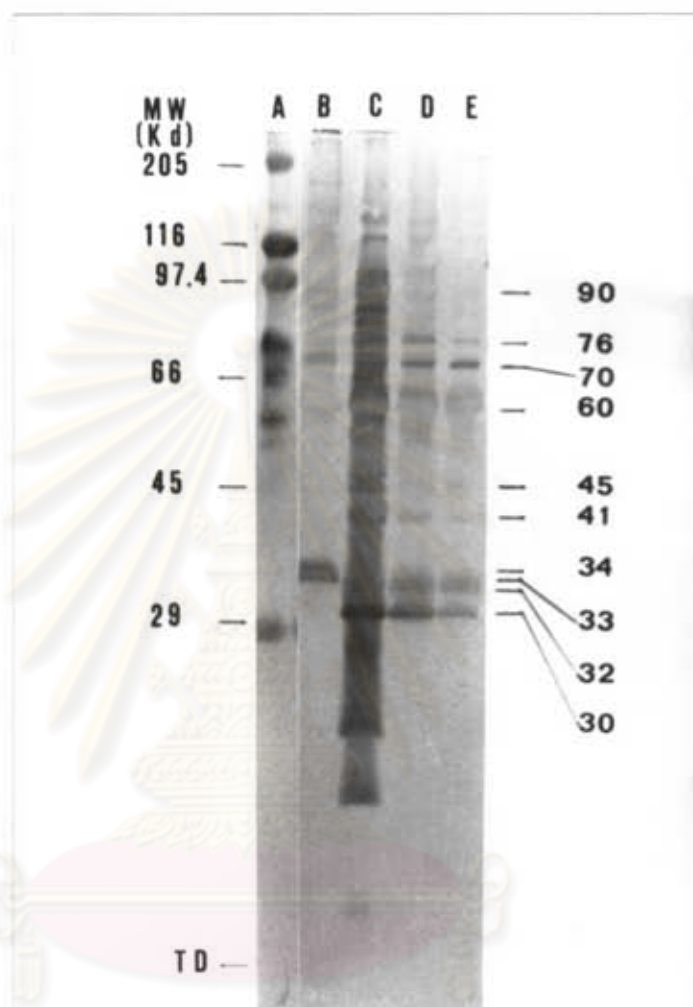


Figure 5 : Immunoblotting of sonic extracted leptospiral antigens with rabbit antiserum against

L. interrogans serovar bataviae. The numbers indicate molecular weight in kilodalton (kd).

lane A : Molecular weight marker proteins.

lane B : *L. biflexa* serovar patoc.

lane C : *L. interrogans* serovar bataviae.

lane D : *L. interrogans* serovar autumnalis.

lane E : *L. interrogans* serovar icterohaemorrhagiae.



4.3.3 Immunostaining with pooled rabbit antiserum against *L. interrogans* serovar autumnalis.

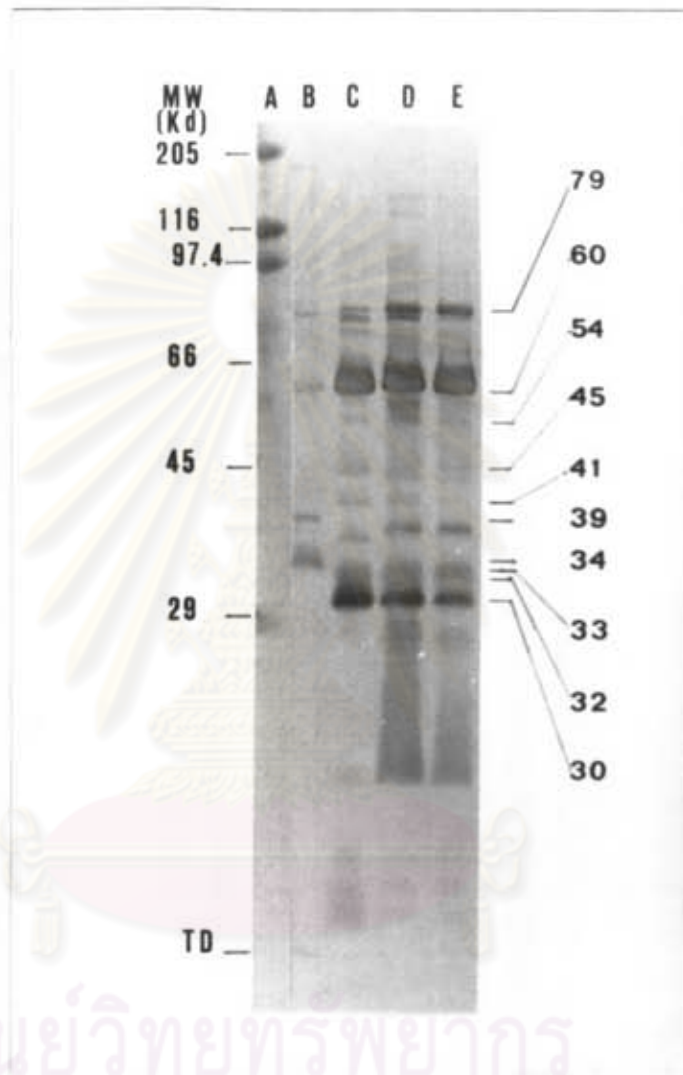


Figure 6 : Immunoblotting of sonic extracted leptospiral antigens with rabbit antiserum against *L. interrogans* serovar autumnalis. The numbers indicate molecular weight in kilodalton (kd).

lane A : Molecular weight marker proteins.

lane B : *L. biflexa* serovar patoc.

lane C : *L. interrogans* serovar bataviae.

lane D : *L. interrogans* serovar autumnalis.

lane E : *L. interrogans* serovar icterohaemorrhagiae.

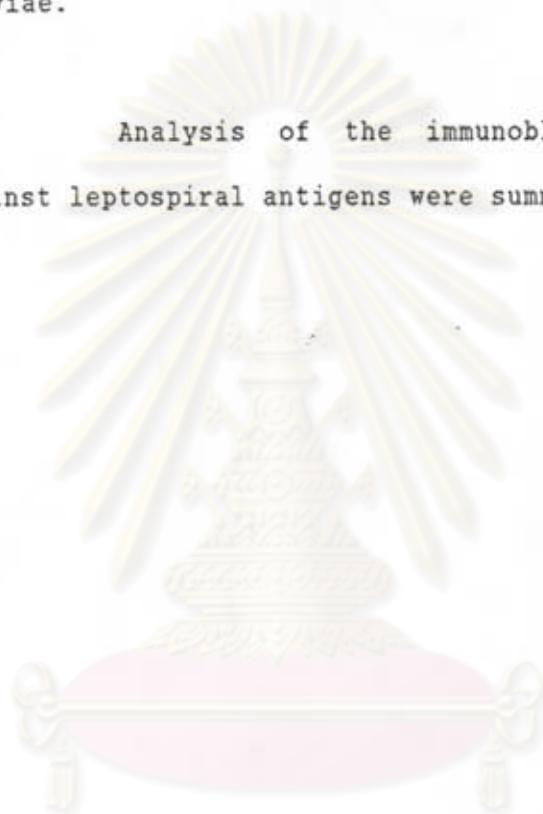
Figure 6 shows the immunoblotting pattern of anti-autumnalis antiserum against sonicated antigens. There were 19 precipitate bands on the homologous antigen in contrast to the 5, 12 and 14 bands on serovar patoc, bataviae and icterohaemorrhagiae. The 33 and 60 kd bands appeared to be common proteins in all serovars tested. Some common antigens observed in the *L. interrogans* sonicates were of 79, 54, 45, 41, 32 and 30 kd, while the cross-reactive bands in serovar patoc were of 39 and 34 kd. The pattern of homologous reaction against serovar autumnalis antigen was similar to that against heterologous serovar icterohaemorrhagiae-antigen suggesting a close antigenic relationship between the two.

4.3.4 Immunostaining with pooled rabbit antiserum against *L. interrogans* serovar icterohaemorrhagiae.

Figure 7 shows the immunoblotting pattern of anti-icterohaemorrhagiae against sonicated antigen revealing 13 precipitate bands on homologous antigen and 11, 12, 13 bands on serovar patoc, bataviae and icterohaemorrhagiae. There were 3 common bands of 33, 60 and 77 kd in all serovars. The bands found specifically in all *L. interrogans* tested, were similar to the specific bands immunostained with rabbit antiserum against serovar autumnalis, namely : bands with MW of 54, 45, 41, 32 and 30 kd. Cross-reactive bands observed on sonicated serovar patoc were of 53 and 34 kd.

As shown in Figure 8, preimmunized rabbit serum with an agglutinating titer of less than 1:100 did not react with any sonicated leptospiral antigens upon immunoblotting except for the nonspecific band of 48 kd against *L. interrogans* serovar bataviae.

Analysis of the immunoblotting of rabbit antisera against leptospiral antigens were summarized in Table 6.



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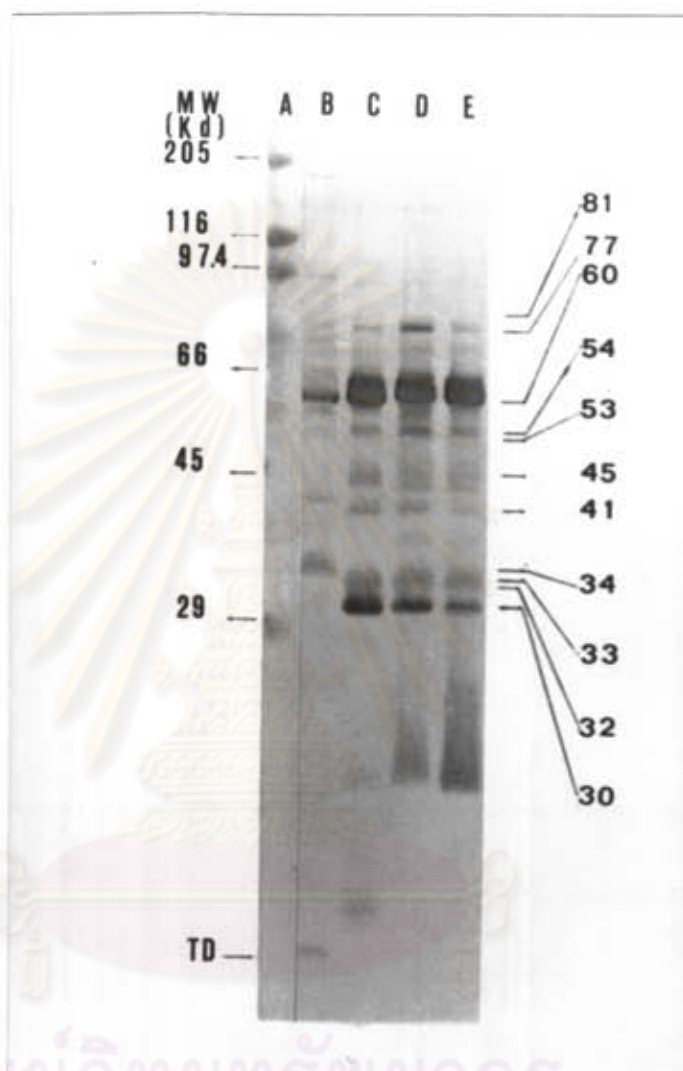


Figure 7 : Immunoblotting of sonic extracted leptospiral antigens with rabbit antiserum against

L. interrogans serovar icterohaemorrhagiae.

lane A : Molecular weight marker proteins.

lane B : *L. biflexa* serovar patoc.

lane C : *L. interrogans* serovar bataviae.

lane D : *L. interrogans* serovar autumnalis.

lane E : *L. interrogans* serovar icterohaemorrhagiae.

The numbers indicate molecular weight in kilodalton (kd).

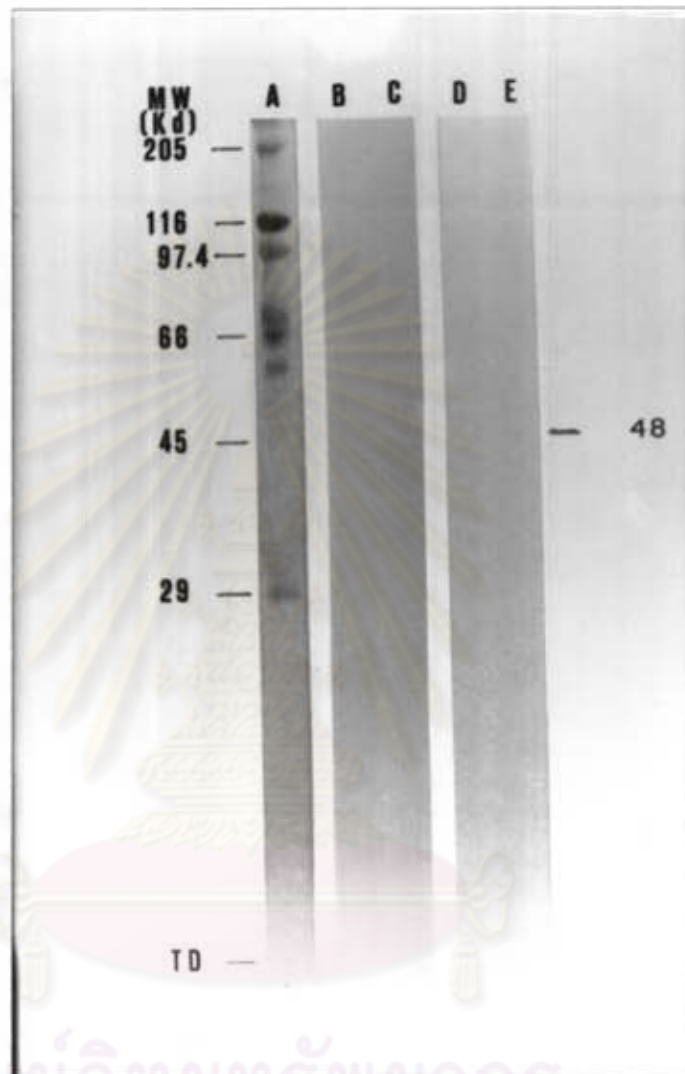


Figure 8 : Immunoblotting of sonic extracted leptospiral antigens with pooled normal rabbit sera.

lane A : Molecular weight marker proteins.

lane B : *L. interrogans* serovar autumnalis.

lane C : *L. interrogans* serovar icterohaemorrhagiae

lane D : *L. biflexa* serovar patoc.

lane E : *L. interrogans* serovar bataviae.

The numbers indicate molecular weight in kilodalton (kd).



Table 6 : A summary of immunoblotting analysis of leptospiral antigens against rabbit anti-leptospiral antibodies.

Molecular weight (kd) of leptospiral antigens revealed by immunoblotting with rabbit antiserum against

serovar patoc	serovar bataviae	serovar autumnalis	serovar icterohaemorrhagiae	normal rabbit serum
C-90	C-90	Sp-79	C-77	
	Sp-76			
	C-70			
C-60	C-60	C-60	C-60	
		Sp-54	Sp-54	
			Sn-53	
Sn-52				
C-49				B-48
Sn-46				
	Sp-45	Sp-45	Sp-45	
	Sp-41	Sp-41	Sp-41	
Sn-39		Sn-39		
Sn-34	Sn-34	Sn-34	Sn-34	
C-33	C-33	C-33	C-33	
Sp-32	Sp-32	Sp-32	Sp-32	
	Sp-30	Sp-30	Sp-30	

C = Common antigen which found in all serovars tested

Sp = Specific pathogenic antigen which found in all *L. interrogans* tested

Sn = Specific antigen which found in non-pathogenic *L. biflexa* serovar patoc

B = *L. interrogans* serovar bataviae

4.4 Analysis of Sonic Extracted Leptospiral Antigens Immunoblotted with Human Antisera against Pathogenic Leptospira.

4.4.1 Immunostaining with human anti-bataviae antiserum.

When leptospiral sonicates were immunoblotted against human serum from a patient with leptospirosis showing an agglutinating titer of 1:3,000 against *L. interrogans* serovar bataviae, several cross-reacting bands were observed (Figure 9). A common 60 kd band was found in all serovars whereas five common precipitate bands (of 80, 73, 68, 43 and 30 kd) were demonstrated among the 3 *L. interrogans* sonicates namely serovar bataviae, autumnalis and icterohaemorrhagiae. Cross-reaction was observed at 71 kd with antigens of the non-pathogenic serovar patoc. A specific band of serovar bataviae antigen was demonstrated at 66 kd.

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4.4.2 Immunostaining with human anti-autumnalis antiserum

Human antiserum with an agglutinating titer of 1:100 against serovar autumnalis reacted to various sonicated antigens revealed a common band of 60 kd on all serovars. Three common antigens of 80, 43 and 30 kd were found on three sonic extracted of species *interrogans*, while a cross-reactive 42 kd band was observed on the serovar patoc antigen (Figure 10).

4.4.3 Immunostaining with human anti-icterohaemorrhagiae antiserum.

Figure 11 shows immunoblotting of sonicated antigens reacted against human serum with leptospirosis with a leptospiral antibody titer of 1:3,000 against serovar icterohaemorrhagiae. A common band, of MW 60 kd was shown on all antigens tested and another specific antigen, MW of 30 kd was found on the three serovars of species *interrogans*.

The reactivity of pooled normal human sera with a leptospiral agglutinin titer less than 1:100 against the four leptospiral antigens was also tested. No immunoprecipitation was observed except for a single band of 89 kd on extracted serovar bataviae antigen (Figure 12).

Analysis of the immunoblotting of human antisera against pathogenic leptospira were summarized in table 7.

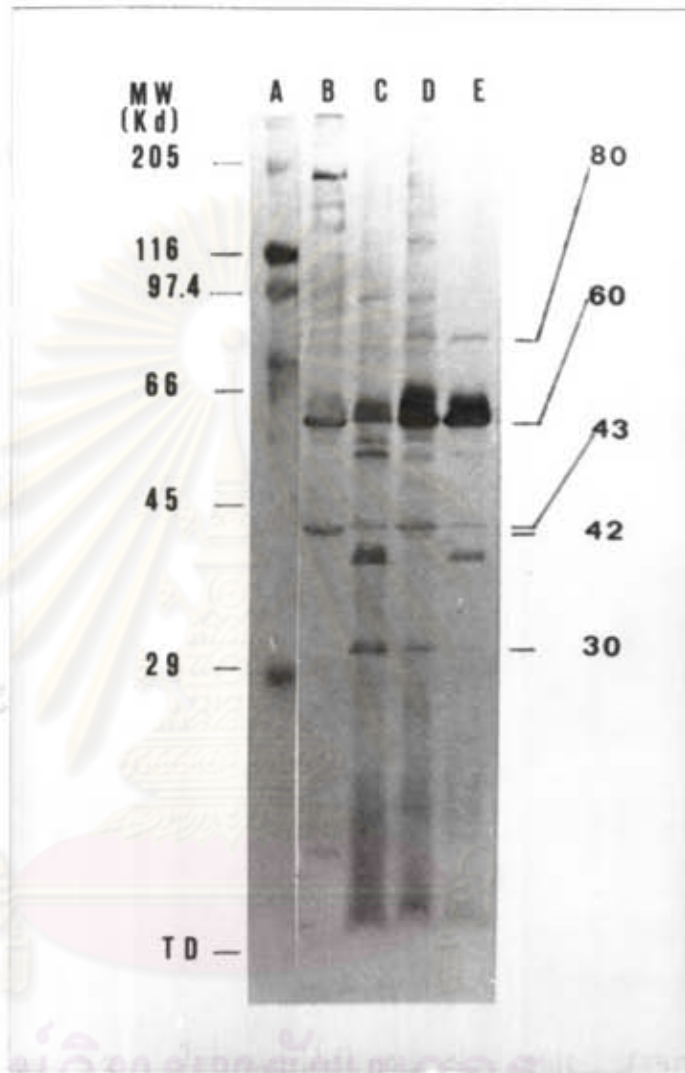


Figure 10: Immunoblotting of sonic extracted leptospiral antigens using human antiserum against

L. interrogans serovar autumnalis.

lane A : Molecular weight marker proteins.

lane B : *L. biflexa* serovar patoc.

lane C : *L. interrogans* serovar bataviae.

lane D : *L. interrogans* serovar autumnalis.

lane E : *L. interrogans* serovar icterohaemorrhagiae.

The numbers indicate molecular weight in kilodalton (kd).

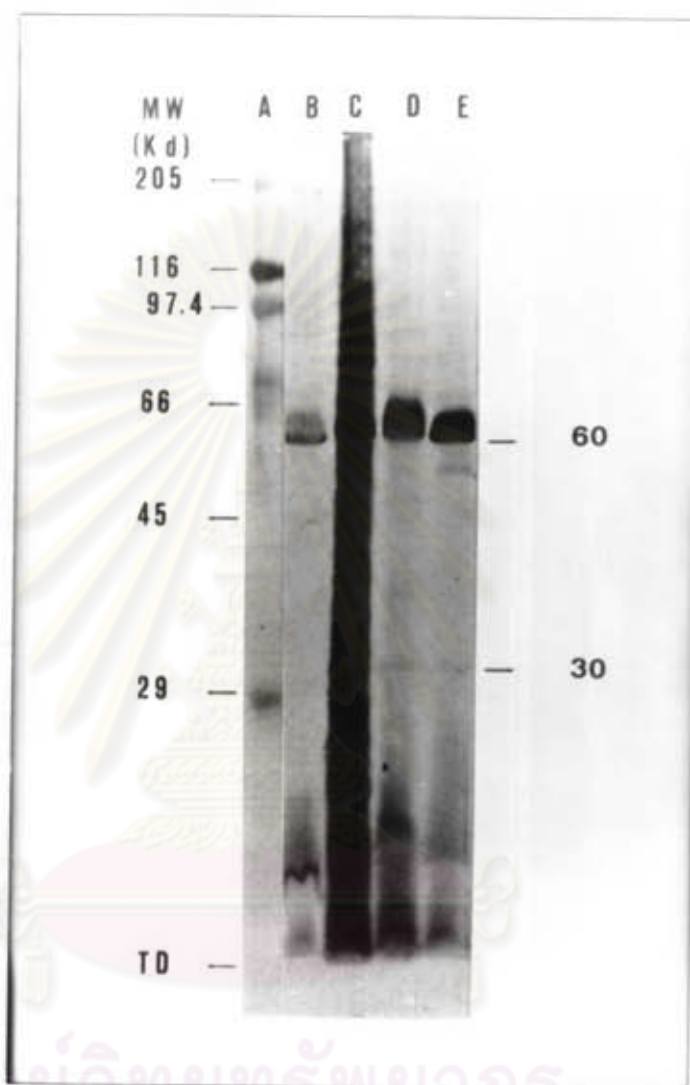


Figure 11: Immunoblotting of sonic extracted leptospiral antigens using human antiserum against *L. interrogans* serovar icterohaemorrhagiae.

lane A : Molecular weight marker proteins.

lane B : *L. biflexa* serovar patoc.

lane C : *L. interrogans* serovar bataviae.

lane D : *L. interrogans* serovar autumnalis.

lane E : *L. interrogans* serovar icterohaemorrhagiae.

The numbers indicate molecular weight in kilodalton (kd).

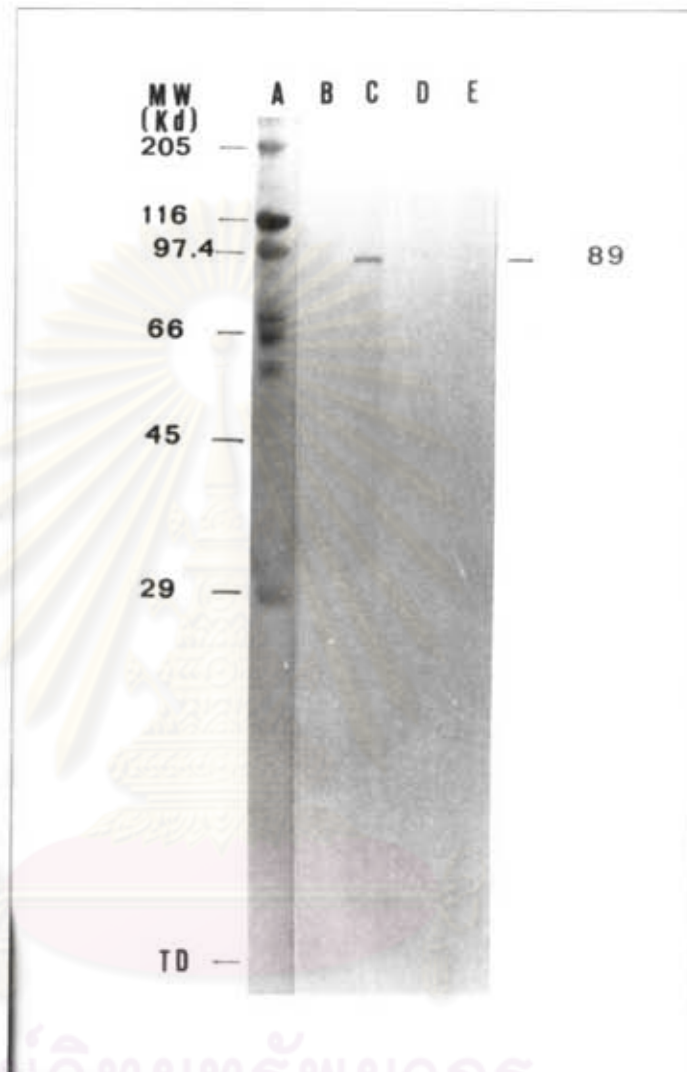


Figure 12: Immunoblotting of sonic extracted leptospiral antigens using pooled normal human sera. The numbers indicate molecular weight in kilodalton (kd).

- lane A : Molecular weight marker proteins.
- lane B : *L. biflexa* serovar patoc.
- lane C : *L. interrogans* serovar bataviae.
- lane D : *L. interrogans* serovar autumnalis.
- lane E : *L. interrogans* serovar icterohaemorrhagiae.

4.5 Cross-reactivity Between Sonic Extracted Leptospiral Antigens and Positive-TPHA Serum.

When the sonic extracted leptospiral antigens were allowed to react with a positive-TPHA human serum, several cross-reacting bands were detected. For instance, there were bands of 89 kd on bataviae and 86 kd on patoc; and four bands of 91, 66, 60 and 48 kd on all serovar tested. This indicates that the 60 kd-common protein found in all leptospira cross-reacts with Treponema antiserum in the immunoblotting technique (Figure 13).



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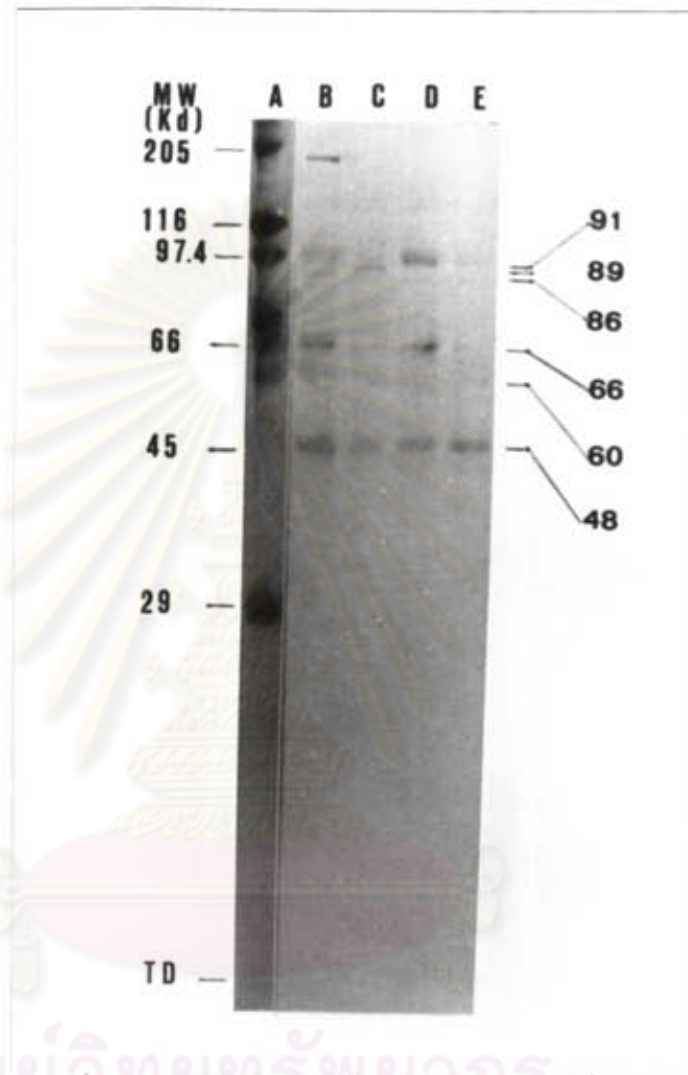


Figure 13: Immunoblotting of sonic extracted leptospiral antigens using positive-TPHA human serum. The numbers indicate molecular weight in kilodalton (kd).

- lane A : Molecular weight marker proteins.
- lane B : *L. biflexa* serovar patoc.
- lane C : *L. interrogans* serovar bataviae.
- lane D : *L. interrogans* serovar autumnalis.
- lane E : *L. interrogans* serovar icterohaemorrhagiae.



Table 7 : Summary of immunoblotting analysis of leptospiral antigens using human antisera against pathogenic leptospira.

Molecular weight (kd) of leptospiral antigens revealed by immunoblotting with human antiserum against				
serovar bataviae	serovar autumnalis	serovar icterohaemorrhagiae	normal human serum	<u>Treponema pallidum</u>
			B-89	C-91 B-89 Sn-86
Sp-80 Sp-73 Sn-71 Sp-68 B-66 C-60	Sp-80			
	C-60	C-60		C-66 C-60 C-48
Sp-43	Sp-43 Sn-42			
Sp-30	Sp-30	Sp-30		

- C = Common antigen which found in all serovar tested
- Sp = Specific pathogenic antigen which found in all *L. interrogans* tested
- Sn = Specific antigen which found in non-pathogenic *L. biflexa* serovar patoc
- B = *L. interrogans* serovar bataviae

Table 8 : Comparison of leptospiral antigen profiles by immunoblotting using rabbit antisera and human antisera.

Molecular weight (kd) of leptospiral antigen profiles revealed by immunoblotting with									
rabbit antiserum against serovar					human antiserum against serovar				human anti- <i>L. pallidum</i> antiserum
patoc	bataviae	autumnalis	ictero- haemorrhagiae	normal rabbit serum	bataviae	autumnalis	ictero- haemorrhagiae	normal human serum	
C-90	C-90							B-89	C-91 B-89 Sn-86
		Sp-79			Sp-80	Sp-80			
	Sp-76		C-77						
	C-70				Sp-73 Sn-71				
					Sp-68 B-66 C-60				C-66 C-60
C-60	C-60	C-60 Sp-54	C-60 Sp-54 Sn-53		C-60	C-60	C-60		
Sn-52 C-49				B-48					C-48
Sn-46	Sp-45	Sp-45	Sp-45		Sp-43	Sp-43 Sn-42			
	Sp-41	Sp-41	Sp-41						
Sn-39		Sn-39							
Sn-34	Sn-34	Sn-34	Sn-34						
C-33	C-33	C-33	C-33						
Sp-32	Sp-32	Sp-32	Sp-32						
	Sp-30	Sp-30	Sp-30		Sp-30	Sp-30	Sp-30		

C = Common antigen which found in all serovar tested

Sp = Specific pathogenic antigen which found in all
L. interrogans tested

Sn = Specific antigen which found in non-pathogenic
L. biflexa serovar patoc

B = *L. interrogans* serovar bataviae